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Award Number: DAMD17-03-1-0554

TITLE: Vectors for Treatment of Metastatic Breast Cancer

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REPORT DATE: August 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01/082006	Final	15 Jul 2003 – 14 Jul 2006

4. TITLE AND SUBTITLE

Vectors for Treatment of Metastatic Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0554

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Sidney Kimmel Cancer Center
San Diego, CA 92121

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT: The objective is to design, build and study vectors which would be able to break tolerance to breast cancer associated TAA and to be used to suppress the recurrence of metastatic breast cancer following surgical resection. The hypothesis is that by fusing the CD40 ligand stripped of its transmembrane domain and intracytoplasmic domains, to a breast cancer TAA such as the extracellular domain of the her-2-neu receptor, or the extracellular tandem repeat peptides of breast cancer associated surface glycoprotein, MUC-1 (both of which have been shown to be capable when loaded on APCs of conferring resistance to engraftment by cancer cells bearing these TAA), one can break tolerance to breast cancer. The subcutaneous injection of this vector creates infected cells as factories to secrete the CD40LTAA into the systemic circulation as well as locally for the activation and antigen loading of APCs, so that they would move to the lymph nodes all over the body to generate the CD8 dependent response against metastatic breast cancer. We also explored boosting of the vector vaccine by TAA/CD40L protein injections. This report summarizes the successful assembly and study of these vectors. These injections break tolerance to tumor associated antigens in mouse models.

15. SUBJECT TERMS
Adenoviral vector, gene therapy, metastatic breast cancer, Her-2-neu, MUC1

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	56	**19b. TELEPHONE NUMBER** *(include area code)*

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Specific Aim #1	**Completed**
Synthesis of vectors and studies of dose and schedule.	
Items a-i:	See Fig. 2 (Section B1a) and publications 2-4 of Section D.
Item j:	See Fig. 5 (Section B1a) and publication 3 of Section D
Item k:	See Fig. 6 (Section B1a) and publication 3 of Section D.
Item l:	See Fig. 4 (Section B1a) and publication 3 of Section D.
Specific Aim #2	**Completed**
Items a-f:	See Fig. 7-9 (Section B3) and publication 2 of Section D.
Specific Aim #3	**Completed**
Items a-c:	See Fig. 7-8 (Section B3) and publications 2-3 of Section D.
Specific Aim #4	**Completed**
Items a-c:	See Fig. 9-10 of Section B4 and publications 2-3 of Section D.

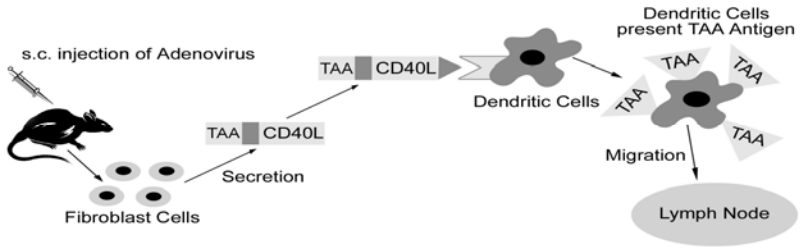
A. Introduction

Details of the Ad-sig-TAA/ecdCD40L Vaccine. The Deisseroth laboratory has developed an in vivo adenoviral vector (Ad-sig-TAA/ecdCD40L) based method of activating and target antigen loading DCs (1-3). This strategy was specifically designed to overcome the defects in CD40L expression in helper CD4 T cells which exist in the immune system of the elderly. The Ad-sig-TAA/ecdCD40L vector carries a transcription unit encoding the extracellular domain (ecd) of the CD40 ligand (CD40L) linked to tumor associated antigens (TAA). The 209 amino acid ecd of the CD40L contains all of the sequences necessary for the formation of the trimer of the CD40L (4). This transcription unit resembles one previously delivered orally in bacteria cells by Xiang et al (5) with the following differences: the arrangement of the TAA and CD40L is reversed from that present in the vaccine of Xiang; a DNA vaccine was used in the work of Xiang et al whereas our laboratory used an adenoviral vector; the bacterial delivered oral DNA vaccine required an antibody targeted cytokine (IL2) boost to break tolerance in all of the test mice, whereas the Ad-sig-TAA/ecdCD40L did not require a cytokine boost to break tolerance to TAA.

The adenoviral system (1-3) has several theoretical advantages over the oral DNA delivery system of Xiang et al (4). The adenoviral vector provides a “danger signal” which contributes to the activation of the immune response. The expression of the TAA/ecdCD40L gene may be at higher levels and for longer periods of time with the adenoviral delivery vector than is the case with the oral DNA vaccine, thereby potentially eliciting a more vigorous immune response. Although immune specific T cells that are elicited post immunization are thought to traffic throughout the body, there is still a propensity for tissue-specific homing by memory T cells to the lymphoid sites draining the natural area of infection. Thus, the sc injection of the adenoviral vector carrying the highly immunogenic TAA/ecdCD40L in the region of the tumor cells may foster the optimal trafficking of sensitized cytotoxic T cells and the generation of memory cells.

The human MUC-1 (hMUC-1), rat Her-2-Neu (rH2N), HPV E7, and the TRP2 melanosome specific antigen have all been in turn linked to a secretory signal peptide (sig) so that the TAA/ecdCD40L protein would be secreted from the vector infected cells at the injection site of the vector continuously over a 10-14 day period. The release of the TAA/ecdCd40L protein from the vector infected cells has been shown to bind to DCs, and to generate activated tumor antigen loaded DCs, which migrate to regional lymph nodes to induce a TAA specific CD8+ T cell response (see Figure 1).

Figure 1



Vaccination of 18 month old aged mice has been shown to increase levels TAA specific CD8 effector cells, and to decrease the level of negative regulatory CD4 FOXP3 positive T cells. Two sc injections of the TAA/ecdCD40L protein boost following the sc administration of the Ad-sig-TAA/ecdCD40L vector expands the magnitude of the cellular and humoral immune response induced by the vector**.** We are now proposing to test the use of the VPP vaccine to the post allograft expansion of TAA specific T cells in the elderly.

B. Body.

B. 1. Statement of Work: Specific Aim #1: Optimization of the Schedule of Administration of the AdVLEDCD40LTAA:

Statement of Work for Specific Aim 1.a.:

a. Amplification and Purification of Vector
b. Injection of 100 million pfu subcutaneously each week X2, X3, X4, X5, X6
c. Repeat of #2
d. Injection of 0.5 million tumor cells subcutaneously in each of the animals
e. Follow-up of animals (5 per dose) for growth of tumor cells
f. Repeat the above with two weekly injections of the vector
g. Then start at 7 days subcutaneous injections of increasing doses of the tumor cells once monthly (0.25, 0.5, 0.75, 1.0,1.5, 2.0, 5 and 10 million
h. Follow animals for immune response and recurrence
i. From the results, determine the optimal schedule of administration on the basis of the endpoints of survival and disease free survival.

B.1.a. Description of Results for Specific Aim #1.a.:

There was not a very steep dose response curve for the vector alone. We had also shown that the sc injection of the TAA/ecdCD40L protein as a booster dramatically increased the magnitude of the immune response over that seen with the vector alone. Therefore we varied the number of days of the protein boost. Since the immune response is known to be diminished in older human beings (in the fifth and sixth decades of life-the peak years for breast cancer), we tested this in old (18 months) as well as in young (2 months) mice.

B.1.a.i. Data on the Immune Response Induced by the V, VP, VPP and VPPP Regimen with the Ad-sig-E7/ecdC40L Vector Prime and E7/ecdCD40L Protein Boost VPP Vaccine in 2 Month Old Mice. We compared the levels of TAA specific splenic CD8 T cells and E7 specific serum antibodies levels of 2 month old mice vaccinated sc with a single Ad-sig-E7/ecdCD40L vector injection (V) or a single vector injection followed by one (VP), two (VPP) or three (VPPP) weekly sc injections of the E7/ecdCD40L protein boost. This vaccine directs an immune response against the human papilloma virus (HPV) E7 antigen in C57BL6 mice.

Figure 2A

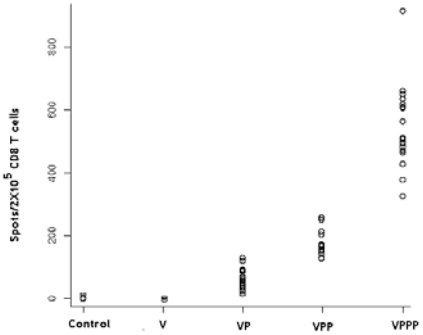
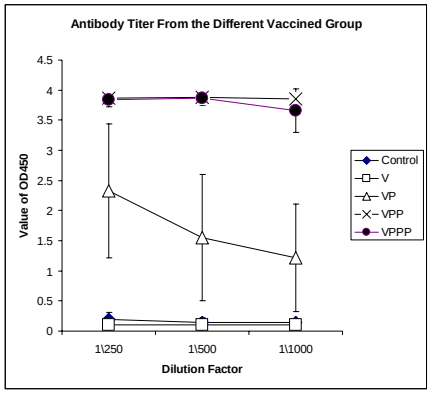


Figure 2B



As shown in Figure 2A, the levels of the E7 specific splenic CD8 T cells of the vaccinated mice increased as one proceeded from V to VP to VPP and to VPPP (all pair wise comparisons among VP, VPP and VPPP are significantly different at the $p<0.05$ level). As shown in Figure 2B, the levels of the E7 specific serum antibodies against the E7 B cell epitope EIDGPAGQAEPDRAHYNIVTFCCKCD of the vaccinated mice increased significantly with 1 (VP), 2 (VPP) and 3 (VPPP) protein boosts after the initial vector injection. At a dilution factor of 1/1000, the difference of the serum antibody levels in the vaccinated vs the control group was statistically significantly different at the $p=0.004$ level for VP mice; at the $p<0.001$ level for the VPP mice; and at the $p<0.0001$ for the VPPP mice.

B.1.a.ii. Data on the Immune Response Induced by the V, VP, VPP and VPPP Regimen with the Ad-sig-E7/ecdC40L Vector Prime and E7/ecdCD40L Protein Boost Vaccine in 18 Month Old Mice. We compared the levels of TAA specific splenic CD8 T cells and E7 specific serum antibodies levels of 18 month old mice vaccinated sc with a single Ad-sig-E7/ecdCD40L vector injection (V) or a single vector injection followed by one (VP), two (VPP) or three (VPPP) weekly sc injections of the E7/ecdCD40L protein boost. This vaccine directs an immune response against the human papilloma virus (HPV) E7 antigen in C57BL6 mice. As shown in Figure 2C, increases in the levels of the E7 specific splenic CD8 T cells of the vaccinated mice were detectable only at the VPP and VPP level. Obviously, this is a result that is different from that seen in the 2 month old mice seen above in Figure 2A in which increases were seen in the level of E7 specific cells at the VP level.

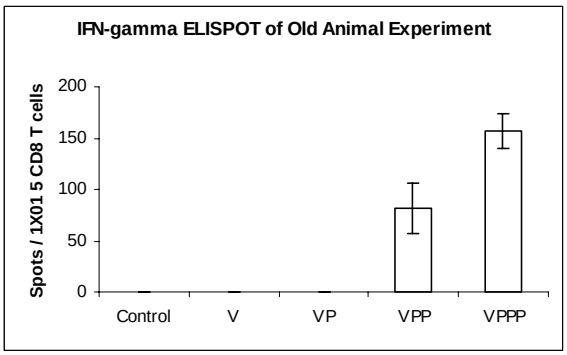


Figure 2C

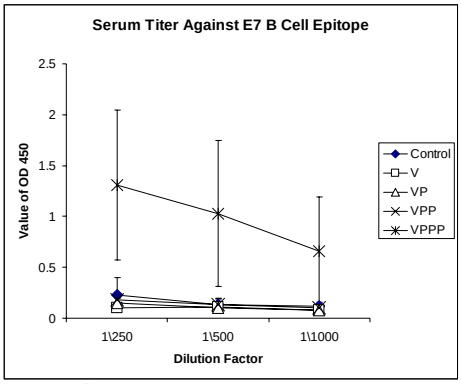


Figure 2D

As shown in Figure 2D, the levels of the E7 specific serum antibodies against the E7 B cell epitope EIDGPAGQAEPDRAHYNIVTFCCKCD of the vaccinated mice increased significantly only with three protein boost injections (VPPP) after the initial vector injection. This result in the old mice was different than that seen in the 2 month old mice (see Figure 2B above) in which significant elevations of E7 specific antibodies were seen with VP, VPP and VPPP.

Conclusions. The results of these experiments show that two protein boosts are needed to induce a humoral and cellular immune response against the E7 antigen in 18 month old mice whereas in 2 month old mice, increases are seen with one protein boost.

B.1.a.iii. Study of the Effect of the Ad-sig-TAA/ecdCD40L Vector Vaccination in Old (18 Month) Mice.

B.1.a.iii.1. Changes in Number and Function of TAA Specific CD8 Effector Cells in Tumor Tissue in 18 Month Old Mice. It is well known that the levels of CD8 effector T cells are decreased in old mice (18 months) and in older human test subjects. In addition, among older human subjects as well as older mice, the level of expression of the CD40L on activated CD4 T cells is lower than that among 2 month old mice or younger human subjects. In order to test the hypothesis that the Ad-sig-E7/ecdCD40L vector prime-E7ecdCD40L protein boost vaccination could circumvent these defects in the immune response system of older individuals, we vaccinated C57Bl/6J mice which were 18 months old with the Ad-sig-E7/ecdCD40L vector (two sc injections 7 days apart). We then measured the changes in the levels of the E7 specific T cells in the tumor tissue by ELISPOT assay. As shown in Figure 3A, the levels of E7 specific T cells was increased to 150-250 antigen specific T cells/100,000 splenic cells by ELISPOT assay. Although it is clear that the magnitude of the induction of antigen specific T cells in the 18 month old mice was less than that seen in the 2 month old mice, the absolute magnitude of the response in the 18 month old mice is sufficient to produce a robust immune response, and is equal to that induced in younger mice with other vaccine strategies. We then tested the changes of the antigen specific T cells in the tumor tissue after vaccination using E7 tetramers. As shown in Figure 3B, the Ad-sig-E7/ecdCD40L vaccine induced the level of antigen specific T cells in the tumor tissue by 10 fold.

B.1.a.iii.2. Changes in the Number of Negative Regulatory CD4 FOXP3 T Cells in Tumor Tissue after Vaccination. Increases of negative regulatory CD4 FOXP3 T cells have been reported to limit the degree to which vaccines suppress the degree of immune response to vaccination. Decreases in the level of negative regulatory FOXP3 CD4 T cells have been reported with vaccination.

Figure 3A

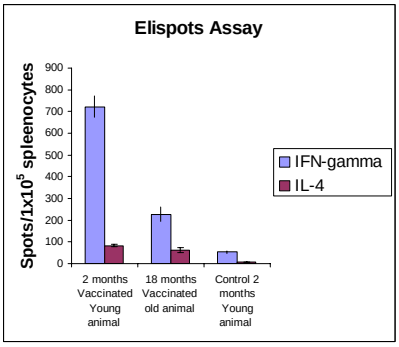


Figure 3B

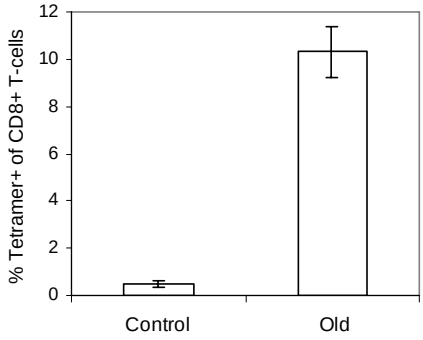
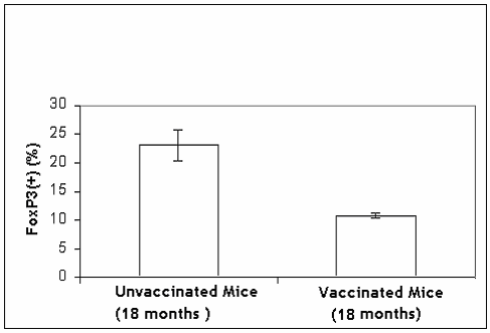


Figure 3C



We therefore measured the level of FOXP3 CD4 T cells in the tumor tissue of the 18 month old mice after vaccination. As shown in Figure 3C, the levels of the negative regulatory CD4 FOXP3 T cells in the vaccinated mice were significantly lower than that of the unvaccinated mice ($p<0.05$). There were 3 mice per group. The VPP vaccine also increased the level of E7 specific CTL in old as well as young test mice.

Statement of Work for Specific Aim #1.b.

j. RTPCR of lymph nodes for the CD40L/TAA junction fragment following subcutaneous injection of AdVLEDCD40LTAA to show that the APCs migrate to the lymph nodes all over the body.
k. ELISA of blood days 2-10 after vector injection for the fusion protein.
l. Study of a AdVCD40LTAA vector in which there is no secretory sequence (LED) and in which the CD40L has the transmembrane domain (TM) to test importance of fusion protein secretion on the development of immunity to tumor cells.

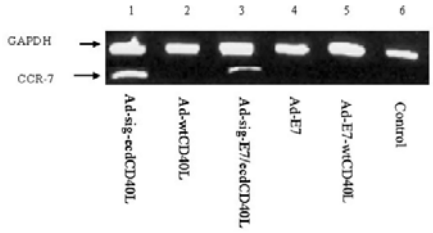
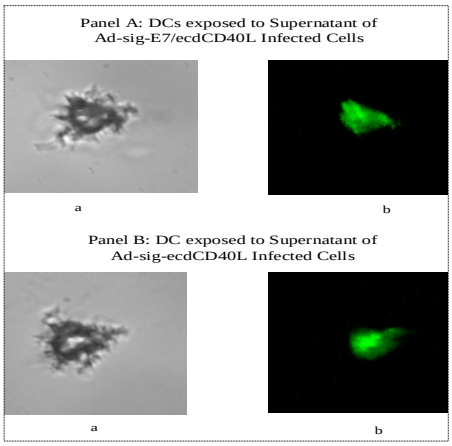


Figure 4A

B.1.b. Description of Results for Specific Aim #1.b.:

B.1.b.i. Exposure of DCs to the Supernatants of Ad-sig-E7/ecdCD40L Vector Infected Cells Increases the Level of the CCR-7 Chemokine mRNA in the DCs. Lymphoid chemokine receptor expression is observed in mature DCs which migrate to regional lymph nodes. Therefore, the effect of exposing DCs to the supernatants of Ad-sig-E7/ecdCD40L vector infected cells on chemokine production by DCs was investigated. Before exposure to the supernatants of cells infected with the Ad-sig-E7/ecdCD40L vector, immature DCs derived from bone marrow cells could not produce CCR-7 mRNA (see Lane 6 of Figure 4A above on the previous page). In contrast, following exposure of the DCs to the Ad-sig-E7/ecdCD40L vector, the level of CCR-7 mRNA was increased by RT-PCR assay (see Lane 3 of Figure 4A above on the previous page). Supernatants from cells infected with vectors the E7/CD40L transcription units of which were not engineered to secrete the E7/CD40L protein did not increase the level of the CCR-7 mRNA (see lanes 2, 4, and 5 in Figure 4A above). As shown in Figure 4A above, the absence of a secretory sequence and the presence of a TM renders the vector inactive.

B.1.b.ii. Study of Binding of the E7/ecdCD40L Protein to DCs.



In order to test if the secretable sig-E7/ecdCD40L fusion protein could bind to the CD40 receptor on DCs, we infected 293 cells with the Ad-sig-E7/ecdCD40L vector, or the Ad-sig-ecdCD40L vectors. We collected the serum free supernatant medium, and labeled the proteins with fluorescein. We then incubated in vitro these proteins with bone marrow derived DCs for 2 hours. Then, the DCs were washed and photographed two ways: once with light microscopy with which one can see the processes of the DCs (left hand panels), and another time with fluorescent microscopy, with which one can see the green fluorescence on the same cells (right hand panels). The results which are presented in Figure 4B (see to the right), show that the secretable form of the sig-E7/ecdCD40L protein in the supernatants of cells infected with the Ad-sig-E7/ecdCD40L vector can bind to the DCs and induce the level of CCR-7 mRNA in the DCs.

Figure 4B

B.1.b.iii. Enhanced DC Migration In Vivo Induced by E7/ecdCD40L Fusion Protein. In order to test if the subcutaneous injection of the Ad-sig-E7/ecdCD40L vector induces migration of the DCs to the regional lymph nodes in vivo, $1X10^6$ DCs were loaded with the CFDA SE dye and exposed in vitro to the Ad-sig-E7/ecdCD40L vector at MOI 200. Then, the dye loaded vector infected DCs were injected into the hind flank of the C57BL/6 mice. Three days after these injections, the mice were sacrificed and the regional axillary lymph nodes on the side of the injection were harvested and studied for the presence of the dye loaded DCs. As shown in left hand panel of Figure 5-Panel A (see below and to the left), CFDA SE stained DC's are detectably present in the regional lymph nodes following injection of the vector carrying the secretable E7/CD40L transcription unit, whereas none of the other vectors which did not encode a secretable E7/ecdCD40L protein was associated with detectable fluorescent labeled DCs in the regional lymph nodes-Figure 5-Panel B (see below in the right hand panel).

Figure 5 - Panel A

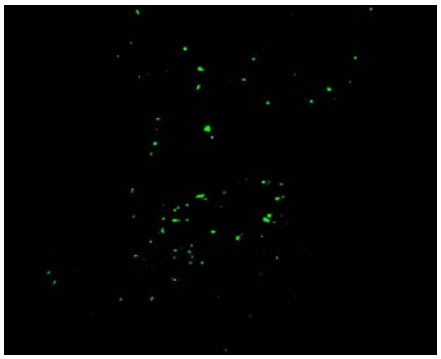
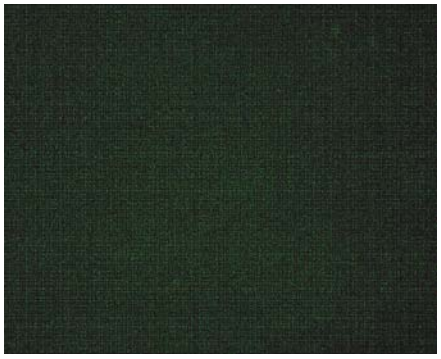


Figure 5 - Panel B



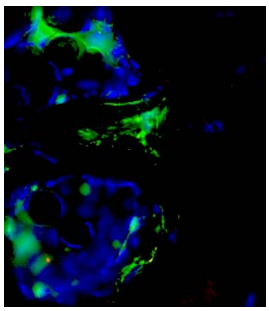
The experimental results summarized in Figures 2-5 show that the Ad-sig-E7/ecdCD40L vector infected cells at the vaccination injection site release a protein which binds to and activates the neighboring DCs so that they increase the expression of the chemokine CCR-7 and migrate to the regional lymph nodes. This data supports the hypothesis outlined above in Figure 1 about the mechanism of action of the Ad-sig-E7/ecdCD40L vector injections.

B.1.b.iv. Study of the Release of the E7/ecdCD40L Protein at the Injection Site of the Ad-sig-E7/ecdCD40L Vector. In order to test whether the secretable sig-E7/ecdCD40L protein is released for 10 days from infected cells, we sectioned the skin at the site of intradermal injection of the Ad-sig-E7/ecdCD40L vector, and double stained these sections with a flurosceinated antibody to the CD40L (which is stained green in Figure 6), and DAPI (which stains nuclear DNA blue in Figure 6). As shown below in Figure 6, a widespread distribution of blue and green double-staining was observed in the subcutaneous space near the injection site from mice vaccinated with the Ad-sig-E7/ecdCD40L vector, which carries the secretable E7/CD40L transcription unit (Panel A of Figure 6 below) at 5 days after the injection, and at 10 days after the injection of the Ad-sig-ecdCD40L vector (Panel B of Figure 6). In contrast, only a few green positive cells were observed in the epidermis following injection of vectors such as the Ad-E7/wtCD40L

(data not shown), and the Ad-wtCD40L (data not shown) vectors which do not release the TAA/CD40L protein from the vector infected cells.

Figure 6 Panel A

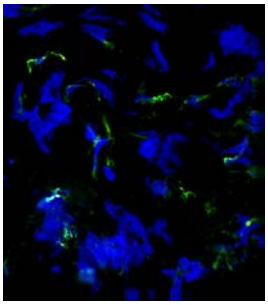
PANEL A



Skin section stained by Anti-CD154
5 days after subcutaneous injection
of Ad-sig-E7/ecdCD40L

Figure 6 Panel B

PANEL B



Skin section stained by Anti-CD154
10 days after subcutaneous injection
of Ad-sig-E7/ecdCD40L

These results show that the injection of the vector carrying the secretable form of E7/CD40L (Ad-sig-E7/ecdCD40L), or the vector which carries the secretable form of the CD40L (Ad-sig-ecdCD40L) can generate protein that binds to the CD40 receptor DCs. The Ad-sig-ecdCD40L vector can produce more of this protein than can the Ad-E7/wtCD40L vectors which encode a non-secretable form of the TAA/CD40L. The use of the vector carrying the sigE7/ecdCD40L transcription unit which encodes a secreted form of the protein is designed to amplify the effect of the vector from the infected cells to a much larger population of cells. The prediction is that the injection of the vector carrying the secretable form of the sigE7/ecdCD40L protein will generate much higher levels of activated DCs loaded with the E7 than injections of vector carrying the non-secretable form of this protein. As shown below, this proved to be the case.

B.2. Statement of Work for Specific Aim #2: Construction, Purification and Cell Based In Vitro Testing of the AdVLEDCD40LTAA vector in which the TAA is either a fragment of the extra cellular domain of the Her-2-Neu Receptor or a peptide from the tandem repeat domain of the MUC-1 protein.

a Construction of a shuttle vector in which a chimeric gene encoding the extra cellular domain of the CD40 ligand linked to either the extra cellular domain of the her-2-neu receptor or MUC-1 peptide is integrated near the site of the packaging signal in the AdV shuttle vector
b. Carry out homologous recombination in bacterial cells in the AdEasy system and isolate plasmid DNA that has the complete vector DNA
c. Transfect into 293 cells and amplify, plaque purify, then amplify again
d. Free/Thaw the cells, clarify the supernatant, purification of the Vector Particles by Cesium Chloride
e. Sequencing of the vector DNA
f. Western blot of proteins from infected cells for CD40L/TAA

Results for Specific Aim #2.

The Ad-sig-rH2N/ecdCD40L and Ad-sig-hMUC-1/ecdCD40L vectors were constructed and shown to express the relevant antigen (data not shown).

B.3. Statement of Work for Specific Aim #3: Compare the vectors made in Specific Aim #2 in conferring resistance in naïve immunocompetent mice to the breast cancer cell line carrying the her-2-neu receptor or the MUC-1 protein. Study the effect of vector injection of survival and disease free survival in mice injected with minimal numbers of breast cancer cells bearing the relevant TAA to simulate the use of the vector for microscopic disease in the adjuvant setting after surgery.

a. Test the effect of vaccination of each AdVCD40LTAA on resistance to engraftment of the relevant breast cancer cell line using the optimal schedule of administration determined above. Carry out functional tests of APC activation and CD8 cell sensitization in these mice.
b. If these experiments are successful, then evaluate the effect of the injection of each AdVLEDCD40LTAA vector at varying time points after injection of 200,000 tumor cells through the tail vein. The tine intervals will be every 5 days after the injection.
c. Repeat the experiment at different doses of the tumor cell line starting at 1 million, 5 million, 10 million, 50 million and 100 million vector particles.

Results for Specific Aim #3.

B.3.a. Vaccination with the Ad-sig-rH2N/ecdCD40L Vector Breaks Anergy to Rat Her-2-Neu (rH2N) Antigen in rH2N.Tg Mice. The first TAA studied in the Ad-sig-TAA/ecdCD40L vector vaccination was the rat Her-2-Neu receptor. The overexpression of the Her-2-Neu (H2N) growth factor receptor in 30% of breast cancers is associated with increased frequency of recurrence after surgery, and shortened survival (24). In order to test if the Ad-sig-TAA/ecdCD40L vaccination strategy could break tolerance for this self antigen, we vaccinated mice which were transgenic for the rat H2N (rH2N) gene (rH2N.Tg mice) and therefore tolerant of rH2N.

The rH2N.Tg mice carry a normal unactivated rH2N gene under the control of the MMTV promoter, which produces overexpression of a non-mutant rH2N receptor, just as is the case in human breast cancer. As shown below in Figure 7A, two sc injections of the Ad-sig-rH2N/ecdCD40L vector induced more complete suppression of the growth of the N202 rH2N positive mouse breast cancer cell line (solid squares in Figure 7A), than did the control ($p<0.05$) whereas one sc injection of the same vector (open triangles in Figure 7A) did not induce sufficient immune response to completely suppress the growth of the rH2N positive N202 cell line.

In Figure 7B, ELISA analysis shows that the level of the rH2N antibody level in the serum of mice vaccinated with two sc injections of the Ad-sig-rH2N/ecdCD40L vector are higher than unvaccinated rH2N.Tg mice. In Figure 7C, ELISPOT assays show that the administration of two sc injections of the Ad-sig-rH2N/ecdCD40L vector 7 days apart induced levels of rH2N specific T cells in the spleens of vaccinated mice which were 10 times higher than the levels of rH2N specific T cells induced in mice following injection of a control vector ($p<0.001$).

Figure 7A

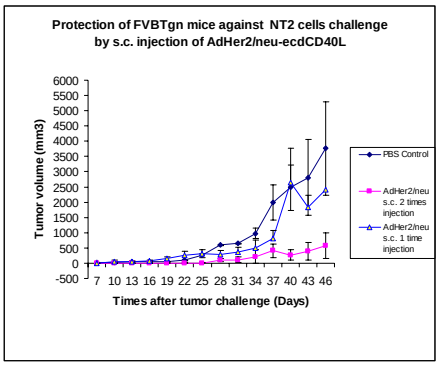


Figure 7B

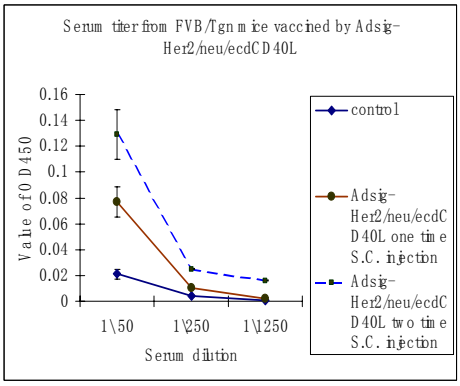
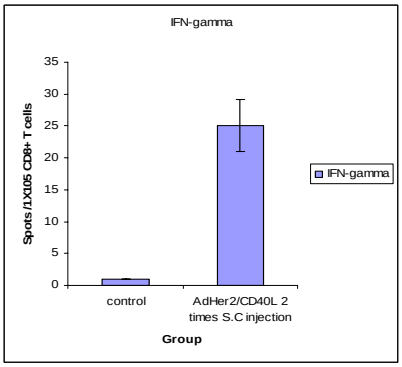


Figure 7C

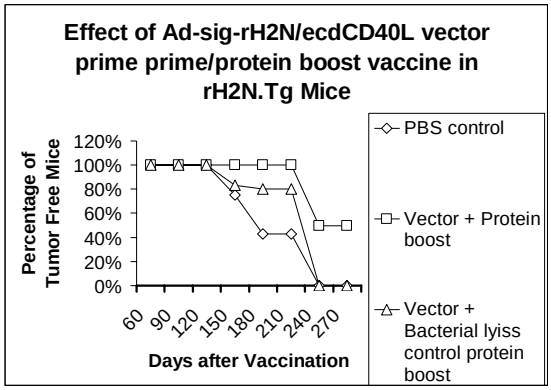


B.3.b. Vector Prime/Protein Boost Suppresses Onset of Spontaneous Breast Cancer in rH2N.Tg Mice.

rH2N.Tg mice were vaccinated starting at 6 weeks of life with the sc injection of the Ad-sig-rH2N/ecdCD40L vector vaccine (once) followed by 5 sc injections (10 micrograms each) of the rH2N/ecdCD40L protein booster (see open squares in Figure 7D). Control mice were injected with PBS instead of the vaccine (see triangles) or the Ad-sig-rH2N/ecdCD40L vector (one sc injection at 6 weeks of age) followed by lysate from the bacterial host strain used to produce the rH2N/ecdCD40L booster protein (open diamonds in Figure 7D). The Ad-sig-rH2N/ecdCD40L vector prime- rH2N/ecdCD40L protein boost prevents the development of

breast cancer for up to 280 days in 50% of the vaccinated mice, whereas all animals have developed breast cancer in the control groups by 245 days of life.

Figure 7D



B.3.c. Vaccination with the Ad-sig-ecdhMUC-1/ecdCD40L Vector Breaks Anergy to Human MUC-1 in hMUC-1.Tg Transgenic Mice. The second TAA chosen for this work was the human MUC-1 (hMUC-1) antigen in hMUC-1.Tg mice which had become tolerant to the hMUC-1 by expressing the gene for this antigen since birth. This antigen was chosen because it is relevant to the treatment of epithelial cancers like breast, lung, colon, ovary and prostate cancer. MUC-1 is also a self protein which is found only focally at low levels on the apical borders of non transformed secretory epithelia. The MUC-1 is a "self antigen" that has been shown to be diffusely over-expressed in carcinomas of the prostate, lung, colon, breast, ovary, and pancreas. Moreover, portions of the ecd of the MUC-1 glycoprotein have been shown to induce an antigen specific immune response when incorporated into vaccines in breast and prostate cancer patients. Mice transgenic for the human MUC-1 have been reported to be unresponsive to stimulation with human MUC-1 antigen. Overexpression of MUC-1 correlates with an aggressive clinical phenotype in epithelial cancer.

Figure 8A

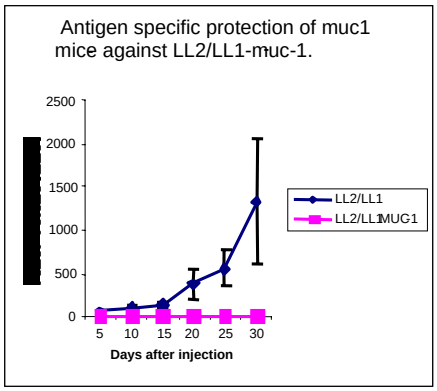
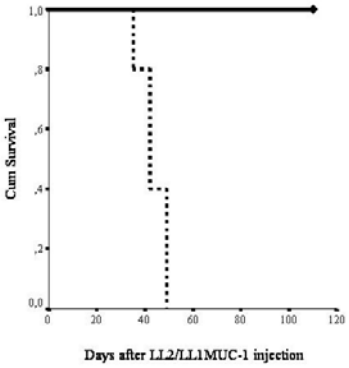


Figure 8B



In order to test if the Ad-sig-ecdhMUC-1/ecdCD40L vector could suppress the growth of the human MUC-1 positive LL2/LL1hMUC-1 mouse cancer cell line and if the immune suppression so generated was antigen specific, the growth of the LL2/LL1hMUC-1 antigen positive cell line in Ad-sig-ecdhMUC-1/ecdCD40L vaccinated hMUC-1.Tg mice was compared with the growth of the hMUC-1 negative LL1/LL2 cell line. As shown in Figure 8A, the growth of the LL2/LL1hMUC-1 cell line (solid squares) was suppressed more than the growth of the LL2/LL1 cell line which is hMUC-1 antigen negative (solid diamonds) ($p<0.01$). This shows that the Ad-sig-ecdhMUC-1/ecdCD40L induces an anti-tumor immune suppressive immune response which is antigen specific. As shown in Figure 8B, the survival of mice injected with the Ad-sig-ecdhMUC-1/ecdCD40L vector is 100% at 120 days after LL2/LL1hMUC-1 tumor cell injections (solid bold line) whereas the survival of uninjected mice is 0% after 50 days (bold broken line).

B.3.d. Testing of the Effect of Subcutaneous Injections of the hMUC-1/ecdCD40L Protein on the Induction of Antigen Specific T Cells by Subcutaneous Injection of the Ad-sig-hMUC-1/ecdCD40L Vector. Use of prime boost vaccination schemes has been shown to expand the magnitude of an initial vector vaccine injection. We next studied the effect of administering subcutaneous injections of the hMUC-1/ecdCD40L protein in hMUC-1.Tg mice following an initial injection of the Ad-sig-hMUC-1/ecdCD40L vector. The plan for this experiment is outlined in Table I below. The hMUC-1/ecdCD40L protein (10 micrograms) was injected sc 7 days after two weekly vector injections (T1), 2 weeks after two weekly vector injections (T2), one week after one vector injection (T3), and two weeks after one vector injection (T4). In T5, two sc protein injections (administered two weeks apart) were given 7 days after a single vector injection. In the "Control", two vector injections were given without protein.

Table I

Testing Group	Week 1	Week 2	Week 3	Week 4
Control	**Vector**	**Vector**	Nothing	Nothing
Treatment 1 (T1)	**Vector**	**Vector**	**Protein**	Nothing
Treatment 2 (T2)	**Vector**	**Vector**	Nothing	**Protein**
Treatment 3 (T3)	**Vector**	**Protein**	Nothing	Nothing
Treatment 4 (T4)	**Vector**	Nothing	**Protein**	Nothing
Treatment 5 (T5)	**Vector**	**Protein**	Nothing	**Protein**
Negative Control	Nothing	Nothing	Nothing	Nothing

As shown in Figure 9A (see below), the increases in the levels of the hMUC-1 specific T cells by the ELISPOT assay for interferon gamma positivity in the spleen cells from the T5 group (two sc protein injections at a 14 day interval which began one week after the initial vector injection) was greater than those induced by no treatment or by one or two vector injections alone ($p<0.01$). The next highest elevation of the frequency of interferon gamma positive T cells was with the T2 group (one protein injection following two vector injections).

We then tested if the subcutaneous injections of the hMUC-1/ecdCD40L protein given before any Ad-sig-hMUC-1/ecdCD40L vector injections could suppress the growth of hMUC-1 positive LL2/LL1hMUC-1 positive syngeneic cancer cells in the hMUC-1.Tg mice which were anergic to the hMUC-1. For this experiment, three combinations of Ad-sig-ecdhMUC-1/ecdCD40L vector and ecdhMUC-1/ecdCD40L protein were administered sc to hMUC-1.Tg mice before sc challenge with LL2/LL1hMUC-1 tumor cells: VVV=three Ad-sig-ecdhMUC-1/ecdCD40L vector subcutaneous injections administered on days 1, 7 and 21; PPP=three ecdhMUC-1/ecdCD40L protein subcutaneous injections administered on days 1, 7 and 21; or VPP=a single Ad-sig-ecdhMUC-1/ecdCD40L vector subcutaneous injection followed at days 7 and 21 by ecdhMUC-1/ecdCD40L protein injections. As shown below in Figure 9B, both the VVV and the VVP induce an immune response that was sufficient to suppress the growth of the hMUC-1 positive mouse cancer cells. In contrast, the administration of the hMUC-1/ecdCD40L (PPP) was insufficient to break anergy in hMUC-1.Tg mice.

Figure 9A

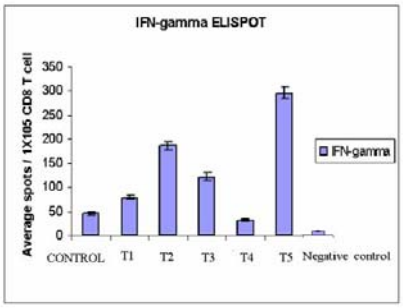


Figure 9B

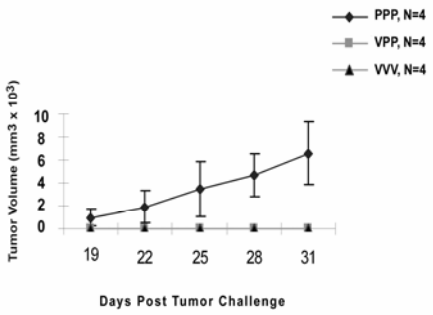
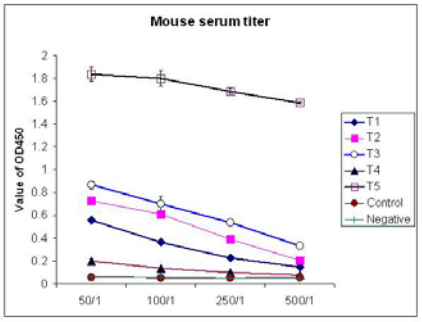


Figure 9C



B.3.e. Levels of hMUC-1 Specific Antibodies in the Serum of Mice Vaccinated with Ad-sig-hMUC-1/ecdCD40L Vector Prime/hMUC-1/ecdCD40L Boost Vaccination. Microwells were coated with the ecdhMUC-1/ecdCD40L protein. Following addition of test mouse serum, a secondary rat anti-mouse antibody conjugated to HRP was added. As shown above in Figure 9C, a dramatic increase in the level of antibodies to the ecdhMUC-1/ecdCD40L fusion protein was generated by the treatment with one vector injection and two protein injections (VPP) spaced at a 14 day interval. The increase in the anti hMUC-1/ecdCD40L antibodies following the T5 treatment was greater than any of the other treatment groups ($p<0.01$). We then tested if the hMUC-1 antibodies induced in the VPP vaccinated mice bind to human breast cancer cells from biopsies. The sections were exposed to the FITC conjugated serum from the Ad-sig-ecdhMUC-1/ecdmCD40L vector and ecdhMUC-1/ecdmCD40L protein vaccinated mice (VPP).

As shown at right in Figure 9D, Panel I, the FITC conjugated serum from the vaccinated mice bound to the breast epithelial cells in the biopsy specimens. There was no binding to the intervening fibroblast or stromal cells in the biopsy specimens (see Panel I of Figure 9D). As shown in Panel II of Figure 9D, the 40 amino acid hMUC-1 peptide used in the Ad-sig-hMUC-1/ecdCD40L vaccine blocked binding of the mouse IgG from the vaccinated mice to the breast cancer epithelial cells. A peptide with the identical amino acid composition but a scrambled amino acid sequence did not block the antibody binding (data not shown). Finally, serum from unvaccinated mice did not bind to human breast cancer cells (see Panel III of Figure 9D).

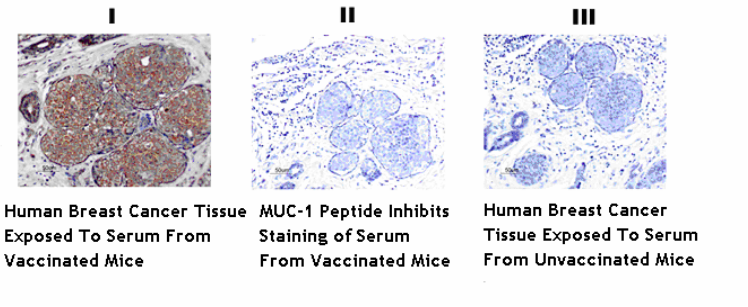


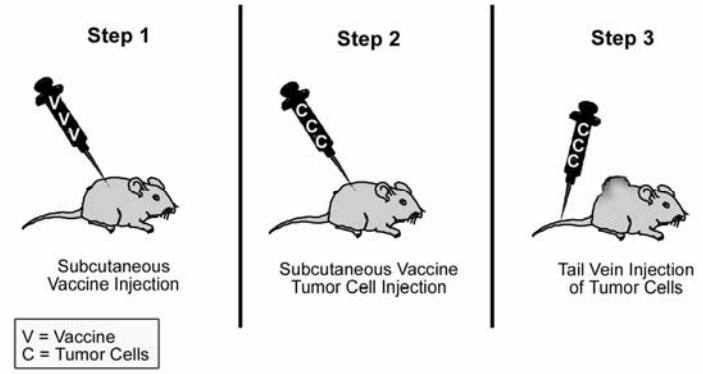
Figure 9D

B.4. Statement of Work for Specific Aim #4: Testing of the effect of each vector on survival and disease free survival in AdVLEDCD40LTAA treated and untreated mice in which there is substantial amounts of pre-existing tumor nodules from the breast cancer cell line which carries the relevant breast cancer TAA (her-2-neu or MUC-1) prior to the vaccination.

a. Inject the mouse breast cancer cell line subcutaneously which carries either TAA at varying doses (0.025, 0.05, 0.075, 0.1, 0.15 and 0.2 million cells).
b. Allow the cells to grow for 5 days and then inject the therapeutic vector for each TAA (with and without the CD40Ligand, with and without the signal sequence and the transmembrane domain)
c. Measure the disease free survival and the survival of the mice for each TAA vector and for mice treated with the control vectors described in the previous step.

Results for Specific Aim #4:

Figure 10A



B.4.a.i. Effect of VPP, VVV, and PPP Vaccination in hMUC-1.Tg Mice On Preventing Engraftment of Subcutaneously Injected hMUC-1 Positive Tumor Nodules and on Pulmonary Metastases From Intravenously Injected LL2/LL1hMUC-1 Cancer Cells ("Prevention Experiment"). As shown in Figure 10A, we injected the vaccine first followed by one sc injection of the LL2/LL1hMUC-1 cell line (which generates a single sc tumor nodule) and then we injected this cell line intravenously which results in multiple lung tumor nodules. We then compared the effect of three sc injections of the Ad-sig-hMUC-1/ecdCD40L vector(VVV), or three sc injections of the hMUC-1/ecdCD40L protein (PPP), or one sc injection of the Ad-sig-hMUC-1/ecdCD40L vector followed by sc injections of the hMUC-1/ecdCD40L protein (VPP) on the ability to establish hMUC-1 positive LL2/LL1 cancer cells by sc injection and intravenous injection following the vaccination ("Prevention" Experiment). As shown above in Figure 9B, three sc injections of the ecdhMUC-1/ecdCD40L protein (PPP) does not completely suppress the growth of the LL2/LL1hMUC-1 tumor cell whereas the VVV or VVP vaccine completely suppresses the growth of the cancer cell line.

B.4.a.ii. Effect of VPP, VVV and PPP Vaccination in hMUC-1.Tg Mice on the Growth of Pre-Existing Subcutaneous Tumor Nodules and Pulmonary Metastases ("Treatment Experiment").

As shown in Figure 10B, we first injected sc the LL2/LL1hMUC-1 cell line, then carried out the test vaccine and last carried out the intravenous injection of the LL2/LL1hMUC-1 cell line. We compared the effect of various schedules of Ad-sig-hMUC-1/ecdCD40L vector and hMUC-1/ecdCD40L protein in hMUC-1.Tg mice with established subcutaneous nodules of hMUC-1 positive LL2/LL1hMUC-1 cancer cells.

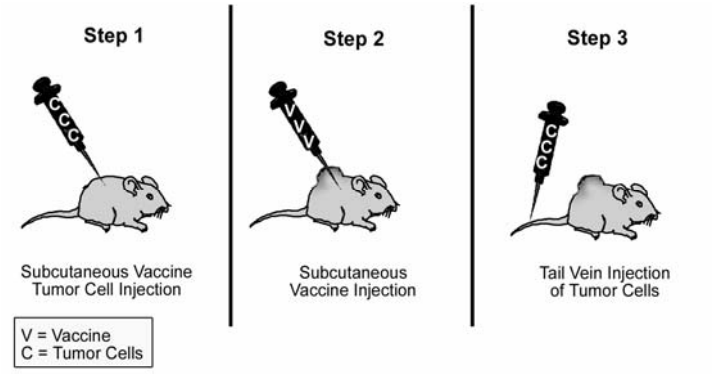
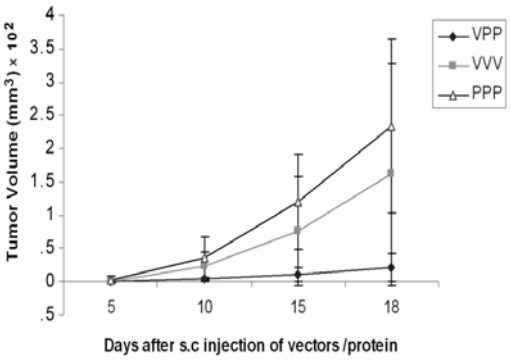


Figure 10B

Figure 10C



As shown in Figure 10C, three sequential sc injections of the hMUC-1/ecdCD40L protein (PPP), without antecedent vector injection, failed to completely suppress the growth of the pre-established sc deposits of the hMUC-1 positive tumor cell line in the hMUC-1.Tg mice. In contrast (see Figure 10C), VPP suppressed the growth of the tumor cells more than PPP ($p<0.02$). VVV was less effective than VPP in suppressing pre-established LL2/LL1hMUC-1 tumor cells.

B.4.b. VPP Vaccination Suppresses Metastatic Disease in the Lung-Engraftment of Intravenously Administered MUC-1 Positive mLL2/LL1hMUC-1 Cancer Cells in the Lungs of hMUC-1.Tg Mice.

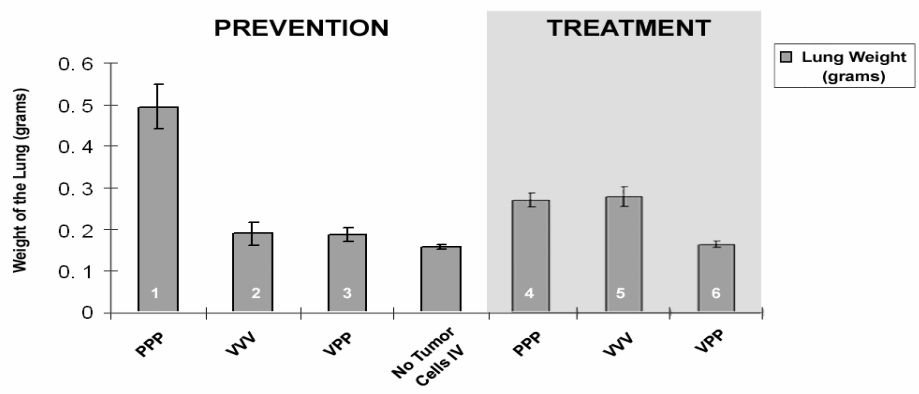
To mimic tumor metastases, we challenged mice by tail vein injection of hMUC-1 positive LL2/LL1hMUC-1 tumor cells following completion of the VPP vaccinations. We injected the LL2/LL1hMUC-1 cells line intravenously following two schedules: “Treatment” and “Prevention”.

B.4.b.i. Prevention. The VPP vaccination was carried out and then the LL2/LL1hMUC-1 was injected sc and then 20 days later, the same cell line injected intravenously (see Figure 10A)**.**

B.4.b.ii. The LL2/LL1hMUC-1 cell line was injected subcutaneously (see Figure 10B), the VPP vaccination was administered, and then 20 days later, the LL2/LL1hMUC-1 cell line was injected intravenously. LL2/LL1hMUC-1 cells engraft in the lung when injected intravenously. Sixty-three days following the initiation of vaccination and intravenous injection of the LL2/LL1hMUC-1 cell line, we sacrificed the mice and then weighed the lungs.

B.4.b.iii. Results-Prevention: As shown below in the left hand side of Figure 10D (which is designed as the “Prevention Experiment” outlined in Figure 10A), in which the mice were vaccinated before sc injection of the hMUC-1 positive LL2/LL1hMUC-1 cancer cells and intravenous administration of the LL2/LL1hMUC-1 tumor cells, the PPP vaccine suppressed the growth of the hMUC-1 positive LL2/LL1hMUC-1 cancer cells in the lungs of the hMUC-1.Tg mice less well than did the VVV or VPP vaccine ($p=0.03$). VVV and VPP almost completely suppressed the engraftment of the lung cancer in the lungs of the vaccinated animals (the lung weights of these animals were almost the same as mice in which no LL2/LL1hMUC-1 tumor cells were injected intravenously-see Figure 10D below).

Figure 10D



B.4.b.iv. Results-Treatment. Next, we measured the lungs of the animals which were vaccinated according to the "Treatment Experiment" plan outlined in Figure 10B in which the sc injection of the LL2/LL1hMUC-1 occurs first followed by sc vaccination and then intravenous injection of the LL2/LL2hMUC-1 cells. In this model, the sc tumor nodules are already growing by the time of the vaccination. Because the subcutaneous tumor nodules were growing to an advanced level after 2 weeks, some of the animals were sacrificed at 2 weeks earlier than in the prevention experiment. As shown above in the right hand side ("Treatment Experiment") of Figure 10D, all three methods of vaccination (VPP, VVV, and PPP) suppressed the growth of the hMUC-1 positive LL2/LL1hMUC-1 tumor cells. Furthermore, these data show that the administration of 3 sc injections of the Ad-sig-hMUC-1/ecdCD40L vector (VVV) generates less of an immune response than did VPP (p=0.03). These results are interesting in that they suggest that an important part of the in vivo suppression of the cancer cells is the induction of increased levels of both the hMUC-1 specific T cells as well as hMUC-1 specific antibodies in the hMUC-1.Tg mice.

C. Key Research Accomplishments

- The Ad-sig-H2N/ecdCD40L and Ad-sig-hMUC-1/ecdCD40L vectors have been shown to break tolerance.
- The TAA/ecdCD40L protein boost has been shown to increase the magnitude of the immune response when injected 7 and 21 days after a single vector injection. This is called VPP.
- VPP has been shown to induce a robust immune response and suppress the growth of tumors in old and young mice.

D. Reportable Outcomes

D.1. Papers

1. Akbulut H, Tang YC, Akbulut KG, Maynard J, Zhang L, and Deisseroth A. Anti-tumor immune response induced by i.t. injection of vector activated dendritic cells and chemotherapy suppresses metastatic breast cancer. Molecular Cancer Therapeutics 5:1975-1985, 2006.
2. Tang YC, Maynard J, Akbulut H, Fang XM, Zhang WW, Xia XQ, Koziol J, Linton P-J, and Deisseroth A. Vaccine overcomes defects acquired during aging and cancer. J. Immunology, 177:5697-5707, 2006.
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D.2. Patent Submissions

Mucin antigen vaccine (10/997,055 and PCT/US2004/39812) November 23, 2004
Methods for generating immunity to antigen (11/009,533 and PCT/US2004/41690) December 10, 2004
CD40 ligand fusion protein vaccine (serial numbers have not been assigned as yet) November 6, 2006

E. Conclusions

We have demonstrated that the Ad-sig-TAA/ecdCD40L vector prime-protein boost vaccine strategy is:

1. Potent
2. Can break anergy in to breast cancer related self antigen transgenic mouse system
3. The immunoprotection lasts for greater than 1 year
4. The immune response is robust in old as well as young test mice
5. The immune response can be induced against viral antigens (E7), breast cancer self antigens (Her-2-Neu and MUC-1) as well as the antigens specific for other cancers (eg melanoma TRP-2).

This suggests that the vaccine is a good candidate for clinical testing. We are proceeding to phase I testing in recurrent breast cancer.

F. References:

1 Zhang L, Tang, Y, Linton PJ, and Deisseroth A. Injection of Ad vector encoding secretable form of TAA/CD40L fusion protein induces T cell dependent immune response for against tumor cells. PNAS 100: 15101-15106, 2003.

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3. Tang YC, Maynard J, Akbulut H, Fang XM, Zhang WW, Xia XQ, Koziol J, Linton P-J, and Deisseroth A. Vaccine overcomes defects acquired during aging and cancer. J. Immunology, 177:5697-5707, 2006.

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G. Appendices

G.1. Tang, et al., Vector Prime/Protein Boost Vaccine that Overcomes Defects Acquired during Aging and Cancer. J. Immunology 177:5697-5707, 2006.
G.2. Akbulut, et al, Antitumor immune response induced by i.t. injection of vector-activated dendritic cells and chemotherapy suppresses metastatic breast cancer. Molecular Cancer Therapetuics 5:1975-1985, 2006.
G.3. Tang, et al., Multistep process through which adenoviral vector vaccine overcomes anergy to tumor-associated antigens. Blood 104:1704-2713, 2004.
G.4. Zhang, et al. An adenoviral vector cancer vaccine that delivers a tumor –associated antigen / CD40 ligand fusion protein to dendritic cells. PNAS 100:15101-15106, 2003.

Vector Prime/Protein Boost Vaccine That Overcomes Defects Acquired during Aging and Cancer[1]

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We showed that the Ad-sig-TAA/ecdCD40L vaccine induces a tumor suppressive immune response to the hMUC-1 and rH2N tumor-associated self Ags (TAA) and to the Annexin A1 tumor vascular Ag, even in mice in which anergy exists to these Ags. When the TAA/ecdCD40L protein is given s.c. as a boost following the Ad-sig-TAA/ecdCD40L vector, the levels of the TAA-specific CD8 T cells and Abs increase dramatically over that seen with vector alone, in young (2-mo-old) as well as old (18-mo-old) mice. The Abs induced against hMUC-1 react with human breast cancer. This vaccine also induces a 4-fold decrement of negative regulatory CD4CD25FOXP3-T cells in the tumor tissue of 18-mo-old mice. These results suggest that the Ad-sig-TAA/ecdCD40L vector prime-TAA/ecdCD40L protein boost vaccine platform may be valuable in reducing postsurgery recurrence in a variety of epithelial neoplasms. ***The Journal of Immunology,*** **2006, 177: 5697–5707.**

The cellular immune response is tolerant of many forms of cancer. This is in part because cancer cells are covered by self Ags that have been present on normal cells from birth. Ag-specific Abs and T cells have difficulty in penetrating the extravascular tumor tissue. In addition, defects are acquired during aging that diminish the immune response to vaccines. One such defect involves the levels of expression of the CD40L on activated CD4 helper cells in older individuals (1, 25). We have designed an Ad-sig-TAA/ecdCD40L adenoviral vector vaccine for the in vivo activation and tumor-associated Ag (TAA)[4] loading of dendritic cells (DCs). Subcutaneous injection of the Ad-sig-TAA/ecdCD40L adenoviral vector (2, 3) results in the secretion for 10 days of a fusion protein composed of a TAA fragment fused to the extracellular domain (ecd) of the CD40L. CD40L is a homo-trimeric protein and is normally found on B cells and helper $CD4^+$ T cell lymphocytes (4, 5). All of the sequences necessary to stabilize this trimeric structure of the protein are contained within the ecd of the CD40L protein (6). The binding of the TAA/ecdCD40L protein to DCs induces migration of these DCs to the regional lymph nodes (2). These DCs carry fragments of TAA bound to surface MHC class I molecules (2).

We tested whether the s.c. injection of the Ad-sig-TAA/ecdCD40L vector can induce a cellular and humoral immune response against two tumor-associated self Ags: the MUC-1 and the Her-2-Neu, the overexpression of which is known to be associated with bad prognosis in human breast cancer (7, 8). The MUC-1 Ag (9) is a structural protein that is expressed at very low levels on the apical surface of normal epithelial cells. The overexpression of the MUC-1 protein in carcinomas of the breast, lung, prostate, ovary, cervix, endometrium, esophagus, stomach, and colon (9) is associated with resistance to therapy and metastases. The Her-2-Neu receptor is a member of the epidermal growth factor family of growth factor receptors. We show that the s.c. injection of the Ad-sig-hMUC-1/ecdCD40L or Ad-sig-rH2N/ecdCD40L vector can induce a hMUC-1- or rH2N-specific immune response that suppresses the growth of hMUC-1- or rH2N-positive cancer cells in hMUC-1.Tg or rH2N.Tg transgenic mice, which are anergic to the hMUC-1 or rH2N Ags (10, 11). Our studies also showed that the s.c. injection of the hMUC-1/ecdCD40L protein at 7 and 21 days after the s.c. injection of the Ad-sig-hMUC-1/ecdCD40L vector increased the levels of the hMUC-1-specific CD8 effector cells and Abs. The hMUC-1-specific Abs were shown to bind to human breast and prostate cancer cells. We also showed that the Ad-sig-TAA/ecdCD40L vector strategy could induce an immune response to the Annexin A1 Ag, which is detected on the luminal membrane of the tumor vascular endothelial cells but not on the luminal surface of vessels in normal tissues (12), and that this suppresses the growth of established cancer cell lines that are negative for the Annexin A1 Ag. These data suggest that the Ad-sig-TAA/ecdCD40L vaccine may be of use for suppression of recurrence of epithelial cancers after surgery and/or radiation therapy.

Finally, we tested the effect of the Ad-sig-TAA/ecdCD40L vector prime/TAA/ecdCD40L protein boost vaccine in 18-mo-old mice and compared the response to that seen in 2-mo-old mice. These studies showed that the VPP vector prime/protein boost schedule dramatically increased the levels of Ag-specific CD8 effector cells in the tumor tissue of 18-mo-old mice. In addition, this vaccine induced a decrease in the level of negative regulatory CD4CD15FOXP3-T cells in the tumor tissue of the 18-mo-old mice. Importantly, the TAA/ecdCD40L protein boost induced complete responses in mice with existing progressive tumor in the 18-mo-old mice.

Materials and Methods

Cell lines

The rH2N-positive NT2 mammary tumor cell line was obtained from DuPont. The LL2/LL1hMUC-1 cell line, which was derived from LL2/LL1

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Received for publication March 27, 2006. Accepted for publication July 25, 2006.

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[1] This work was supported by the Breast Cancer Research Foundation, the Department of Defense Breast Cancer Research Program (DAMD 17-99-9457, DAMD 18-99-9477, and BC022063), and National Institutes of Health Grant SBIR 2003-2. Support was also received from the Kimmel Foundation, the Anthony Dewitt Frost Foundation, and the George and the Barbara Bush Leukemia Research Fund.

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[4] Abbreviations used in this paper: TAA, tumor-associated Ag; DC, dendritic cell; ecd, extracellular domain; AnxA1, Annexin A1; MMC, mitomycin C.

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0022-1767/06/$02.00

(American Type Culture Collection catalog no. CRL-1642), was genetically modified to express hMUC-1 by transfection with the plasmid pcDNA3-hMUC-1 and selected by growth in medium supplemented with 1 mg/ml G418.

Construction of TAA/ecdCD40L plasmids and vectors

The Ad-sig-ecdhMUC-1/ecdCD40L plasmid expression vector was constructed as described previously (2, 3). K/ratHer2/Neu with the upstream κ signal sequence was generated by four rounds of PCR amplification (first round: primers 4 + 5; second round: primer 3 + 5; third round: primer 2 + 5; fourth round: primer 1 + 5). The signal peptide encoding the mouse IgG κ chain METDTLLLWVLLLWVPG was added before Her2/Neu cDNA by PCR amplification, which encodes the mouse IgG κ chain signal sequence METDTLLLWVLLLWVPGSTGD. The primers are as follows: 1) the forward primer 1 is 5′-CCACC ATG GAG ACA GAC ACA CTC CTG CTA TGG GTA CTG CTG-3′; 2) the forward primer 2 is 5′-TC CTG CTA TGG GTA CTG CTG CTC TGG GTT CCA GGT TC-3′; 3) the forward primer 3 is 5′-TG CTC TGG GTT CCA GGT TCC ACT GGT GAC GAA CTC-3′; 4) the forward primer for the rH2N extracellular domain 4 is 5′-TCC ACT GGT GAC CCA GAC AGT CTC CGT GAC CTC-3′; and 5) the reverse primer for the rH2N extracellular domain 5 is 5′-GGAG CTC GAG GAC CAC CAC TAA GAT CAG GAA CAG-3′.

The K/rH2N encoding DNA was cloned into the pcDNA 3.1 TOPO vector (Invitrogen Life Technologies) forming pcDNA-K/.rH2N. The ecd of the mouse CD40L was amplified from the template of pshuttle-hMUC1/ecdCD40L, which was inserted into the plasmid pcDNA-K/rH2N after restriction endonuclease digestion with *Xba*I and *Not*I. The primers for CD40L are as follows: 5′-GGAAGATCTCCCAAGCTTCCTCCAGTC CACAATGTCACCCTC-3′ and 5′-TTGCGGCCGCTCAGAGTTTGAG TAAGCCAAAAGATGAG-3′. The K/rH2N/ecdCD40L encoding DNA was cut from the pCDNA3TOPO vector using *Hin*dIII-*Not*I restriction endonuclease digestion and inserted into the pShuttle-CMV downstream of the CMV promoter. The recombinant adenoviral vectors were generated using the AdEasy vector system (13). Briefly, the resulting plasmid pShuttle-CMV K/rH2N/ecdCD40L was linearized by PME I digestion and then cotransformed into *Escherichia coli* strain BJ5183 together with pAdEasy-1 (13).

Assembly of the Ad-sig-AnxA1/ecdCD40L vector

The plasmid pShuttle-CMV K/rH2N/ecdCD40L generated in the synthesis of the Ad-sig-rH2N/ecdCD40L was linearized using PME I digestion. The Ad-sig-Anx1A/ecdCD40L vector was constructed as described above for the Ad sig-rH2N/ecdCD40L vector, except that the pair 4, 5 was changed to the following primer pair sequence for Annexin A1: 5′-TCCACTGGT GACCCAGCTCAGTTTGATGCGATG-3′, and 5′-GGAGCTCGAGCTT CTCGGCAAAGAAAGCTGGAGTG-3′.

Production of hMUC-1/ecdCD40L protein

The hMUC1/ecdCD40L cDNA was amplified from the template pshuttle hMUC-1/ecdCD40L with the primers 5′-GGAAGATCTTCCCCACCAT GGAGACAGACACACTCC-3′ and 5′-TTGCGGCCGCTCAGAGTTTG AGTAAGCCAAAAGATGAG-3′. The product was inserted into the pTriEx-2 hygro Vectors (Novagen) following *Bgl*II and *Not*I digestion. Following incubation in isopropyl β-D-thiogalactoside-supplemented medium for 4 h, the cell lysate was prepared by the CellyticB Plus kit (Sigma-Aldrich). The hMUC-1/ecdCD40L protein was purified from the soluble fraction by HIS-select Nickel Affinity Gel (Sigma-Aldrich). Then, the protein was concentrated and desalted by centrifugation through an Ultrafree-15 Biomax-50 filter (Millipore) and eluted with PBS.

ELISPOT assays for IFN-γ-positive Ag-specific T cells following Ad-sig-TAA/ecdCD40L vector vaccination

The presence of Ag-specific effector T cells in the immunized mice was assessed by ELISPOT assays, as previously described (2, 3).

Study of effect of Ad-sig-TAA/ecdCD40L vector prime and TAA/ecdCD40L protein boost in TAA transgenic mice

Mice (four per group) that were transgenic for the rH2N or hMUC-1 genes were vaccinated via s.c. injection with 1×10^8 PFU of the Ad-sig-rH2N/ecdCD40L vector. One week later, mice were boosted with the same adenoviral vector injection or with an s.c. injection of the TAA/ecdCD40L protein at 7 and 21 days after the vector vaccination. One week after the last vaccination, TAA.Tg mice were challenged by s.c. injection of 5×10^5 TAA-positive cancer cells/mouse. The volumes of tumor nodules were measured by caliper. The tumor volume was calculated as follows: tumor volume = length × (width^2)/2, assuming an ellipse. Two types of experiments were conducted: 1) the "prevention experiment," in which the vaccination precedes the s.c. injection of the target TAA-positive tumor cell line, and 2) the "therapy experiment," in which the vaccination is delivered s.c. following the s.c. injection of the TAA-positive tumor cell line.

Study of Ab levels before and after vaccination

Blood was collected from test mice before and 1 wk after the last Ad-sig-TAA/ecdCD40L vaccination. Serum samples were titrated for the presence of TAA-specific Ab by ELISA as reported previously (2, 3).

Study of the changes of the patterns of gene expression in tumor-infiltrating effector T cells following Ad-sig-TAA/ecdCD40L vaccination

Tumor tissue was harvested 7 days following vaccination, minced, treated with collagenase, and strained through gauze to develop a suspension of single cells. CD8 effector T cells were purified from this population using the FACSAria preparative cell sorter. The cells were then enriched for the following phenotypes using fluorescent-conjugated Abs that recognize the following immunophenotype: $CD8^{high}$, $CD44^{high}$ and $LY6C^{high}$, and $CD62L^{low}$. RNA was purified from these cells, and cDNA libraries were made. We then conducted an analysis of the expression of genes that exhibited increases of >5-fold or more following vaccination by methods described in the Affymetrix manual. Both supervised pathway analysis and unsupervised cluster analysis were conducted.

Statistical analysis

Unless otherwise noted, data comparing differences between two groups were assessed using unpaired Student's *t* test. Differences were considered significant when $p < 0.05$. Data are presented as mean ± SE.

Results

Subcutaneous injection of the Ad-sig-hMUC-1/ecdCD40L vector vaccine confers resistance to subsequent engraftment of hMUC-1-positive cancer cells (prevention experiment)

The MUC-1 protein consists of two subunits. Subunit I consists of a large extracellular protein, which carries a large but variable (up to 90) number of 20-aa highly glycosylated repeat domains (9). Subunit II has a transmembrane domain with a 65-aa cytoplasmic domain, and a 69-aa extracellular domain. Subunits I and II bind to each other through noncovalent interactions. We used the LL2/LL1hMUC-1 mouse cancer cell line, which had been transfected with hMUC-1 as a target of the vaccine in the hMUC-1.Tg mice. These mice had been shown by Gendler and colleagues (10) to be anergic to the hMUC-1 Ag. In these experiments, we administered the vaccine before s.c. injection of the hMUC-1-positive LL2/LL1hMUC-1 tumor cell line. This is called the "prevention experiment." We conducted two s.c. injections at 7-day intervals of the Ad-sig-hMUC-1/ecdCD40L vector into hMUC-1.Tg mice (see Fig. 1, *A*, ■, and *B*, ■). This vector encodes two 20-aa tandem repeats from an epitope of subunit I linked to CD40L (see Fig. 1*A*) or an epitope of subunit II of the MUC-1 Ag linked to the CD40L (see 1*B*). As shown in Fig. 1, the vector vaccine suppresses the in vivo growth of hMUC-1-positive cancer cells more than do the control injections ($p < 0.01$).

Boosting the immune response by s.c. injection of the hMUC-1/ecdCD40L protein before s.c. injection of cancer cells (prevention experiment)

Clinical trials have shown that the s.c. injection of a vector as a prime and a second vector as a boost expands the magnitude of the Ag-specific immune response (14, 15). We compared the in vivo growth of hMUC-1-positive cancer cells 7 days following three s.c. injections of the Ad-sig-hMUC-1/ecdCD40L vector (VVV), or three s.c. injections of the hMUC-1/ecdCD40L protein (PPP), or when the s.c. injection of the Ad-sig-hMUC-1/ecdCD40L vector was followed in 7 and 21 days by s.c. injections of a TAA/ecdCD40L protein boost (VPP) in hMUC-1.Tg mice (four mice per group). As

shown in Fig. 1*C*, three s.c. injections of the ecdhMUC-1/ecdCD40L protein (PPP) without antecedent injection of the Ad-sig-hMUC-1/ecdCD40L vector do not completely suppress the growth of the LL2/LL1hMUC-1 tumor cell line. In contrast, the administration of three s.c. injections of the Ad-sig-hMUC-1/ecdCD40L vector (VVV) or the administration of one s.c. Ad-sig-hMUC-1/ecdCD40L vector injection followed by two hMUC-1/ecdCD40L s.c. protein boost injections (VPP) completely suppress the growth of the hMUC-1-positive cancer cell line in hMUC-1.Tg mice.

We next studied the effect of various schedules of the Ad-sig-hMUC-1/ecdCD40L vector and the hMUC-1/ecdCD40L protein boost (subunit I), as outlined in Table I. We measured the effect of the vector prime/protein boost vaccine on the levels of the hMUC-1-specific splenic T cells in the vaccinated animals (four mice per group). As shown in Fig. 1*D*, the levels of Ag-specific CD8 cells in the spleen following two vector prime injections followed by one protein boost (T2) mice were significantly different from the control group (two vector injections) at the $p = 0.001$ level). The level of hMUC-1-specific T cells was highest following a single Ad-sig-hMUC-1/ecdCD40L vector injection followed by two hMUC-1/ecdCD40L protein injections (VPP), which is group T5 in Fig. 1*D*. This was six times as high as the level of Ag-specific T cells following two vector injections, designated as control in Fig. 1*D* ($p = 0.00003$). Since it is known that hyperglycosylation of MUC-1 reduces the immune response to MUC-1, the unglycosylated form of the protein used for the booster injections may have induced such high levels of Ag-specific T cells. We will refer to this schedule of vaccination as VPP.

VPP induces anti-hMUC-1 Abs in hMUC-1.Tg mice, which bind to human breast cancer cells

As shown in Fig. 1*E*, the VPP regimen (T5) induced levels of hMUC-1-specific Ab, which were greater than any of the other combinations of vector and protein ($p < 0.01$). We then tested whether the hMUC-1 Abs induced in the hMUC-1.Tg mice by the Ad-sig-hMUC-1/ecdCD40L vector prime and hMUC-1/ecdCD40L protein boost (subunit I) VPP vaccination would bind to human breast cancer epithelial cells. The Abs from the Ad-sig-hMUC-1/ecdCD40L vaccinated mice bound to 54 of the 100 of the breast cancer specimens tested (see Fig. 1*F*, I). In addition, exposure of the mouse serum to the specific hMUC-1 20-aa repeat peptide encoded by the vector or protein transcription units blocked completely the binding of the mouse Abs to the breast cancer cells (see Fig. 1*F*, II). Serum from unvaccinated mice did not bind to the human breast cancer cells (see Fig. 1*F*, III). The amino acid sequence of the hMUC-1 peptide was then scrambled so that the order of the amino acids was randomized but the composition of amino acids remained the same. This peptide did not block the binding of the serum from the vaccinated hMUC-1.Tg mice (data not shown).

VPP and VVV induce regression of existing tumor nodules (therapy experiment)

We compared the effect of various schedules VVV, VPP, and PPP vaccines of Ad-sig-hMUC-1/ecdCD40L vector and hMUC-1/ecdCD40L protein (subunit I) in hMUC-1.Tg mice (four mice per treatment group) with established s.c. nodules of hMUC-1-positive LL2/LL1hMUC-1 cancer cells. These vaccinations were conducted within 3 days after the injection of the tumor cells. These s.c. nodules were established by injecting 500,000 LL2/LL1hMUC-1 tumor cells under the skin. There is extensive experience with this cell line to show that, by 3 days after the injection of these cells, 100% of the mice so injected will die from the progressive growth of these tumor cells. This is called the "therapy experiment." VPP suppressed the growth of the tumor cells the most (see Fig. 1*G*, ◇), whereas VVV (*G*, □) was less effective. In contrast, the PPP vaccine (see Fig. 1*G*, ▲), without antecedent vector injection, suppressed the growth of the hMUC-1-positive tumor cell line in the hMUC-1.Tg mice less than was the case for VVV or VPP. The differences between the VPP and the PPP groups in terms of tumor growth were significant at the $p = 0.02$ level.

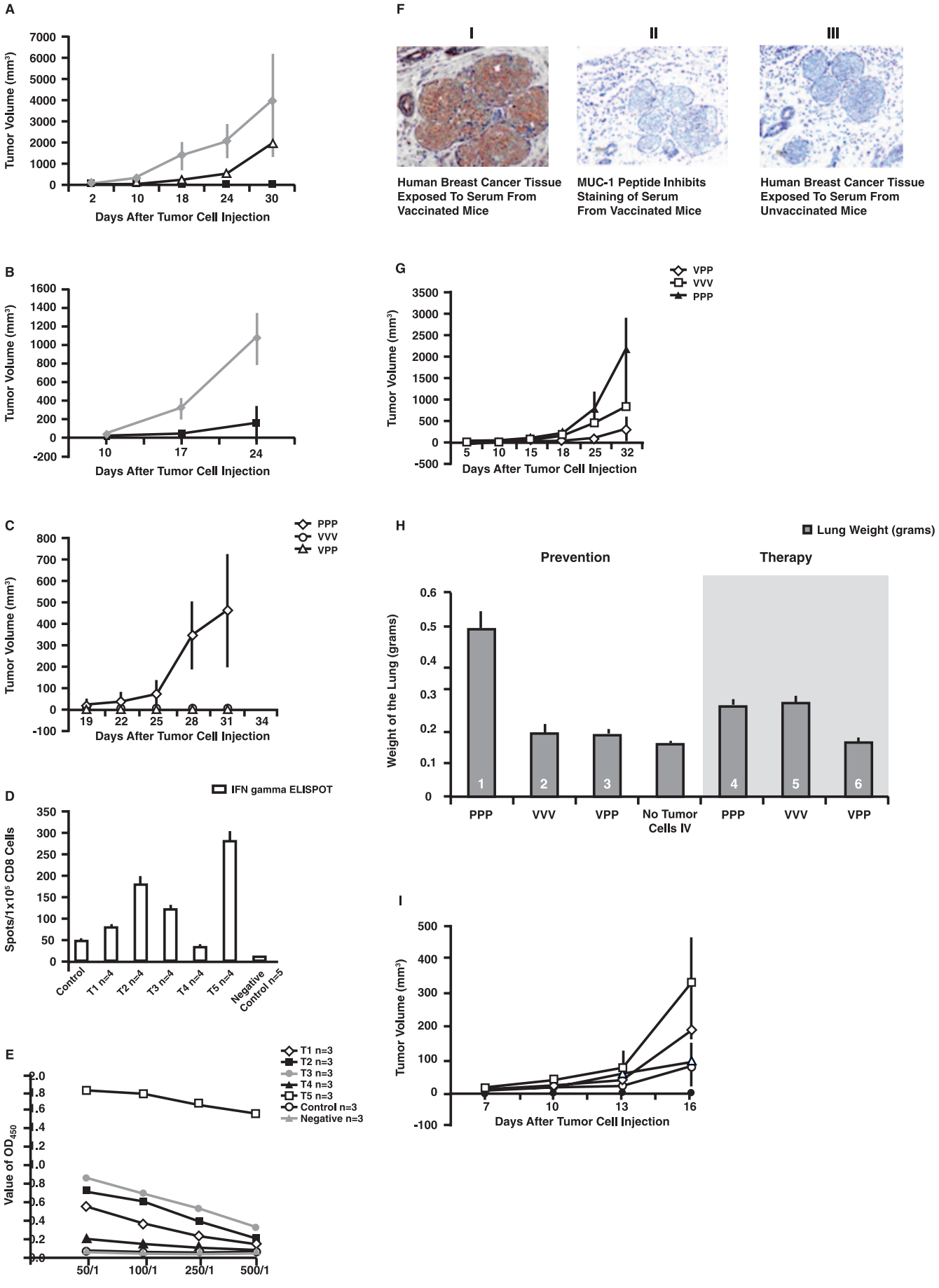
VPP suppresses the growth of i.v. administered MUC-1-positive cancer cells in the lungs of hMUC-1.Tg mice

To mimic tumor metastases, we challenged hMUC-1.Tg mice (three mice per group) by tail vein injection of hMUC-1-positive LL2/LL1hMUC-1 tumor cells following completion of the vaccinations. We then weighed the lungs of mice sacrificed 63 days following the initiation of vaccination. As shown in Fig. 1*H* (prevention side), the weight of the lungs in mice injected with PPP was 2.5 times the weight of the lungs in mice not injected i.v. with the LL2/LL1hMUC-1 cell line. In contrast, the weight of the lungs in mice injected s.c. with three successive Ad-sig-hMUC-1/ecdCD40L vector injections (see VVV on prevention side in Fig. 1*H*), or the single Ad-sig-hMUC-1/ecdCD40L vector s.c. injection followed by two successive s.c. injections of the hMUC-1/ecdCD40L protein at 7 and 21 days (VPP), was within the margin of error of the weight of the lungs in mice not injected i.v. with the LL2/LL1hMUC-1 cancer cells (see *left* side of Fig. 1*H*). The differences between the weights of the lungs in mice injected with PPP vs VPP were different at the $p = 0.03$ level.

We next tested the vaccines in hMUC-1.Tg mice carrying hMUC-1-positive s.c. tumor nodules (three mice per treatment group), which were established 3 days before the vaccination, which is called the therapy experiment. As shown on the *right* side of Fig. 1*H* (therapy experiment), VPP completely suppressed the growth of the tumor cells in the lungs, whereas PPP did not (the differences between the lung weights of the VPP and the PPP groups was significant at the $p = 0.03$ level). Furthermore, VVV was less effective than VPP.

Both the MUC-1 Ag and the CD40L are required for the hMUC-1/ecdCD40L protein boost

We compared the growth of hMUC-1-positive tumor cells in the hMUC-1.Tg mice that had been vaccinated with VPP (●), or the hMUC-1/KLH-the hMUC-1 antigenic peptide linked to the KLH stabilizing molecule (△), or the hMUC-1/KLH) with IFA (○), or an extract of the bacterial host strain used to produce the hMUC-1/ecdCD40L—without the hMUC-1/ecdCD40L protein (◇) or PBS (□). The vaccination was conducted before the injection of the tumor cells. As shown in Fig. 1*I*, the hMUC-1/KLH with (see Fig. 1*I*, ○) or without (see *I*, △), Freund's adjuvant failed to boost the immune response induced by the Ad-sig-hMUC-1/ecdCD40L vector sufficiently to completely suppress the growth of the hMUC-1 tumor cells in the hMUC-1.Tg mice. In contrast, the s.c. injection of the hMUC-1/ecdCD40L protein (see Fig. 1*I*, ●) as a boost to the Ad-sig-hMUC-1/ecdCD40L vector suppressed the growth of the hMUC-1-positive tumor cells to a greater degree than did other types of boosters tested. Because the hMUC-1/ecdCD40L protein used for the boost was derived from a bacterial expression vector, we also tested the effect of injecting the lysate from bacterial cells not containing the hMUC-1/ecdCD40L protein. As shown in Fig. 1*I*, neither the bacterial cell lysate (◇) nor PBS (□) boosted the effect of the Ad-sig-hMUC-1/ecdCD40L sufficiently to suppress the in vivo growth of hMUC-1-positive tumor cells.


A
Tumor Volume (mm³)
Days After Tumor Cell Injection
B
Tumor Volume (mm³)
Days After Tumor Cell Injection
C
PPP
VVV
VPP
Tumor Volume (mm³)
Days After Tumor Cell Injection
D
IFN gamma ELISPOT
Spots/1x10⁵ CD8 Cells
Control
T1 n=4
T2 n=4
T3 n=4
T4 n=4
T5 n=4
Negative Control n=5
E
T1 n=3
T2 n=3
T3 n=3
T4 n=3
T5 n=3
Control n=3
Negative n=3
Value of OD450
50/1
100/1
250/1
500/1
F
I
II
III
Human Breast Cancer Tissue Exposed To Serum From Vaccinated Mice
MUC-1 Peptide Inhibits Staining of Serum From Vaccinated Mice
Human Breast Cancer Tissue Exposed To Serum From Unvaccinated Mice
G
VPP
VVV
PPP
Tumor Volume (mm³)
Days After Tumor Cell Injection
H
Lung Weight (grams)
Prevention
Therapy
Weight of the Lung (grams)
PPP
VVV
VPP
No Tumor Cells IV
PPP
VVV
VPP
I
Tumor Volume (mm³)
Days After Tumor Cell Injection

Testing of the Ad-sig-TAA/ecdCD40L platform against the Her-2-Neu(H2N)Ag

We next tested whether the Ad-sig-TAA/ecdCD40L vaccination strategy could be used to induce immunity against the H2N receptor, which is associated with poor prognosis in human breast cancer (8). We therefore constructed the Ad-sig-rH2N/ecdCD40L vector, which carried a transcription unit encoding an epitope from the ecd of the rat H2N (rH2N) receptor linked to the ecdCD40L. We injected s.c. the Ad-sig-rH2N/ecdCD40L vector one or two times at 7-day intervals in rH2N.Tg mice (four mice per treatment group) to test whether an immune response could be induced against the rH2N Ag. Seven days following completion of the vaccination, we injected the rH2N-positive breast cancer cells (500,000) s.c. As shown in Fig. 2*A*, two s.c. injections of the Ad-sig-rH2N/ecdCD40L vector induced complete suppression of the in vivo growth of the rH2N-positive mouse breast cancer cell line in the rH2N.Tg mice (Fig. 2*A*, □), whereas one s.c. injection of the same vector (Fig. 2*A*, △) only partially suppressed the growth of the rH2N-positive mouse breast cancer cell line. At day 46 after tumor cell injection, the difference in the tumor cell growth between the mice vaccinated twice with the Ad-sig-rH2N/ecdCD40L vector (□) and untreated (◇) mice was significant at the $p = 0.047$ level.

Table I. *Vector then protein boost*

Testing Group	Week 1	Week 2	Week 3	Week 4
Control	**Vector**	**Vector**	Nothing	Nothing
Treatment 1 (T1)	**Vector**	**Vector**	**Protein**	Nothing
Treatment 2 (T2)	**Vector**	**Vector**	Nothing	**Protein**
Treatment 3 (T3)	**Vector**	**Protein**	Nothing	Nothing
Treatment 4 (T4)	**Vector**	Nothing	**Protein**	Nothing
Treatment 5 (T5)	**Vector**	**Protein**	Nothing	**Protein**
Negative control	Nothing	Nothing	Nothing	Nothing

We also measured the rH2N-specific Ab levels in mice vaccinated following one or two s.c. injections of the Ad-sig-rH2N/ecdCD40L vector. As shown in Fig. 2*B*, the levels of the rH2N-specific Ab levels were higher following two s.c. injections (■) than following a single s.c. injection (●) of the Ad-sig-rH2N/ecdCD40L vector. As shown in Fig. 2*C*, ELISPOT assays showed that the administration of two s.c. injections of the Ad-sig-rH2N/ecdCD40L vector 7 days apart induced levels of rH2N-specific T cells in the spleens of vaccinated mice that were 10 times higher than the levels of rH2N-specific T cells induced in unvaccinated mice (three mice per group). The difference in the level of spots in the control vs the vaccinated groups was significant at the $p = 0.0006$ level.

FIGURE 1. *A*, Ad-sig-hMUC-1/ecdCD40L vector vaccine which encodes epitope for subunit I (all extracellular) of the hMUC-1 linked to the ecd of the CD40L suppresses growth of the LL2/LL1hMUC-1 cell line in hMUC-1.Tg mice. Test mice were injected s.c. twice at 7-day intervals with the Ad-sig-hMUC-1/ecdCD40L vector prime and hMUC-1/ecdCD40L protein boost vaccine (epitope of subunit I of hMUC-1 linked to CD40L), and then 7 days later injected s.c. with the LL2/LL1hMUC-1 tumor cells (prevention experiment). We then measured the size of the s.c. nodule that developed at the s.c. injection site of 500,000 LL2/LL1hMUC-1 tumor cells in hMUC-1.Tg mice that had been vaccinated with the Ad-sig-hMUC-1/ecdCD40L vector. This vector contains a 40-aa epitope from subunit I of hMUC-1, which is totally extracellular. The following was used for the vaccination: no vaccination (◆); Ad-sig-hMUC-1/ecdCD40L subunit I vector (■); Ad-sig-hMUC-1 subunit I vector (△). *B*, Two s.c. injections at 7-day interval of the Ad-sig-hIIMUC-1/ecdCD40L vector vaccine that encodes epitope for subunit II (the subunit embedded in the membrane) of hMUC-1 linked to ecd of CD40L suppresses growth of the LL2/LL1hMUC-1 cell line in hMUC-1.Tg mice. Test mice were injected twice s.c. with the Ad-sig-hMUC-1/ecdCD40L vector (epitope of subunit II of hMUC-1 linked to CD40L) and then 7 days later injected s.c. with the LL2/LL1hMUC-1 tumor cells (prevention experiment). We then measured the size of the s.c. nodule that developed at the s.c. injection site of 500,000 LL2/LL1hMUC-1 tumor cells in hMUC-1.Tg mice that had been vaccinated with the Ad-sig-hMUC-1/ecdCD40L subunit II vector. This vector contains an epitope from the ecd of subunit II of hMUC-1. Subunit II is the subunit in which there is a transmembrane protein with both an ecd and a cytoplasmic domain. The following was used for the vaccination: nothing (◆); Ad-sig-hMUC-1/ecdCD40L subunit II vector (■). *C*, Effect of the VVV, VPP, and PPP vaccination on the growth of s.c. nodules of hMUC-1-positive LL2/LL1hMUC-1 cancer cells when the s.c. vaccination utilizing subunit I of hMUC-1 linked to ecd of CD40L precedes the s.c. injection of the LL2/LL1hMUC-1 cancer cells (prevention experiment). The growth of s.c. nodules of hMUC-1-positive LL2/LL1hMUC-1 cancer cells as s.c. nodules was measured in hMUC-1.Tg mice, which had been injected s.c. with 500,000 hMUC-1-positive LL2/LL1hMUC-1 cancer cells after administration of one of the following vaccination schedules: VVV (○), VPP (△), or PPP (◇). V, Ad-sig-hMUC-1/ecdCD40L subunit I vector; P, hMUC-1/ecdCD40L subunit I protein. All injections were at 7-day intervals. *D*, The effect of various schedules of the Ad-sig-hMUC-1/ecdCD40L vector and the hMUC-1/ecdCD40L protein (subunit I) on the level of IFN-γ-positive T cells in the spleen of hMUC-1.Tg mice before and after vaccination. The following combinations of the Ad-sig-hMUC-1/ecdCD40L subunit I vector (V) and hMUC-1/ecdCD40L subunit I protein (P) were injected s.c. into the hMUC-1.Tg mice: each s.c. administration was conducted at 7-day intervals. Control, VVNN; T1, VVPN; T2, VVNP; T3, VPNN; T4, VNPN; T5, VPNP; negative control, NNNN. N, Nothing. All injections (V, P, or N) are separated at 7-day intervals. V, Vector; P, protein; N, nothing. *E*, The effect of various schedules of the s.c. injection of the Ad-sig-hMUC-1/ecdCD40L subunit I vector and the hMUC-1/ecdCD40L subunit I protein on the level of hMUC-1-specific Abs in ecdhMUC-1.Tg mice. The following combinations of the Ad-sig-hMUC-1/ecdCD40L subunit I vector (V) and hMUC-1/ecdCD40L subunit I protein (P) were injected s.c. in the hMUC-1.Tg mice: control, VVNN (○); T1, VVPN (◇); T2, VVNP (■); T3, VPNN (light filled in circle); T4, VNPN (▲); T5, VPNP (□); negative control, NNNN (light filled in triangle). All injections are at 7-day intervals. V, Ad-sig-hMUC-1/ecdCD40L subunit I vector; P, hMUC-1/ecdCD40L subunit I protein. *F*, Binding of Abs from the serum of Ad-sig-hMUC-1/ecdCD40L subunit I vector vaccinated mice to human breast cancer cells. Serum collected from hMUC-1.Tg mice following vaccination with the Ad-sig-hMUC-1/ecdCD40L vector and hMUC-1/ecdCD40L protein (subunit I) was applied to sections from human breast cancer clinical specimens. I, Abs from vaccinated mice. II, Abs from vaccinated mice that were exposed to the hMUC-1-specific peptide used in the vaccination before applying the mouse serum to the sections. III, Serum from unvaccinated mice. *G*, Effect of the VVV, VPP, and PPP vaccination on the growth of s.c. nodules of the LL2/LL1hMUC-1 cancer cells when the s.c. injection of the LL2/LL1hMUC-1 cells precedes the Ad-sig-hMUC-1/ecdCD40L subunit I vector vaccination (therapy experiment). The growth of s.c. nodules of hMUC-1-positive LL2/LL1hMUC-1 cancer cells was measured in hMUC-1.Tg mice that were injected s.c. with the hMUC-1-positive LL2/LL1hMUC-1 cells 3 days before being vaccinated with one of the following regimens: VVV (□), VPP (◇), or PPP (▲). V, Ad-sig-hMUC-1/ecdCD40L subunit I vector. P, hMUC-1/ecdCD40L subunit I protein. *H*, The effect of Ad-sig-hMUC-1/ecdCD40L subunit I vector vaccination on the growth of the hMUC-1-positive LL2/LL1hMUC-1 cancer cells in the lungs of hMUC-1.Tg mice. We weighed the lungs of hMUC-1.Tg mice that were vaccinated before the s.c. and i.v. injection of the hMUC-1-positive LL2/LL1hMUC-1 cancer cells (*left panel*, prevention) or in mice vaccinated after the s.c. injection of the LL2/LL1hMUC-1 cancer cells (*right panel*, therapy). *I*, Testing of boosting proteins composed of hMUC-1 TAA without CD40L. To test the importance of the presence of both the CD40L and the hMUC-1 to the boosting of the immune response, induced by the Ad-sig-hMUC-1/ecdCD40L vector (subunit I), we compared the effect of the following boosts with the hMUC-1/ecdCD40L protein (●): bacterial cell lysate (◇), keyhole limpet hemocyanin (KLH)-conjugated hMUC-1 Ag with (○) and without (△) IFA, and PBS (□).

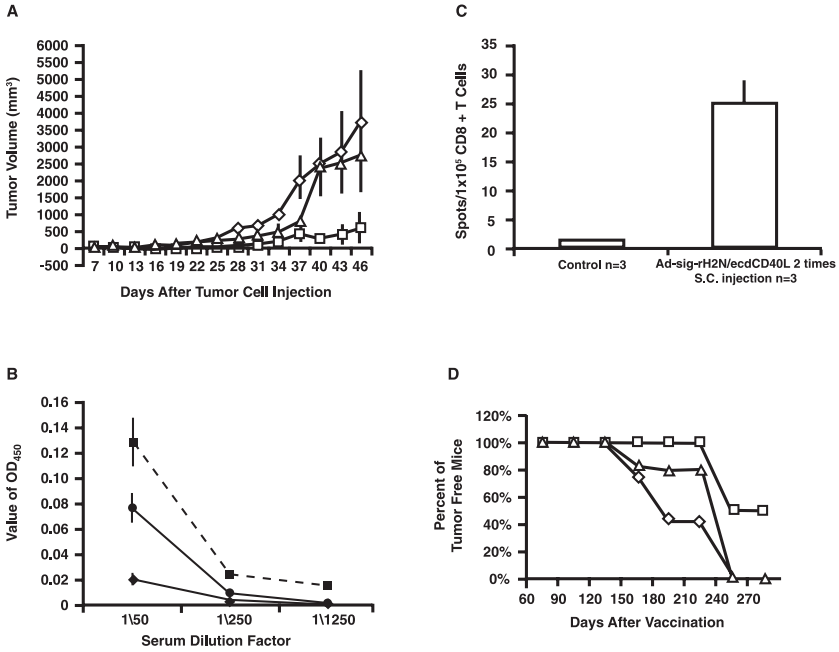


FIGURE 2. *A*, Effect of the Ad-sig-rH2N/ecdCD40L vector on the growth of rH2N-positive NT2 cells. The following vectors were injected s.c. twice at a 7-day interval: no vector (◇); two s.c. injections of the Ad-sig-rH2N/ecdCD40L vector (□), and one s.c. injection of the Ad-sig-rH2N/ecdCD40L vector (△). *B*, Effect of the Ad-sig-rH2N/ecdCD40L vector on the induction of rH2N-specific Abs against rH2N-positive NT2 cells. The following vectors were injected s.c. twice at a 7-day interval: no vector (♦); two s.c. injections of the Ad-sig-rH2N/ecdCD40L vector (■), and one s.c. injection of the Ad-sig-rH2N/ecdCD40L vector (●). *C*, Effect of the Ad-sig-rH2N/ecdCD40L vector on the induction of rH2N-specific T cells as measured by the ELISPOT assay. The ELISPOT assay was used to measure the level of the IFN-γ-positive T cells/1 $\times$ 10^5 spleen cells following in vitro exposure to mitomycin C (MMC)-treated rH2N tumor cell lines. The T cells were collected from the spleens of mice before and after vaccination two s.c. injections of the Ad-sig-rH2N/ecdCD40L vector or no vaccination (control). *D*, Protection of Her-2-Neu transgenic mice against development of spontaneous breast cancer. rH2N.Tg mice were injected s.c. with the Ad-sig-rH2N/ecdCD40L vector at 6 wk. Following this, a s.c. injection of 10 μg of the rH2N/ecdCD40L protein was conducted at 7, 13, 18, and 21 wk of life. The mice were followed for the appearance of palpable tumors in the mammary glands. Mice were injected with the vector prime-protein booster (□); unvaccinated (◇); bacterial lysate (△).

Ad-sig-rH2N/ecdCD40L vector prime/protein boost vaccine suppresses onset of spontaneous breast cancer in rH2N.Tg mice

The rH2N.Tg mice were vaccinated starting at 6 wk of life with the s.c. injection of the Ad-sig-rH2N/ecdCD40L vector vaccine (once) followed by four s.c. injections of the rH2N/ecdCD40L protein booster injections (10 μg) over the duration of the experiment (see Fig. 2*D*, □). Control mice were injected with PBS instead of the vaccine (see Fig. 2*D*, ◇) or the Ad-sig-rH2N/ecdCD40L vector (one s.c. injection at 6 wk of age) followed by lysate from the bacterial host strain used to produce the rH2N/ecdCD40L booster protein (Fig. 2*D*, △). As shown in Fig. 2*D*, the Ad-sig-rH2N/ecdCD40L vector prime/rH2N/ecdCD40L protein boost prevents the development of breast cancer for up to 280 days in 50% of the vaccinated mice, whereas all animals have developed breast cancer in the control groups by 245 days of life. Although still early (10 mo) in the life of these spontaneous rH2N.Tg breast cancer mice, the results are suggesting a protective effect of the anti-Her-2-Neu vaccine strategy.

Induction of an immune response against Ags on tumor vascular endothelial cells

A recent paper (12) reported that the Annexin A1 (AnxA1) protein was present on the luminal surface of the endothelial cells of tumor vasculature but was not detectable on the luminal surface of the vascular endothelial cells of normal tissues. We therefore decided to test whether the s.c. injection of the Ad-sig-AnxA1/ecdCD40L vector would suppress the growth of the hMUC-1-positive LL2/LL1hMUC-1 cancer cell line.

To test specifically whether the immune response generated by the Ad-sig-AnxA1/ecdCD40L vector is directed against the Annexin A1 Ag, serum was taken from a mouse that had been injected s.c. twice with the Ad-sig-AnxA1/ecdCD40L vector and tested by ELISA for the presence of Abs against the Annexin A1 Ag. As shown in Fig. 3*A*, Abs that bind Annexin A1 are induced in the serum of the Ad-sig-AnxA1/ecdCD40L vaccinated hMUC-1.Tg mice (there were three mice per group). The difference in the levels of AnxA1 Abs in the vaccinated (♦) vs the unvaccinated (■) mice was significant at the $p = 0.00003$ level.

To directly test whether these Abs are binding to the tumor vasculature, multiparameter fluorescence confocal microscopy was conducted on frozen sections of hMUC-1-positive, Annexin A1-negative tumor tissue. As shown in Fig. 3*B*, the binding of the FITC-conjugated (green staining) serum Abs against Annexin A1 generated in the mice injected s.c. with the Ad-sig-AnxA1/ecdCD40L vector bind to the tumor vasculature as shown by the yellow spots in the *right panel* in Fig. 3*B*. The red color of the anti-CD31 vascular binding Ab (PE conjugated) coincides with the binding of the FITC-conjugated serum (stains tissue green) from the Ad-sig-AnxA1/ecdCD40L vaccinated mouse. No yellow color (or green color) appears in the *left panel* of Fig. 3*B* in which frozen sections of tumor tissue were exposed to FITC-conjugated serum from unvaccinated mice and the PE-conjugated anti-CD31 Abs. These results suggest that the Ad-sig-TAA/ecdCD40L vaccine strategy can induce an immune response against tumor vascular endothelial cells, and thereby potentially suppressing the growth of the tumor tissue, which depended on the Anx1A-positive tumor vasculature.

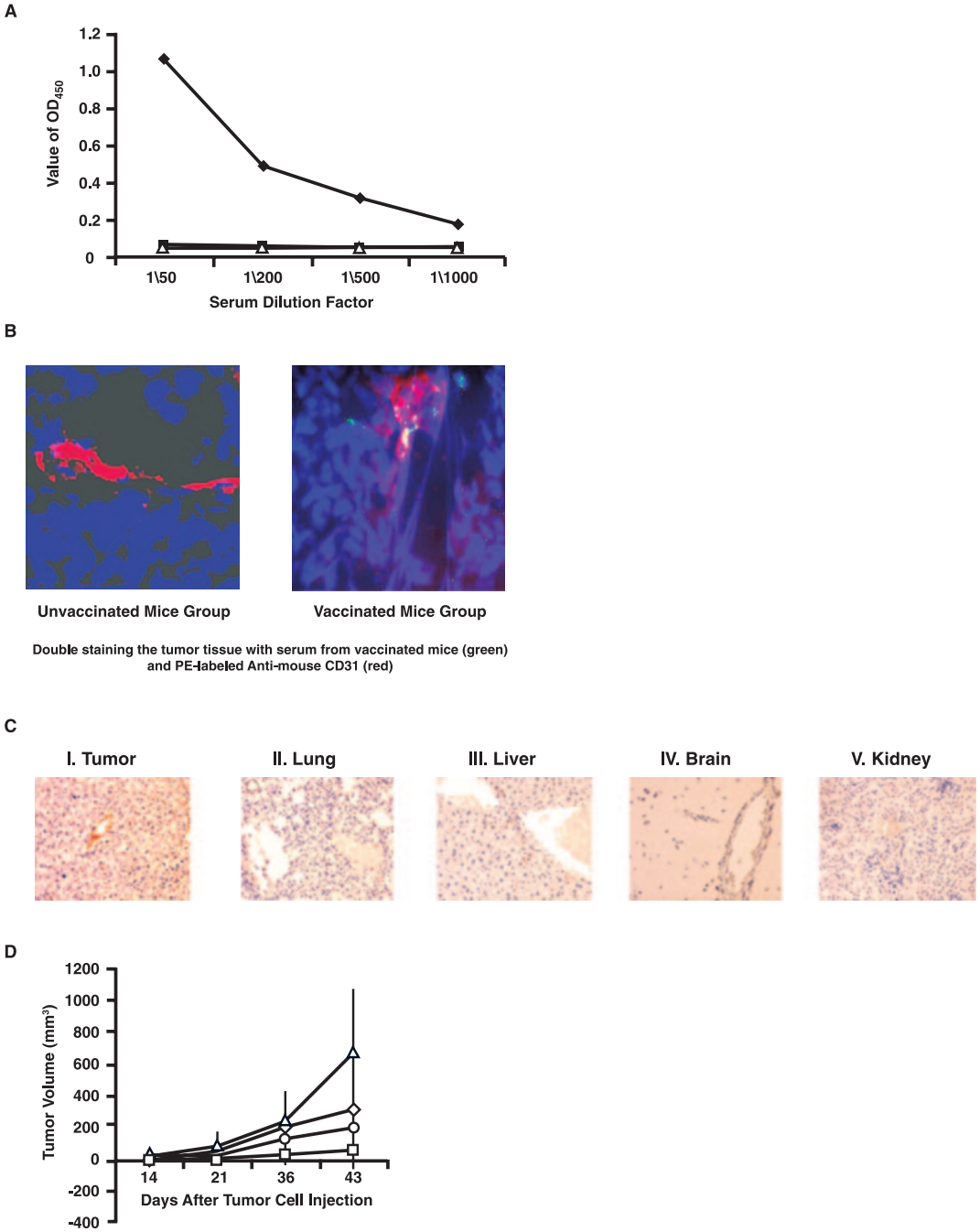


FIGURE 3. *A*, The serum level of Annexin A1-specific Abs before and after vaccination with the Ad-sig-AnxA1/ecdCD40L vector. To test specifically whether the immune response generated by the Ad-sig-AnxA1/ecdCD40L vector is directed against the Annexin A1 Ag, serum was taken from a mouse that had been injected s.c. twice with the Ad-sig-AnxA1/ecdCD40L vector, and was tested by ELISA for the presence of Abs against the Annexin A1 Ag. The following was used for experiment: No serum added (△); serum from Ad-sig-AnxA1/ecdCD40L vaccinated mice (◆); serum from unvaccinated mice (■). *B*, The binding of FITC-labeled serum proteins from test mice vaccinated with the Ad-sig-AnxA1/ecdCD40L vector to tumor vasculature. To specifically test whether these Abs are binding to the tumor vasculature, multiparameter fluorescence in situ confocal microscopy was conducted on frozen sections of hMUC-1-positive Annexin A1-negative tumor tissue. *Right panel*, FITC-conjugated (green staining) serum Abs against Annexin A1 generated in the mice injected s.c. with the Ad-sig-AnxA1/ecdCD40L vector were applied to frozen sections of s.c. tumor nodules of a cell line that was Annexin A1 negative. Anti-CD31 vascular binding Ab (PE conjugated) was also applied to this frozen section. The yellow dots indicate coincident binding of the FITC label Abs from the serum of the vaccinated mice and the PE-conjugated CD31 Ab. *Left panel*, The same experiment as described in the *right panel* except that the FITC-conjugated serum came from unvaccinated mice. *C*, The binding of serum proteins from test mice vaccinated with the Ad-sig-AnxA1/ecdCD40L vector to the vessels in tumor tissue and normal tissues. Serum was taken from the Ad-sig-AnxA1/ecdCD40L vector-vaccinated mice and added to formalin-fixed paraffin-embedded sections from s.c. tumor nodules (I), normal lung (II), normal liver (III), normal brain (IV), and normal kidney (V). Then, the sections were exposed to a secondary anti-mouse Ab conjugated with HRP and the sections were stained. *D*, The effect of combining the Ad-sig-rH2N/ecdCD40L and Ad-sig-AnxA1/ecdCD40L vaccines. The mice were vaccinated with a combination of the two vector vaccines and then challenged with an Annexin A1-negative rH2N-positive cell line. The growth of the tumor in mice vaccinated with the combination of the two vaccines and the two vaccines used independently was measured and compared with the tumor growth in unvaccinated mice. The following vaccination groups were studied: control (△); Ad-sig-AnxA1/ecdCD40L (◇); Ad-sig-rH2N/ecdCD40L (○); Ad-sig-AnxA1/ecdCD40L and Ad-sig-rH2N/ecdCD40L together in combination (□).

Annexin A1 is a cytosolic protein in normal ciliated tissues, the CNS, and endothelial cells. It is involved in the inflammatory response as well. Therefore, to evaluate the feasibility of using the Ad-sig-AnxA1/ecdCD40L immunization, it was important to test the selectivity of the humoral immune response induced by the Ad-sig-AnxA1/ecdCD40L vaccination. This would in part be dependent on the distribution of Annexin A1, which is intracellular in normal cells but may be available to the extracellular environment in endothelial cells in neoplastic tissue. We therefore tested the binding of serum from the bloodstream of Ad-sig-AnxA1/ecdCD40L-vaccinated mice to paraffin-embedded formalin-fixed sections of tumor tissue (Fig. 3*C*, I), normal lung—a ciliated tissue (*C*, II), liver (*C*, III), normal CNS (*C*, IV), and normal kidney (*C*, V). As shown in Fig. 3*C*, HRP-conjugated secondary anti-mouse Abs produced positive staining in the vessels of tumor tissue but not in the vessels of normal lung, liver, brain, or kidney.

To test whether the combination of the Ad-sig-TAA/ecdCD40L anti-cancer cell vaccine with the Ad-sig-TVECA/ecdCD40L antitumor vascular endothelial cell vaccine would produce a tumor-suppressive effect that is greater than either vaccine alone, we vaccinated rH2N.Tg mice s.c. with the Ad-sig-rH2N/ecdCD40L anti-Her-2-Neu breast cancer cell vaccine and with the Ad-sig-AnxA1/ecdCD40L antitumor vascular endothelial cell vaccine. It is noteworthy that the rH2N-positive breast cancer cells injected s.c. in the vaccinated mice were AnxA1 negative. As shown in Fig. 3*D*, the growth of the rH2N-positive tumor cells in the rH2N.Tg mice vaccinated with the combination of the Ad-sig-rH2N/ecdCD40L and the Ad-sig-AnxA1/ecdCD40L vaccines (□) was significantly less than the tumor growth in unvaccinated (△) mice ($p = 0.00007$). The growth of the rH2N-positive tumor cells in unvaccinated mice (Fig. 3*D*, △) was significantly greater than in Ad-sig-rH2N/ecdCD40L vaccinated (◇) mice ($p = 0.01$) or the Ad-sig-AnxA1/ecdCD40L vaccinated (○) mice ($p = 0.006$). The difference among the vaccinated groups was not significant at the $p < 0.05$ level.

Level of CD8 T cells infiltrating the tumor tissue increased after vaccination with the Ad-sig-rH2N/ecdCD40L vector

We had shown previously (2, 3) that the s.c. injection of the Ad-sig-TAA/ecdCD40l vector activated the tumor Ag-loaded DCs and promoted their migration to the regional lymph nodes, resulting in an increase in the levels of the TAA-specific T cells there. One question left unresolved by these earlier studies was whether these TAA-specific effector CD8 T cells reached the tumor tissue in the extravascular space. One of the predictions that could be made on the basis of previous work is that the levels of CD8 effector T cells in the tumor tissue will be increased following vaccination with the Ad-sig-rH2N/ecdCD40L vector. We therefore minced s.c. tumor nodules of rH2N.Tg mice before and after two s.c. injections of the Ad-sig-rH2N/ecdCD40L vector. Single-cell suspensions were generated from the tumor tissue after mincing, treatment with 0.03% DNase, treatment with 0.14% collagenase I, and filtration through nylon mesh. There were six mice per treatment group. We found that the percentage of CD8 T cells with the immunophenotype of effector T cells ($CD8^{high}$, $CD44^{high}$, $LY6C^{high}$, and $CD62L^{low}$) isolated from the tumor tissue after vaccination ranged from 3.5 to 9.5%, whereas it was no higher than 2.5% in unvaccinated mice as shown in Fig. 4. The difference in the levels of the CD8 effector T cells in the tumor tissue of the control and the vaccinated mice was significant at the $p = 0.01$ level. These data suggest that the suppression of the growth of the rH2N-positive tumor cells in the rH2N.Tg mice following Ad-sig-rH2N/ecdCD40L vaccination is mediated in part by an increase in the trafficking of effector T cells into the tumor tissue.

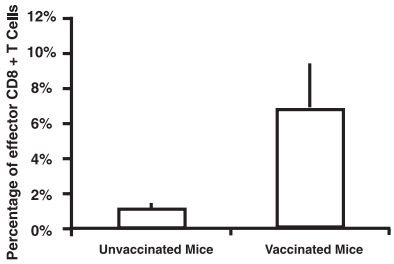


FIGURE 4. Infiltration of CD8 effector cells following Ad-sig-TAA/ecdCD40L vaccination. We minced the s.c. tumor nodules of rH2N.Tg mice before and after two s.c. injections of the Ad-sig-rH2N/ecdCD40L vector. Single-cell suspensions were isolated and treated with 0.03% DNase and 0.14% collagenase I, and then filtered through nylon mesh. The resulting cell suspension was then characterized for the percentage of the cells with the immunophenotype of effector T cells ($CD8^{high}$, $CD44^{high}$, $LY6C^{high}$, and $CD62L^{low}$).

Changes in gene expression in effector CD8 T cells that infiltrate tumor tissue following vaccination

RNA was isolated from the tumor-infiltrating CD8 effector T cells and the pattern of gene expression was compared before and after vaccination using the Affymetrix gene expression system. We also examined the expression level of the 21 known chemokine receptors and ligands in the effector T cells that were infiltrating the tumor tissue. The chemokine pathway plays a major role in the trafficking of effector and memory T cells from the lymph nodes draining sites of vaccination or infection to the tissue sites harboring inflammation or infection (16, 17). The CCL3 (2.8-fold increase) and CCR5 (16-fold increase), which are involved in the targeting of T cells to the extravascular sites of tissue inflammation, were increased in the tumor-infiltrating CD8 effector T cells in vaccinated mice but not in unvaccinated mice.

Study changes in number of TAA-specific CD8 effector cells after Ad-sig-TAA/ecdCD40L vector prime/protein boost vaccination in old (18-mo-old) mice

It is well known that the immune response to vaccines is subject to acquired quantitative defects in both CD8 and CD4 T cells and acquired defects in CD4 T cells (1, 25) during the aging process in mice (18, 19) and in humans (20, 21). We therefore injected the Ad-sig-E7/ecdCD40L vector two times s.c. in 2-mo and 18-mo-old mice. We chose the E7 protein as the TAA target because it was a foreign Ag (from human papilloma virus) so that we would be testing the effect of aging separated from the effects of tolerance. We then measured the level of the E7-specific T cells by ELISPOT assay. We chose the HPV E7 Ag for the initial vaccination studies in the old mice, because it was a foreign viral Ag. As shown in Fig. 5*A*, the levels of E7-specific T cells in the spleen of old mice was increased to 230 Ag-specific T cells per 100,000 CD8 splenic T cells by ELISPOT assay. Although it is clear that the magnitude of the induction of Ag-specific T cells in the 18-mo-old mice (230) was less than that seen in the 2-mo-old mice (230), the absolute magnitude of the response in the 18-mo-old mice (230) is in the range induced by most other vaccines in young mice and is clearly sufficient to produce a robust immune response.

We then measured the increase of the percentage that Ag-specific T cells constituted of total CD8 T cells in the tumor tissue before and after vaccination using E7 tetramers. As shown in Fig. 5*B*, the Ad-sig-E1/ecdCD40L vaccine induced the level of Ag-specific T cells in the tumor tissue by 10-fold. We also measured the increase of the T cells as a percentage of the total number cells in the tumor tissue following vaccination in the old mice. As

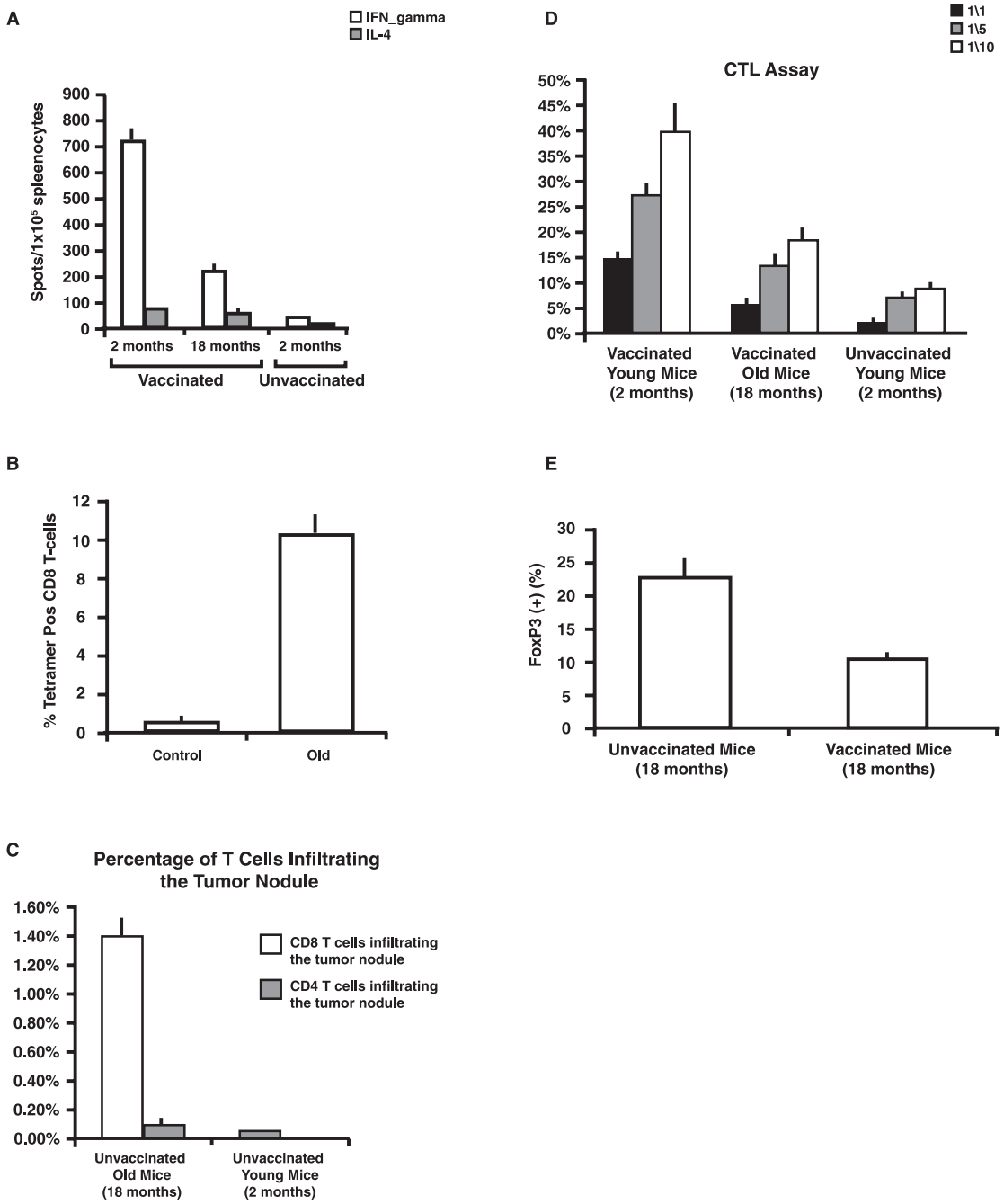


FIGURE 5. *A*, Effect of the Ad-sig-E7/ecdCD40L vector on the induction of E7 specific T cells as measured by the ELISPOT assay in 18- and 2-mo-old mice. The ELISPOT assay was used to measure the level of the IFN-γ- (▨) or IL-4- (■) positive T cells/1 × 10^5 spleen cells following in vitro exposure to MMC-treated E7-positive TC-1 tumor cell lines. The T cells were collected from the spleens of mice before and after vaccination with one s.c. injection of the Ad-sig-E7/ecdCD40L vector followed by two s.c. E7/ecdCD40L protein boost injections. *B*, Effect of the Ad-sig-E7/ecdCD40L vector prime-E7/ecdCD40L protein boost vaccine on E7-specific CD8 effector T cells in tumor E7-positive TC-1 s.c. nodules. We measured the increase of the percentage that Ag-specific T cells constituted of total CD8 T cells in the tumor tissue before (control) and after vaccination using E7 tetramers in 18-mo-old C57BL/6J mice. Tumor tissue was minced, treated with DNase I and collagenase, and the resulting cells were filtered through nylon gauze. Then, FACS analysis was conducted with the FACSCalibur to determine the number of E7-specific CD8 effector cells by tetramer assays as described previously (2). *C*, Effect of the Ad-sig-E7/ecdCD40L vector prime-E7/ecdCD40L protein (3×) boost (VPPP) vaccine on the percentage of the total number of cells composed by T cells in tumor E7-positive TC-1 s.c. nodules. We processed tumor nodules following VPPP vaccination as described above, and then measured the increase of the T cells as a percentage of the total number cells in the tumor tissue following vaccination in the old (18 mo) and young (2 mo) C57BL/6J mice. *D*, Effect of the Ad-sig-E7/ecdCD40L vector prime-E7/ecdCD40L protein (3×) boost (VPPP) vaccine on the induction of cytotoxic T cells. The cytotoxicity assay was used to measure the level of cytotoxic lymphocytes (CTLs) in the spleen following in vitro exposure to MMC-treated E7-positive TC-1 tumor cell lines following the VPP vaccination. The T cells were collected from the spleens of mice before and after vaccination with one s.c. injection of the Ad-sig-E7/ecdCD40L vector followed by two s.c. E7/ecdCD40L protein boost injections. CTL cells were measured by release of lactate dehydrogenase as outlined previously (3) in 18- and 2-mo-old mice at varying E:T ratios which were as follows: 1/1, ▧; 1/5, ■; 1/10, □. The ordinate is percent cytotoxicity. *E*, Effect of the Ad-sig-E7/ecdCD40L vector prime-E7/ecdCD40L protein (3×) boost (VPPP) vaccination in old mice on the levels of negative regulatory CD4CD25FOXP3-T cells in E7-positive tumor tissue. We used the FACSCalibur to measure the level of FOXP3CD25CD4 T cells in E7-positive TC-1 tumor tissue before and after vaccination in 18-mo-old C57BL/6J mice. The tumor tissue was processed as outlined above in Fig. 4. The results measure the level of CD4CD25FOXP3-positive cells.

shown in Fig. 5*C*, the increase of the percentage of T cells increased over 10-fold after the vaccination in the old mice. We then tested the level of increase of Ag-specific CTLs induced by vaccination in 2-mo- and 18-mo-old mice. The results presented in Fig. 5*D* show impressive increases in Ag-specific CTLs following vaccination in the old as well as the young animals. Again, the level of the increase of the CTLs seen in the 18-mo-old mice was less than that seen in the 2-mo-old mice, but the absolute magnitude of the induction was impressive in the 18-mo-aged mice.

Effect of the Ad-sig-TAA/ecdCD40L vector vaccination in old mice on the levels of negative regulatory CD4 FOXP3-positive T cells in tumor tissue

Increases in negative regulatory CD4 FOXP3-positive T cells have been reported to limit the degree to which vaccines induce the immune response in old mice. Decreases in the level of negative regulatory FOXP3-CD4 T cells have been reported with vaccination. We therefore measured the level of FOXP3 CD4 T cells in the tumor tissue before and after vaccination. As shown in Fig. 5*E*, the vaccination decreased the level of the CD4 FOXP3-positive T cells in the tumor tissue by 3-fold in 18-mo-old mice.

Effect of the Ad-sig-TAA/ecdCD40L vector vaccine against viral Ag in old mice on growth of cell positive for viral Ag

As shown in Fig. 6*A*, the suppression of E7-positive tumor growth in the 18-mo-old mice (○) was almost equal to the level of suppression of the tumor growth in 2-mo-old mice (◇). We then tested the effect of the protein boosts on the induction of the immune response induced by the Ad-sig-E7/ecdCD40L vector. The endpoint of these studies was in vivo suppression of the E7 tumor growth in C57BL/6J mice, as measured by the percentage of mice that remained tumor free. As shown in Fig. 6*B*, the s.c. injection of the E7/ecdCD40L protein induced complete regressions of existing tumor and converted tumor-positive mice to tumor-negative mice (see Fig. 6*B*, ▲). These data suggested that the protein boost could induce complete regressions in existing tumor that was progressive in 18-mo-old mice.

Discussion

We have successfully used two transgenic mouse models in which anergy exists to TAA to show that the s.c. injection of the Ad-sig-TAA/ecdCD40L vector induces a cellular and humoral immune response to the rH2N and hMUC-1 Ags. The results also suggest that the Ad-sig-TAA/ecdCD40L adenoviral vector induces an immune response that is more forceful than previous studies involving bacterial cells to deliver the TAA/ecdCD40L gene (22), because the oral DNA vaccine used in these latter studies (22) required an IL-2 cytokine boost. In contrast, the Ad-sig-TAA/ecdCD40L vector s.c. injections completely suppressed the growth of the TAA-positive tumor cells without any boosts.

The addition of hMUC-1/ecdCD40L and rH2N/ecdCD40L protein booster s.c. injections to the s.c. injection of the Ad-sig-hMUC-1/ecdCD40L and Ad-sig-rH2N/ecdCD40L adenoviral vectors further increased the level of Ag-specific T cells and Abs induced by the vector vaccination. It is clear from the results shown in Fig. 1, *C–F*, that the hMUC-1/ecdCD40L protein, when administered without antecedent vector injection, is less effective than the Ad-sig-hMUC-1/ecdCD40L vector or the vector prime-protein (VPP) boost vaccine. These results suggested that the vector prime/protein boost vaccination strategies may be useful for the development of vaccines for cancers of the breast, lung, colon, ovary, prostate, endometrium, and cervix, because >90% of these epithelial neoplasms exhibit overexpression of the hMUC-1 protein (23).

One of the most challenging aspects of activating and maintaining an immune response against cancer cells, is the barrier that must be overcome to deliver the Ag-specific Abs and T cells to the tumor cells into the extravascular space. One obvious solution to this problem is to change the target of the vaccine induced immune response from the cancer cells themselves to the markers that are uniquely expressed on the luminal membrane of tumor vascular endothelial cells. Some of the most interesting of these tumor vascular endothelial markers are those that do not appear on the vessels in normal tissue and may not even appear on the growing vasculature of normal tissue undergoing the process of repair and healing after injury.

The preliminary experimental results obtained with the Ad-sig-AnxA1/ecdCD40L vector vaccine are therefore very exciting. The fact that the growth of rH2N-positive tumor cells, which were negative for the AnxA1 tumor vascular Ag, were suppressed in their growth by the vaccine, suggests that an immune response directed to Ags not present on the tumor cells, but present on the tumor vascular endothelial cells, can suppress the growth of the cancer. The fact that the vascular cells are genetically stable, and not capable of the immunological escape mechanisms constantly at play when an immune response is directed to the tumor tissue, may turn out to be an important advantage in the use of this vaccine to control breast cancer.

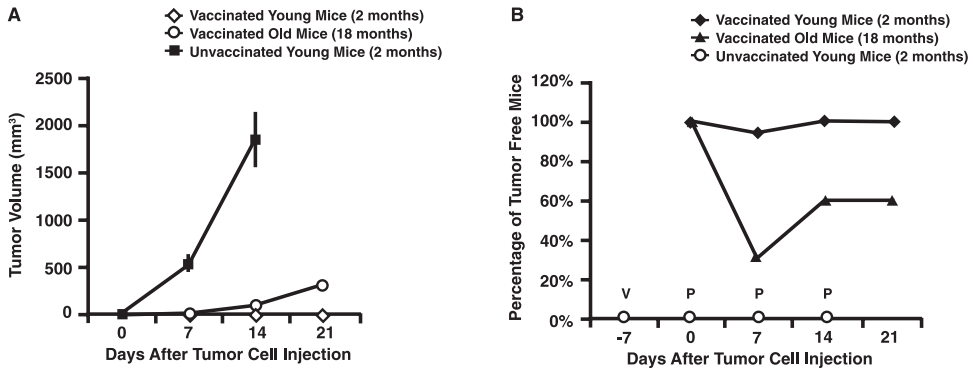


FIGURE 6. Effect of the Ad-sig-E7/ecdCD40L vector prime-E7/ecdCD40L protein (3×) boost (VPPP) vaccine against the E7 viral Ag in old (18 mo) and young (2 mo) mice on growth of E7-positive TC-1 tumor cells. C57BL/6J mice were injected once s.c. with the Ad-sig-E7/ecdCD40L vector and then three times s.c. with E7/ecdCD40L protein injections (every 7 days) starting 7 days after the vector injection. Ten micrograms of the E7/ecdCD40L protein boost were used for each injection. The tests were conducted in 18-mo-old mice, or 2-mo-old mice. The results are expressed as the change in the volume of the s.c. nodules of the TC-1 cells (*A*) or the percentage of mice that are tumor free at any point following the tumor injection and vaccination (*B*). V = Ad-sig-E7/CD40L vector; P = E7/ecdCD40L protein.

The experimental results showed that the levels of effector T cells in the tumor tissues are increased 3-fold following the Ad-sig-hMUC-1/ecdCD40L vector injection. Moreover, we show that these T cells are releasing the CCL3 chemokine ligand, which attracts CCR5-positive effector T cells into the tumor tissue. This result shows that there are increased levels of the effector T cells in the tumor tissue after vaccination, and that these cells are programmed to attract additional T cells into the tumor tissue.

Many workers have shown that, as mice age, although the total number of T cells stays the same, the ratio of naive/memory CD8 cells decreases. This may be due to the involution of the thymus gland, which is associated with the failure to maintain adequate levels of IL-7, and hormonal changes in puberty. This results in a reduction of the repertoire of CD8 T cells available for the immune response. Aged mice will also show oligoclonal expansion of T cells during immunostimulation. In addition, growth of tumor cell lines in mice for >5 days has been reported to be associated with the emergence of anergy to tumor cell Ags.

Previous studies (24) have indicated that the number of IFN-γ-secreting effector CD8 T cells induced by vaccination as well as CD4 cells (25) are decreased in the elderly vs young test subjects after vaccination. In addition, the kinetics of development of the immune response as measured by the peak day of the IFN-γ-secreting effector CD8 T cell level is slower in older animals and in elderly human subjects than in young test subjects (24).

It has been reported that the level of CD154 (CD40L) on CD4 T cells is lower in older mice and test subjects following exposure to vaccination than is the case in younger test subjects (1, 25). The presence of the CD40L on the TAA/ecdCD40L protein serves to replace the need for CD40L on CD4 cells. However, we do not know to what extent the Ad-sig-TAA/ecdCD40L vaccine is also indirectly inducing increases in the level of CD40L on CD4 T cells, thus overcoming the functional defect of these cells in older mice or test subjects.

Previous studies from other laboratories have shown that the levels of negative regulatory CD4 FOXP3-positive T cells is higher in the tumor tissue of older mice than is the case in young mice. We have shown that the Ad-sig-E7/ecdCD40L vector vaccine can induce a three times decrease in the level of the negative regulatory CD4 FOXP3-T cells in 18-mo-old mice for a foreign Ag. The combination of increased effector CD8 T cells and diminished levels of negative regulatory CD4 FOXP3-T cells in the tumor tissue induced by the vaccination is undoubtedly responsible for the conversion of 18-mo-old mice with tumor progression into tumor-free mice. These data (see Fig. 6*B*) suggest that the vector prime-protein boost vaccine strategy can overcome tolerance to TAA in tumor progressor mice in 18-mo-old mice.

On the basis of the results reported in this paper, arrangements have been made for a phase I clinical trial of the Ad-sig-hMUC-1/ecdCD40L vaccine in breast cancer patients whose disease has recurred following initial local therapy. Ultimately, this vaccine could be of use in reducing the recurrence rate in patients at high risk of recurrence following definitive local therapy in these and other epithelial neoplasms.

Acknowledgments

Many thanks to Dr. Sandra Gendler of the Mayo Clinic Research Laboratories (Scottsdale, AZ) for the gift of the hMUC-1.Tg mice and many informative discussions. We acknowledge Gosia Czarny for expert support with confocal microscopy and Carina Rumold for assistance with use of the FACS ARIA preparative sorting of T cell subsets. Many thanks to Dr. Amy K. Stout of the National Institutes of Health Tetramer Core Facility at Emory University for providing the tetramers in Fig. 5*B*.

Disclosures

The authors have no financial conflict of interest.

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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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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

Received 1/26/06; revised 3/17/06; accepted 6/15/06.

Grant support: Sidney Kimmel Cancer Center; Breast Cancer Research Foundation, U.S. Army Breast Cancer Research Program grants DAMD17999457 and BC022063, Bush Leukemia Research Foundation, Anthony Dewitt Frost Melanoma Research Fund, and Audrey Demas Melanoma Research Fund (A. Deisseroth); and Turkish Academy of Sciences grant TUBA-GEBIP-2001-1 and National Cancer Institute (H. Akbulut).

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doi:10.1158/1535-7163.MCT-06-0049

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

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)	

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*

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.

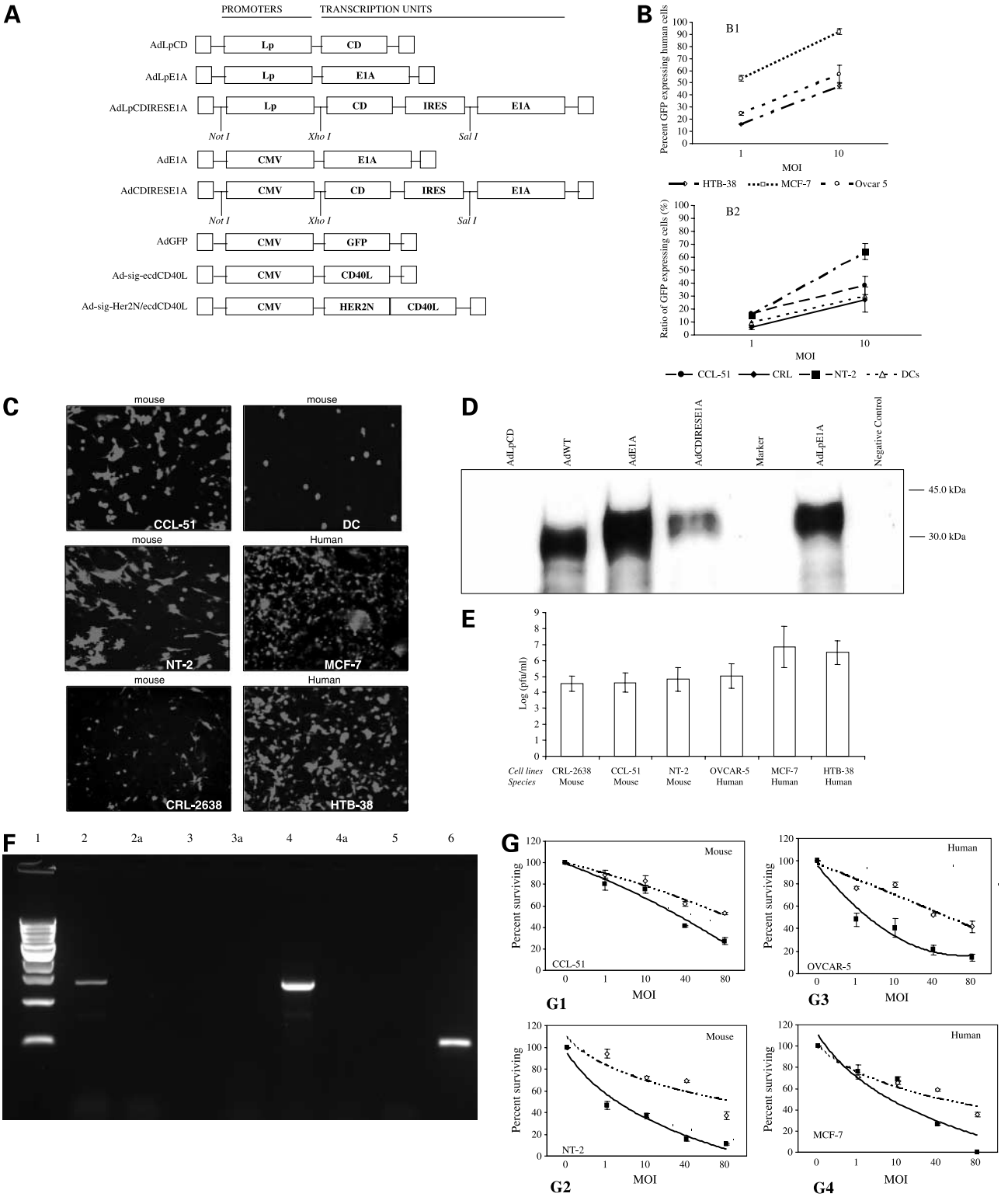
Cell-Mediated Cytotoxicity Assay of Splenic T Cells from the Vaccinated Mice. Cell-mediated cytotoxicity was assayed by an antibody to the apoptosis-associated caspase-3 by flow cytometry. Seven days after the injection of dendritic cells, the spleens of the mice were removed and $CD8^+$ cells were isolated. $CD8^+$ 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

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).



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*).

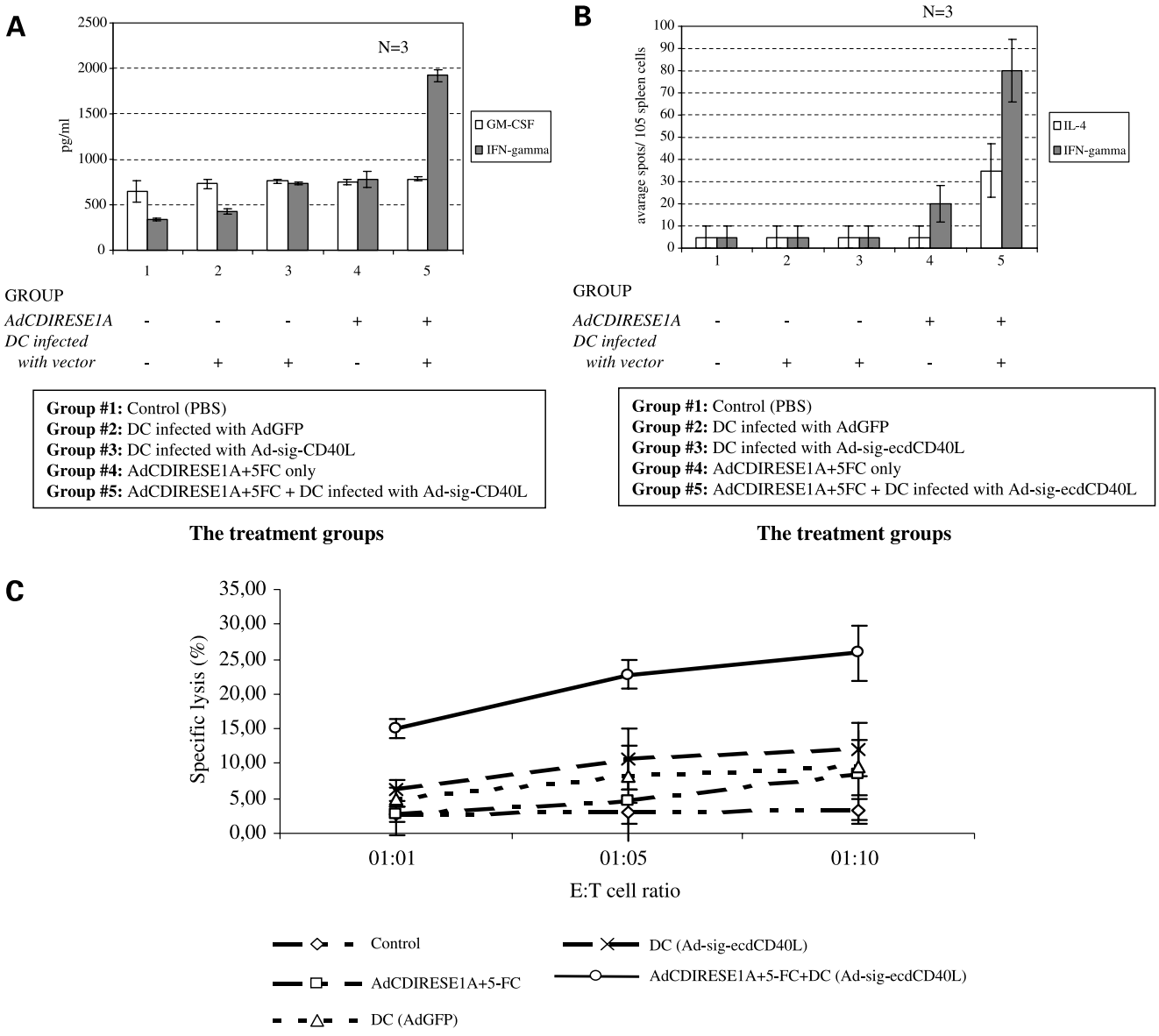


Figure 2. Induction of specific immunity by the i.t. injected dendritic cells. **A,** cytokine release from the activated splenic T cells of BALB/c mice. The T cells from the dendritic cell + gene therapy with a replication-competent vector carrying CD transcription unit released significantly more IFN-γ 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

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

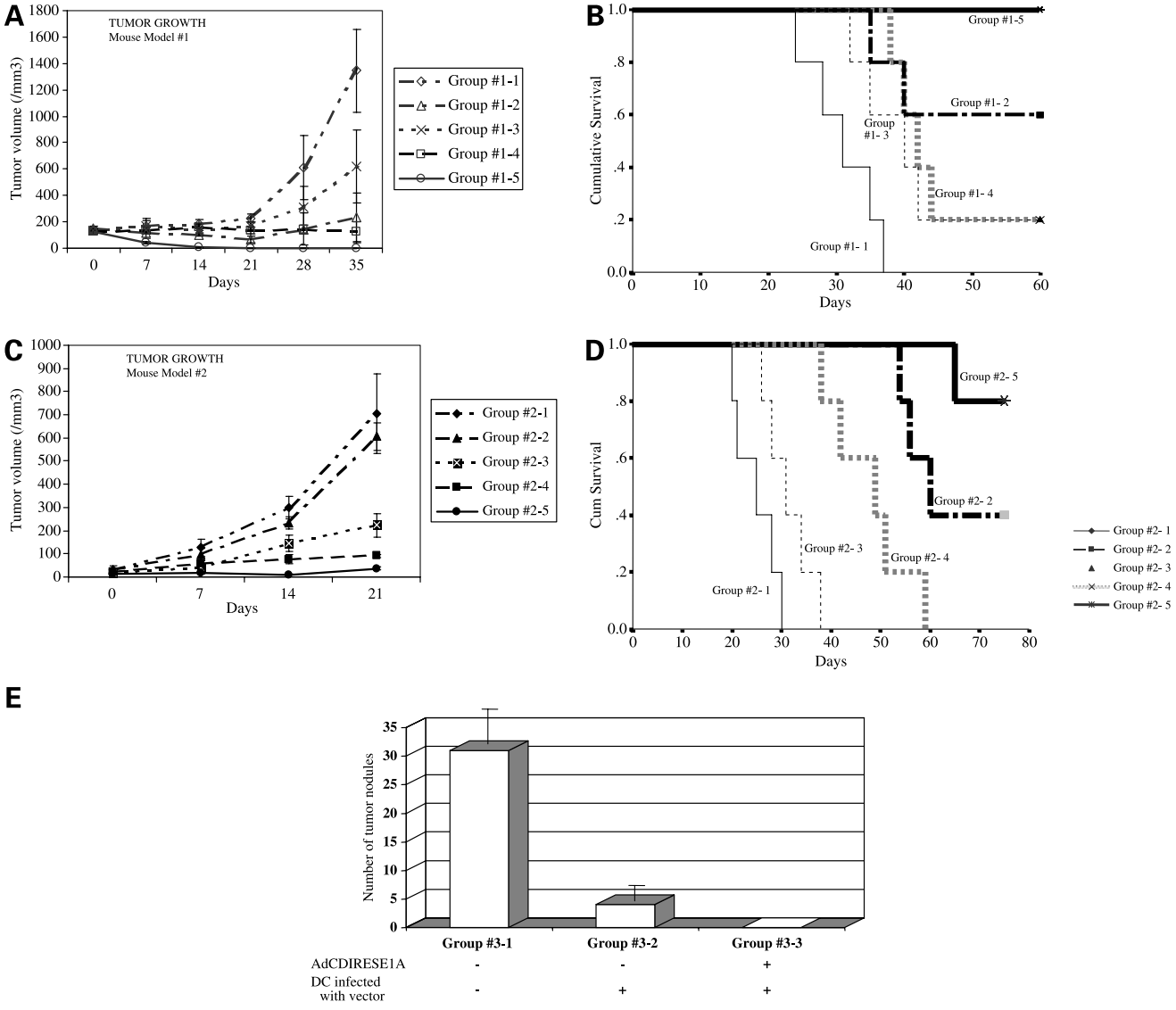


Figure 3. *In vivo* efficacy of the combination of i.t. injection of AdCDIRESE1A + 5-FC system and dendritic cells. **A,** effect of vaccine on tumor cell growth in BALB/c mice injected s.c. with CCL-51 cells (mouse model 1). In mice treated with combined i.t. injection of vector-infected dendritic cells 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).

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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Multistep process through which adenoviral vector vaccine overcomes anergy to tumor-associated antigens

Yucheng Tang, Lixin Zhang, Jing Yuan, Hakan Akbulut, Jonathan Maynard, Phyllis-Jean Linton, and Albert Deisseroth

Our goal in the present work was to characterize the multiple steps involved in overcoming the anergy that exists in tumor hosts to tumor-associated antigen (TAA). Our studies showed that the subcutaneous injection of the Ad-sig-TAA/ecdCD40L vector resulted in secretion of the TAA/ecdCD40L protein for at least 10 days from infected cells. Binding of the TAA/ecdCD40L protein to dendritic cells (DCs) resulted in the induction of CCR-7 chemokine receptor expression and cytokine release. This was followed by migration of the DCs to regional lymph nodes. Tetramer staining, enzyme-linked immunospot (ELISPOT) assay, and cytotoxicity assay all showed that the Ad-sig-TAA/ecdCD40L vector increased the levels of splenic $CD8^+$ T cells specific for the 2 TAAs (human MUC1 [hMUC1] and HPV E7) tested. Vaccination with the Ad-sig-hMUC1/ecdCD40L vector suppressed the growth of hMUC1 antigen–positive tumor cells in 100% of the test mice that were previously anergic to the hMUC1 antigen. These data suggest that Ad-sig-TAA-ecd/ecdCD40L vector injections may be of value in treating the many epithelial malignancies in which TAA-like hMUC1 is overexpressed. (Blood. 2004;104:2704-2713)

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Introduction

We previously reported that subcutaneous injection of the Ad-sig-TAA/ecdCD40L vector can overcome the anergy in tumor hosts against tumor-associated antigen (TAA).[1] Dendritic cells (DCs) are specialized cells of the immune system responsible for the initiation and regulation of cellular and humoral responses. The ability of DCs to regulate immunity is dependent on DC maturation. In the absence of costimulatory molecule expression on the DC surface, the presentation of TAA to naive T cells can lead to T-cell anergy caused by the induction of apoptosis in the T cells.[2]

Human DCs require multiple activation signals for the efficient generation of tumor antigen–specific T lymphocytes.[3,4] These changes endow DCs with the ability to costimulate antigen-specific $CD8^+$ and $CD4^+$ T-cell responses and to foster $CD8^+$ T-cell differentiation into cytotoxic lymphocytes (CTLs).[5,6] The fact that antigen-loaded DCs can generate antitumor immune responses capable of eradicating established tumors in vivo has been documented in a number of animal tumor models. Strategies for loading DCs with TAA include the pulsing of tumor cell RNA into DCs, the mixing of tumor cell lysates with DCs, and the in vitro addition of recombinant peptides of proven binding capability to DCs.[7-13] DC vaccination leads to tumor regression in selected patients with advanced cancer, but the weight of clinical trial data suggests that in vivo activation and tumor antigen loading of DCs might provide an advantage over in vitro activation strategies.

To develop an in vitro strategy of activation and tumor antigen–loading of DCs with which to overcome anergy to TAA, we built on the oral DNA vaccine/interleukin-2 (IL-2) targeting strategy of Xiang et al[14] to create an adenoviral vector (Ad-sig-TAA/ecdCD40L) vaccine. The Ad-sig-TAA/ecdCD40L adenoviral vector encodes a secretable (sig) form of a TAA fused to the extracellular domain (ecd) of the CD40 ligand (CD40L). The ecd of CD40L contains all the sequences necessary to form a functional trimeric CD40L.[15] Our previous studies with this vector show that subcutaneous injection of the Ad-sig-TAA/ecdCD40L vector induced immune resistance to the growth of TAA-positive cancer cells for more than 1 year.[1]

In the present work, we sought to characterize the multiple steps through which the Ad-sig-TAA/ecdCD40L vector induces an immune response to TAA in anergic animals. As shown in Figure 1A, this involves secretion of the TAA/ecdCD40L protein from the Ad-sig-TAA/ecdCD40L vector–infected cells near the subcutaneous injection site for more than 10 days. Binding of the TAA/ecdCD40L protein to the DCs resulted in activated cytokine release, increased levels of the CCR-7 chemokine, and increased membrane levels of the CD80 and CD86 receptors. This induced migration of DCs, which displayed TAA peptides on their surface major histocompatibility complex (MHC) class I molecules, and resulted in increases in the number of TAA-specific $CD8^+$ T cells competent to recognize and kill cancer cells bearing the TAA.[7,16]

We studied 2 types of TAA in this vector vaccination strategy: the human papillomavirus (HPV) E7 foreign antigen, which has been shown to be a strong stimulus of the cellular immune response,[17-20] and the ecd of the human Mucin-1 (hMUC1) self-antigen, which is expressed focally at low levels on normal epithelial cellular surfaces.[21-24] The MUC1 antigen is expressed at high levels diffusely in neoplastic epithelial mucosal cells, thereby

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Submitted December 30, 2003; accepted June 2, 2004. Prepublished online as *Blood* First Edition Paper, July 6, 2004; DOI 10.1182/blood-2003-12-4319.

Supported by the Vaccine Development Fund of the Sidney Kimmel Cancer Center, the George and Barbara Bush Leukemia Research Fund, The Breast Cancer Research Program of the United States Army (DAMD 17 99 9457 and BC022063), and the Breast Cancer Research Foundation of New York City. We recognize the support of Sidney Kimmel to our cancer center, which made this work possible. We thank the following for their support: the Brian Schultz Cancer Research Fund, the Audrey Demas Melanoma Research Fund, and the Anthony Dewitt Frost Melanoma Research Fund.

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An Inside *Blood* analysis of this article appears in the front of this issue.

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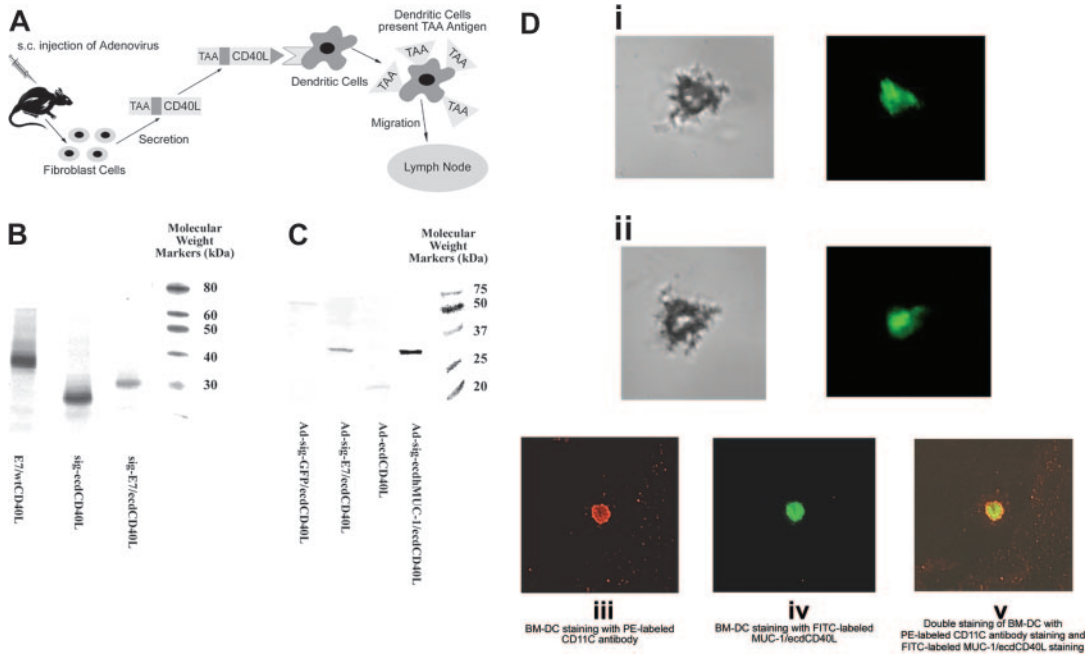

Figure 1. TAA/ecdCD40L protein produced by Ad-sig-TAA/ecdCD40–infected cells binds to DCs. (A) Proposed mechanism for induction of immune response by the Ad-sig-TAA/CD40L vector. Injecting Ad-sig-TAA/ecdCD40L induces in vivo activation and tumor-antigen loading of DCs, migration of the DCs to regional lymph nodes, and activation of $CD8^+$ cytotoxic T cells, which are specific for cells carrying the tumor antigen. (B) In vitro expression of the E7/ecdCD40L transcription unit. Plasmid expression vectors encoding the nonsecretable E7/wtCD40 ligands (lane 1), the secretable ecd of the CD40 ligand (sig-ecdCD40L) alone (lane 2), and the secretable sig-E7/ecdCD40 ligand protein (lane 3) produced in a cell-free transcription/translation system are as predicted: lane 1, E7/wtCD40L is 39 kDa; lane 2, sig-ecdCD40L is 22 kDa; and lane 3, sig-E7/ecdCD40L is 32 kDa. Molecular weight markers are in the extreme right lane. (C) Western blot analysis of the expression of E7/ecdCD40L protein in 293 cells. Molecular weights of the TAA/ecdCD40L proteins produced from 293 cells infected by the Ad-sig-TAA/ecdCD40L vectors adenoviral vectors were as predicted: lane 1, lysates from cells infected with the Ad-sig-GFP/ecdCD40L vector; lane 2, lysates from cells infected with the Ad-sig-E7/ecdCD40L vector; lane 3, lysates from cells infected with the Ad-sig-ecdCD40L vector; and lane 4, lysates from the Ad-sig-ecdhMUC1/ecdCD40L vector. Molecular weight markers are in the extreme right lane. (D) Secretory form of TAA/ecdCD40L binds in vitro to DCs. Bone marrow–derived DCs were fractionated to 78% purity. (i-ii) FITC-labeled E7/ecdCD40L recombinant proteins released from Ad-sig-E7/ecdCD40L–infected 293 cells were incubated with bone marrow–derived DCs. Cells were portioned with light microscopy (left panels) to demonstrate the morphology of the DCs and then with fluorescence microscopy (right portion panels) to detect the binding of the fluoresceinated proteins. (i) DCs incubated with FITC-labeled proteins from the supernatant of cells infected with the Ad-sig-E7/ecdCD40L. (ii) DCs incubated with FITC-labeled proteins from the supernatants of cells infected with the Ad-sig-ecdCD40L vector. (iii-v) Proteins released from Ad-sig-ecdhMUC-1/ecdCD40L-infected 293 cells were fractionated on a Nickel column to purify the His-tagged ecdhMUC-1/ecdCD40L proteins. These proteins were fluorescein labeled, as outlined in "Materials and methods." FITC-labeled ecdhMUC-1/ecdCD40L proteins and a PE-conjugated rat antimouse CD11C antibody were added to the purified DCs. (iii) Cells exposed to a laser excitatory for phycoerythrin. (iv) Cells exposed to a laser excitatory for FITC. (v) Overlay of the images from subpanels iii and iv. A Nikon Eclipse TE-2000-U microscope, which was equipped with a Perkin Elmer UltraView R55 spinning disk confocal attachment, was used at 20 × N.A. 0.5. Adobe Photoshop was the software used.

disrupting the regulation of anchorage-dependent growth, which leads to metastases.[22,23] The MUC1 antigen is a self-protein overexpressed in carcinomas of the breast, ovary, lung, prostate, colon, and pancreas, among other carcinomas.[21] Overexpression in epithelial cancers is thought to disrupt E-cadherin function, leading to anchorage-independent growth and metastases.[22] Although non-MHC–restricted cytotoxic T-cell responses to MUC1 have been reported in patients with breast cancer,[23] hMUC1 transgenic mice (MUC1.Tg) have been reported to be unresponsive to stimulation with hMUC1 antigen.[24]

Our results show that immunizing hMUC1 transgenic mice, which are anergic to the hMUC1 antigen,[24] with the Ad-sig-hMUC1/ecdCD40L vector induces a $CD8^+$ T cell–dependent systemic T-helper 1 (T_H1) immune response that is antigen specific and HLA restricted and that overcomes the block in proliferation that exists in T cells in anergic hosts. Vaccination increases the frequency of hMUC1-specific T cells in the spleens of injected mice. This response requires the Ad-sig-ecdhMUC1/ecdCD40L adenoviral vector and cannot be produced by subcutaneous injection of the hMUC1/ecdCD40L protein alone. Using a similar vector system, but with the E7 antigen in place of the hMUC1 antigen, we showed that the Ad-sig-E7/ecdCD40L vector injection induced immune responses against E7-positive TC-1 tumor cells in 100% of the injected mice for up to 1 year. These results suggest that Ad-sig-TAA/ecdCD40L vector injections induce a memory cell response against TAA-positive tumor cells without the need for additional cytokine boosting treatments.

Materials and methods

Mice and cell lines

Six- to 8-week-old C57BL/6 mice were purchased from Harlan. MUC1 transgenic mice-C57/BL6/human MUC1[24] were obtained from Dr S. Gendler of Mayo Clinic Scottsdale and were bred on site.

Construction of recombinant adenoviruses

The *E7/ecdCD40L* fusion gene was constructed by ligating the amino terminal end of the ecd of CD40L to an octapeptide linker (NDAQAPKS), which was linked in turn to the carboxyl terminal end of a TAA, the amino terminal end of which was linked to a secretory signal sequence. The oligonucleotide for E7 was 5′-TGG GTT CCA GGT TCC ACT GGT GAC ATG CAT GGA G AT ACA CCT AC-3′ and 5′-CCG CTC GAG TGG TTT CTG AGA ACA GAT GGG GCA C -3′. This oligonucleotide was cloned to the pcDNA3TOPO vector. Coding sequences for the full-length mouse CD40 ligand were generated by using the following primers: 5′-GAGAC CTC GAG AAC GAC GCA CAA GCA CCA AAA AGC ATG ATA GAA ACA TAC AGC CAA C-3′ and 5′-CCG CGC CCC AAG CTT ATC AGA GTT TGA GTA AGC CAA AAG-3′. The CD40L template is the plasmid pDC406-mCD40L (American Type Culture Collection, Manassas, VA). Polymerase chain reaction (PCR) conditions are as per protocol from Tgo

DNA polymerase kit (Roche Diagnostics, Mannheim, Germany): 94°C for 3 minutes, 25 cycles at 94°C for 30 seconds, 56°C for 45 seconds,72°C for 45 seconds, and 1 cycle at 72°C for 7 minutes. The PCR fragment was inserted into the plasmid pcDNA3-E7 after restriction endonuclease digestion with *Xba*I (TCTAGA) and *Xho*I (CTCGAG). This vector was named pCDNA3CE7/wtCD40L. The E7/wt encoding DNA was cut from pCDNA3CE7/wtCD40L using *Hin*dIII-*Xba*I restriction endonuclease digestion that was then inserted into pShuttle-cytomegalovirus (CMV) downstream of the CMV promoter. This plasmid is designated pShuttle-E7/wtCD40L.

The ecdCD40L fragment for pShuttle-ecdCD40L was generated by PCR encoding the mouse immunoglobulin G (IgG) κ chain by 4 rounds of PCR amplification (first round, primers 1 and 5; second round, primers 2 and 5; third round, primers 3 and 5; fourth round, primers 4 and 5). Primers were as follows: (1) 5′-CTG CTCTGG GTT CCA GGT TCC ACT GGT GAC AAG GTC GAA GAG GAA GTA AAC C-3′; (2) 5′-TG CTC TGG GTT CCA GGT TCC ACT GGT GAC ATG CAT G-3′; (3) 5′-TC CTG CTA TGG GTA CTG CTG CTC TGG GTT CCA GGT TC3′; (4) 5′-ACG ATG GAG ACA GAC ACA C TC CTG CTA TGG GTA CTG CTG-3′; (5) 5′-CCG CGC CCC TCT AGA ATC AGA GTT TGA GTA AGC CAA AAG-3′.

The CD40L template is the plasmid pDC406-mCD40L (American Type Culture Collection). PCR conditions are per protocol from Tgo DNA polymerase kit (Roche Diagnostics). Conditions are the same as given earlier in this section. Fragments of ecdCD40L were cloned into the pcDNA3.1TOPO vector (Invitrogen, Carlsbad, CA), then cut from the pCDNA3-hMUC1/ecdCD40L vector using *Hin*dIII-*Xba*I restriction endonuclease digestion and inserted into pShuttle-CMV downstream of the CMV promoter and named pShuttle-ecdCD40L.

A transcription unit that included DNA encoding the signal sequence of the mouse IgG κ chain gene upstream of DNA encoding hMUC-1 was generated by PCR using plasmid pcDNA3-hMUC-1 (gift of O.J. Finn, University of Pittsburgh School of Medicine, PA) and the following primers. DNA encoding the mouse IgG κ chain METDTLLLWVLLLWVPGSTGD (single-letter amino acid code) was prepared by PCR amplification to generate the full 21–amino acid mouse IgG κ chain signal sequence: (1) 5′-CCACC ATG GAG ACA GAC ACA CTC CTG CTA TGG GTA CTG CTG-3′; (2) 5′-TC CTG CTA TGG GTA CTG CTG CTC TGG GTT CCA GGT TC-3′; (3) 5′-TG CTC TGG GTT CCA GGT TCC ACT GGT GAC GAT G -3′; (4) 5′-GGT TCC ACT GGT GAC GAT GTC ACC TCG GTC CCA GTC-3′; (5) 5′-GAG CTC GAG ATT GTG GAC TGG AGG GGC GGT G-3′. K/hMUC-1 with the upstream κ signal sequence was generated by 4 rounds of PCR amplification (first round, primers 4 and 5; second round, primers 3 and 5; third round, primers 2 and 5; fourth round, primers 1 and 5). PCR conditions are the same as given earlier in this section. The hMUC-1 encoding DNA was cloned into the pcDNA3.1TOPO vector (Invitrogen) forming pcDNA-hMUC-1. A pair of PCR primers was designed for ecdCD40L without the cytoplasmic and transmembrane domains: 5′-CCG CTC GAG AAC GAC GCA CAA GCA CCA AAA TCA AAG GTC GAA GAG GAA GTA -3′; 5′-GCG GGC CCG CGG CCG CCG CTA GTC TAG AGA GTT TGA GTA AGC CAA AAG ATG AG-3′. The CD40L template is the plasmid pDC406-mCD40L (American Type Culture Collection). PCR conditions are as per protocol from the Tgo DNA polymerase kit (Roche Diagnostics), which are the same as earlier in this section. The PCR fragment was inserted into the plasmid pcDNA-hMUC-1 after restriction endonuclease digestion with *Xba*I (TCTAGA) and *Xho*I (CTCGAG). This vector was named pCDNA3-hMUC1/ecdCD40L. The hMUC1/ecdCD40L encoding DNA was cut from the pCDNA3-hMUC1/ecdCD40L vector using *Hin*dIII-*Xba*I restriction endonuclease digestion and was inserted into pShuttle-CMV downstream of the CMV promoter. The plasmid is designated pShuttle-hMUC1/ecdCD40L.

Coding sequences for the full-length mouse CD40L were generated by using the following primers: 5′-GAG ACC TCG AGA ACG ACG CAC AAG CAC CAA AAA GCA TGA TAG AAA CAT ACA GCC AAC-3′ and 5′-CCG CGC CCC AAG CTT ATC AGA GTT TGA GTA AGC CAA AAG-3′. The CD40L template is the plasmid pDC4mCD40L (American Type Culture Collection). PCR conditions as per protocol from the Tgo DNA polymerase kit (Roche Diagnostics) are the same as given earlier in this section. Using PCR methods, in some vectors, we added the mouse HSF1 trimer domain between MUC-1 and CD40L and a His tag at the end of the CD40L. Fragments of the TAA/CD40L fusion were inserted downstream of the CMV promoter in the pShuttleCMV expression vector using the *Xho*I and *Xba*I restriction sites. The ecd of the CD40L and the full-length-wtCD40L was amplified by PCR primers and cloned into the pShuttleCMV plasmid using the *Hin*dIII and *Xba*I restriction endonuclease sites. Recombinant adenoviral vectors were generated using the AdEasy vector system.[25]

All populations of vector particles used in the experiments described in this paper were shown to contain fewer than 5 replication-competent adenoviral particles (RCAs) per 1×10^{10} viral particles (VPs).

Western blotting and in vitro expression of the E7/ecdCD40L transcription unit

Western blotting and in vitro cell-free transcription/translation were used to analyze protein expression from the vector transcription units as described previously.[30] The coupled in vitro transcription–translation system of reticulocyte lysate (RRL) (TNT kits; Promega, Madison, WI) was used to synthesize the protein products of the transgenes of the following vectors: Ad-sig-E7/ecdCD40L, Ad-E7/wtCD40L (where wt indicates the full-length or wild-type *CD40L* gene), Ad-sig-ecdCD40L, Ad-wtCD40L, and Ad-sig-ecdhMUC1/ecdCD40L. The protein cell lysate derived from 293 cells infected by each adenoviral vector described in the preceding sentence at a multiplicity of infection (MOI) of 40 was fractionated on a 10% reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). After blocking with 5% nonfat milk for 2 hours at room temperature, the membrane was probed with an antibody against the specific mouse CD40L (mCD40LM; eBioscience, San Diego, CA) in TBS-T buffer (20 mM Tris-HCl [pH 7.6], 137 mM NaCl, and 0.5% Tween 20) in the presence of 2% bovine serum albumin (BSA) overnight. After 4 washes with TBS-T buffer, the blot was incubated with a goat antihamster alkaline phosphatase–conjugated antibody (Jackson ImmunoResearch, Bar Harbor, ME) for 1 hour. Immunoreactive bands were visualized on membranes by using the ProtoBlot II AP system (Promega).

Assay for binding of the TAA/CD40L protein to DCs

DCs were derived from incubation of bone marrow mononuclear cells in granulocyte macrophage–colony-stimulating factor (GM-CSF) and IL-4 for 7 days, followed by purification to a purity of 78% DCs. The TAA/CD40L proteins were generated by exposing 293 cells to either the Ad-sig-E7/ecdCD40L vector (Figure 1Di-ii) or the Ad-sig-ecdhMUC-1/ecdCD40L(His-tagged) vector (Figure 1Diii-v). In Figure 1Di-ii, no purification of the proteins was carried out, whereas in panel C, nickel column purification of the ecdhMUC-1/ecdCD40L proteins was carried out. The TAA/CD40L proteins were fluorescently labeled with the Fluoreporter fluorescein isothiocyanate (FITC)–protein labeling kit (Molecular Probes), added to the DCs at a final concentration of 10 μg/mL, and incubated for 30 minutes. Cells were then washed 3 times with cold medium, fixed with 1% paraformaldehyde, and observed under a fluorescence microscope.

Assay for activation of bone marrow–derived DCs

DCs were incubated with the supernatant from 293 cells infected by Ad-sig-TAA/ecdCD40L adenoviral vectors, and then plated in 24-well plates at 2×10^5 cells/mL. After incubation for 24 hours and 48 hours at 37°C, the supernatant fluid (1 mL) was harvested and centrifuged to remove debris. The level of murine IL-12 or interferon-γ (IFN-γ) released into the culture medium from vector-infected cells was assessed by enzyme-linked immunoadsorbent assay (ELISA), using mouse IL-12 p70 or IFN-γ (R&D Systems, Minneapolis, MN), respectively. Bone marrow cells were incubated for 5 days in GM-CSF and IL-4. DCs were purified with the SpinSep Mouse Dendritic Cell enrichment kit (Stem Cell Technologies, Vancouver, BC, Canada). Forward and side scatter analyses of the populations before and after fractionation are given in Figure 1B-C. We then stained the bone marrow–derived DCs before and after fractionation with phycoerythrin (PE)–labeled CD11c antibody, incubating nonenriched and enriched cells for 10 minutes on ice with 5% normal rat serum to block the nonspecific

background before adding fluorochrome-conjugated antibodies. Then we stained DC fractions with PE-labeled CD11c antibody.

Detection of CCR-7 mRNA by RT-PCR

Total RNA extracted from DCs was analyzed for CCR-7 mRNA as described previously.[26] Primers for detecting CCR7 and the GAPDH control were as follows: for CCR7 sense, 5′-TCC TCC TAA TTC TTC CCT TC-3′; for CCR7 antisense, 5′-AAA CTC ATA GCC AGC ATA GG-3′); for GAPDH sense, 5′-TTG TGA TGG GTG AAC CAC-3′; and for GAPDH antisense, 5′-CCA TGT AGG CCA TGA AGT CC-3′. Expected sizes of the amplified fragments were 400 bp for CCR7 and 525 bp for GAPDH. Amplified samples were resolved on ethidium bromide–stained agarose gels. Total cellular RNA was extracted using the Trizol reagent (Life Technologies, Burlington, ON, Canada). Reverse transcription–polymerase chain reaction (RT-PCR) was performed on 5 μg RNA for the reverse transcription reaction. Half of each cDNA product was used to amplify CCR-7 and GAPDH.

DC migration assays

Bone marrow–derived DCs were loaded with the carboxyfluorescein diacetate succinimidyl ester (CFDA SE) supravital dye for 15 minutes at 37°C (Molecular Probes, Eugene, OR). Rinsed DCs were mixed with each recombinant adenoviral vector at an MOI of 200 and were injected into the left flank of the test mouse. Three days later, axillary lymph nodes draining the region of the injection site for the DCs were removed, and frozen tissue sections were made and observed under the fluorescence microscope.

Immunohistochemical staining

Immunized mice were killed 3 and 10 days after injection of the Ad-sig-E7/ecdCD40 vector. Skin at each site of subcutaneous vector injection was subjected to biopsy, embedded in optimum cutting temperature (OCT) solution, and cut into 5-μm sections. Slides were incubated with rat anti-CD40L antibody (eBioscience) and exposed to biotinylated goat anti–rat IgG antibody (1:200 dilution) and avidin-biotin complex (Vector Laboratories, Burlingame, CA). Stained slides were then mounted and studied under a fluorescence microscope.

Tetramer and ELISPOT assays

PE-labeled H-2D^b tetramers containing HPV16 E7$_{49\text{-}57}$ peptide (RAHYNIVTF) were purchased from Beckman Coulter (Hialeah, FL) and were used for the fluorescence-activated cell sorter (FACS) analysis of peptide-specific CTL immunity. Tetramer-positive and CD8$^+$ cells are shown as percentages of total spleen cells. The presence of E7- and hMUC1-specific effector T cells in the immunized mice was also assessed by carrying out enzyme-linked immunospot (ELISPOT) assays, as previously described.[27]

Cytotoxicity assay

E7-positive TC-1 target cells or LL2/LL1hMUC1-positive target cells (5×10^3) were incubated with splenic mononuclear cells (effector cells) at varying effector-target ratios (100:1, 20:1, and 5:1) for 4 hours at 37°C, in culture media containing 5% fetal bovine serum (FBS). Effector cells had been prestimulated with the TAA-positive cancer cells for 5 days in vitro before the in vitro cytotoxicity assay. Cell-mediated cytotoxicity was determined using a nonradioactive lactate dehydrogenase (LDH) release assay. Student unpaired *t* test was used to determine differences among the various groups in cytotoxicity assays. Statistical significance was defined by the *P* less than .05 level.

In vivo efficacy experiment in mouse model

Mice (5 or 10 per group) were vaccinated through subcutaneous injection with 1×10^8 plaque-forming units (pfus) of the Ad-sig-TAA/ecdCD40L, Ad-TAA, Ad-TAA/wtCD40L, Ad-sig-CD40L, Ad-wtCD40L, or Ad-sig-ecdhMUC1/ecdCD40L vectors. One week later, mice were boosted with the same adenoviral vector regimen as the first vaccination. One week after the last vaccination, mice were challenged by subcutaneous injection of 5×10^5 TAA-positive cancer cells. Tumor volumes were measured in centimeters by caliper, and the volumes were calculated as tumor volume = length × (width^2)/2 (this assumes an elliptical shape).

Analysis of p44/p42 mitogen-activated protein kinase and SAPK/JNK phosphorylation

Western blot analysis of p44/p42 and SAPK/JNK was carried out with kits (no. 9100 for p44/p42 and no. 9250 for SAPK/JNK) from New England Biolabs (Beverly, MA). Responder splenocytes were isolated from vaccinated mice and enriched in CD8$^+$ cells using a murine CD8 T-cell enrichment kit (catalog 13033; StemCell Technologies, Vancouver, BC, Canada). Bone marrow–derived DCs were infected with Ad-sig-ecdlMUC1/ecdCD40L for 2 hours, then washed with phosphate buffered saline and incubated for 48 hours.[28] Responder cells were mixed in a 1:1 ratio with Ad-sig-ecdhMUC1/ecdCD40L infected antigen-presenting cells (APCs), and Western blot analysis was performed at the indicated time points.

Statistics

All parameters were analyzed using Student *t* test or analysis of variance (ANOVA), followed by the Scheffé procedure for multiple comparisons as post hoc analysis. All data shown are presented as mean ± SEM.

Results

TAA/ecdCD40L protein binds to DCs

Cell free–coupled transcription/translation and Western blot analysis of the E7/ecdCD40L, E7, ecdCD40L, E7/wtCD40L, and wtCD40L proteins were used to study the molecular weights of the proteins produced in cells infected by the Ad-sig-E7/ecdCD40L, Ad-sig-E7, Ad-sig-ecdCD40L, Ad-E7/wtCD40L, and Ad-wtCD40L vectors, respectively. As shown in Figure 1B-C, the molecular weights of these proteins are those predicted.

We then collected the TAA/ecdCD40L proteins from vector-infected 293 cells and labeled these proteins with fluorescein (see "Materials and methods"). These proteins were then incubated in vitro with bone marrow–derived DCs (fractionated to 78% purity) for 30 minutes at 4°C. The DCs were washed and portioned once using light microscopy and again using fluorescence microscopy. As shown in Figure 1Di-ii, the secretable form of E7/ecdCD40L can bind to the DCs.

A second experiment was carried out in which 293 cells were infected with the Ad-sig-ecdhMUC-1/ecdCD40L vector (His tag present), and the proteins were fluorescein labeled after purification of the MUC-1/ecdCD40L proteins on a Nickel column. The cells were exposed to a PE-conjugated anti-CD11C antibody and to the FITC-conjugated ecdhMUC-1/ecdCD40L proteins. The results (Figure 1Diii-v) show that the DCs bind the ecdhMUC-1/ecdCD40L proteins.

E7/ecdCD40L protein can be detected in vivo for up to 10 days in vivo after subcutaneous injection of the Ad-sig-E7/ecdCD40L vector

We then sectioned the skin at the site of intradermal injection of the Ad-sig-E7/ecdCD40L vector to determine when the secretable sig-E7/ecdCD40L protein was released from vector-infected cells. We double stained these sections with an FITC-labeled antibody to the CD40L (CD154), which stained green (Figure 2A), and DAPI, which stained the nuclear DNA blue (Figure 2A). As indicated in Figure 2A, double staining showed that the TAA/CD40L protein bound in vivo to cells near the vector-infected cells for up to 10 days after subcutaneous injection with the Ad-sig-E7/ecdCD40L vector, which carried the secretable TAA/ecdCD40L transcription unit. In contrast, a lower level of double-stained positive cells was

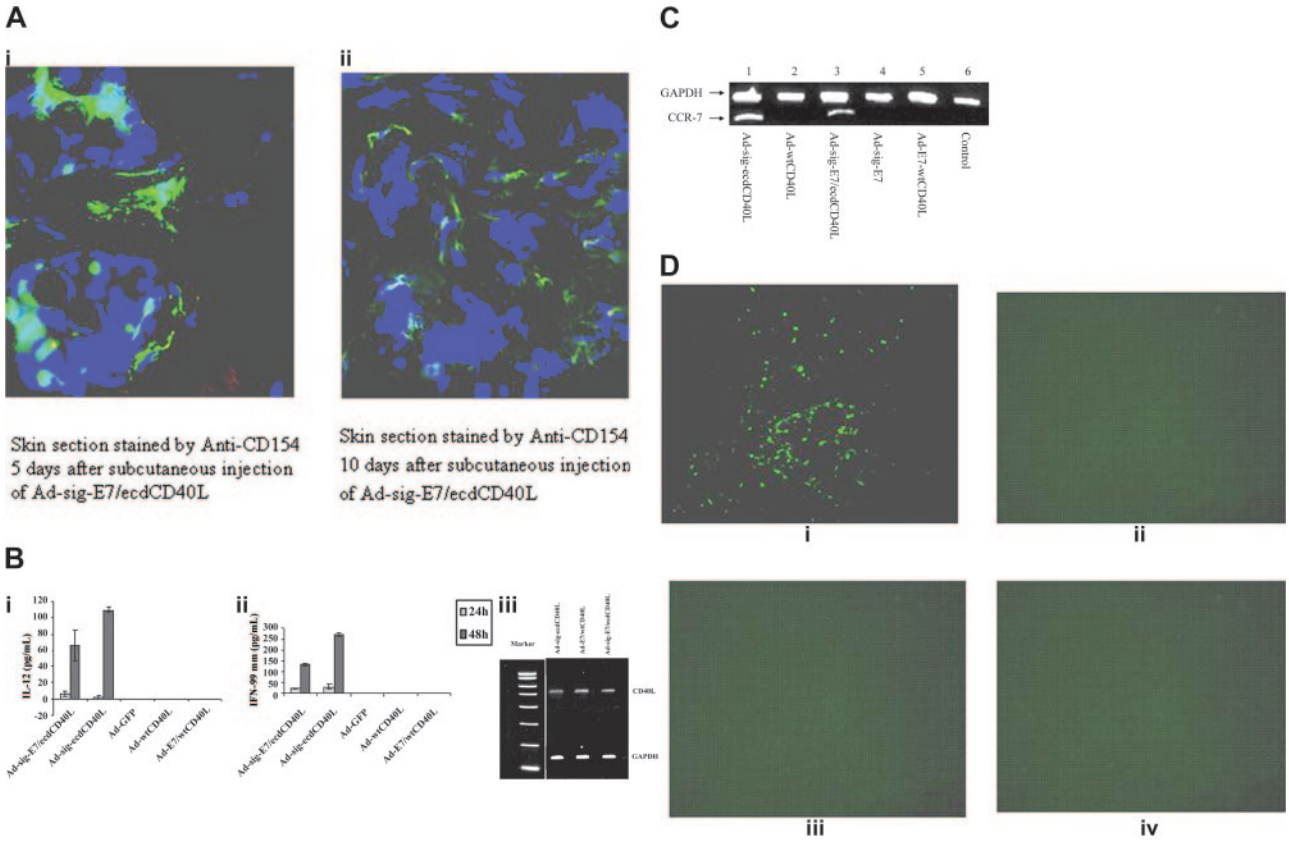

Figure 2. TAA/ecdCD40L protein from Ad-sig-TAA/ecdCD40L vector–infected cells binds to and activates DCs, which induce migration to regional lymphoid tissue. (A) Injection of the Ad-sig-E7/ecdCD40L vector generates the release of the E7/ecdCD40L protein around the vector injection site for up to 10 days. Skin section stained by anti-CD154 and DAPI 5 days (i) and 10 days (ii) after injection of the Ad-sig-E7/ecdCD40L vector. (B) Bone marrow–derived DCs release IL-12 and IFN-γ after exposure to the Ad-sig-E7/CD40L Vector. IL-12 (i) or IFN-γ (ii) released by vector-infected DCs into the supernatant medium was measured by ELISA in DCs stimulated for 24 hours (light gray bars) and 48 hours (dark gray bars) with the adenoviral vectors Ad-sig-E7/ecdCD40L, Ad-ecdCD40L, Ad-GFP, Ad-wtCD40L, and AD-E7/wtCD40L. (iii) Semiquantitative RT-PCR reaction was used to measure the levels of E7/CD40L RNA in 293 cells exposed to the Ad-sig-eE7/ecdCD40L vector or the Ad-E7/wtCD40L vector. 293 cells were infected with the vectors Ad-sig-ecdCD40L, Ad-E7/wtCD40L, and Ad-sig-E7/ecdCD40L at an MOI of 10. Then the RNA was isolated and PCR was carried out with primers specific for E7/CD40L mRNA. The cDNA generated was then fractionated on a molecular-weight gel. The electrophoretic species corresponding to the predicted molecular weight of the PCR product from the E7/CD40L template is indicated in the right-hand margin of the gel by the CD40L label. Electrophoretic mobility of a PCR cDNA product using the same RNA but primers specific for GAPDH (loading control) is indicated in the right-hand margin by glyceraldehyde phosphate dehydrogenase (GAPDH). (C) Up-regulation of CCR-7 mRNA in DCs exposed to the Ad-sig- E7/ecdCD40L vector. Lane 1: the Ad-sig-ecdCD40L vector. Lane 2: the Ad-wtCD40L vector. Lane 3: the Ad-sig-E7/ecdCD40L vector. Lane 4: the Ad-E7 vector. Lane 5: the Ad-E7-wtCD40L vector. Lane 6: uninfected cells (control). (D) In vivo study of migration of DCs to regional lymph nodes after loading of DCs with CFDA SE dye and infection with the Ad-sig-E7/ecdCD40L vector. Bone marrow–derived DCs were loaded in vitro with the CFDA SE supravital dye, exposed in vitro to the following vectors at an MOI of 200. (i) Ad-sig-E7/ecdCD40L. (ii) Ad-ecdCD40L. (iii) Ad-E7/wtCD40L. (iv) Ad-wtCD40L. DCs were then injected subcutaneously into the hind flanks of the test mice. Two days later, regional lymph nodes were dissected and frozen sections were studied under a fluorescence microscope. Color micrographs were obtained.

observed in the epidermis 3 days after injection of the Ad-E7/wtCD40L, which contained a nonsecretable CD40L transcription unit (data not shown).

Activation of DCs by the Ad-sig-E7/ecdCD40L vector

As shown in Figure 2Bi, there was a statistically significant increase in the level of induction of IL-12 production after in vitro exposure of the DCs to the supernatant of Ad-sig-E7/ecdCD40L vector–infected 293 cells. This vector carried a transcription unit encoding a secretable TAA/CD40L protein as in Figure 1. The results were compared with vectors encoding a nonsecretable TAA/CD40L protein, such as the Ad-E7/wtCD40L vector ($P < .0001$). IL-12 (6 ± 3 pg/2×10^5 cells per milliliter per 24 hours or 66 ± 18 pg/2×10^5 cells per milliliter per 48 hours) was produced by DCs exposed to the Ad-sig-E7/ecdCD40L vector supernatant, whereas exposing DCs to the Ad-E7/wtCD40L vector supernatant resulted in no measurable IL-12 at 24 hours or 48 hours.

Similarly, there was a statistically significant increase in the IFN-γ released from DCs exposed to the supernatant from the Ad-sig-E7/ecdCD40L vector–infected cells: 24 ± 3 pg in the first 24 hours and 132 ± 6 pg during the next 24 hours, compared with 0 pg released from DCs exposed to supernatant from 293 cells infected with nonsecretable CD40L vectors or other control vectors (Figure 2Bii). These experimental data suggest that the TAA/ecdCD40L fusion protein secreted from the Ad-sig-TAA/ecdCD40L-infected cells bound to the CD40 receptor on DCs to generate the observed effect on cytokine release.

Differences between the cytokine release induced in bone marrow–derived DCs exposed to the supernatant from 293 cells infected with CD40L secretable or nonsecretable transcription units could be attributed to the E7/CD40L RNA levels generated by the Ad-sig-E7/ecdCD40L (encoding the secretable E7/CD40L protein) compared with the Ad-E7/wtCD40L (encoding the nonsecretable E7/CD40L protein). Another possibility is that one vector encodes a secretable or a nonsecretable protein. To test this question, RNA was extracted from 293 cells that had been infected by either the Ad-sig-E7/ecdCD40L vector or the Ad-E7/wtCD40L vector at an MOI of 10. The cDNA was synthesized by using the superscript first-strand system (Invitrogen, Carlsbad, CA). RT-PCR was performed using 5 μg total RNA extracted from the vector-infected cells and the reverse transcription reaction with a random primer. The cDNA product was split into 2 halves; one half was

used as a template for a PCR reaction with primers specific for the E7/CD40L cDNA, and the other half was used to prime a PCR reaction with primers specific for GAPDH as a control. Results shown Figure 2Biii, indicate no difference in the E7/CD40L mRNA levels using the secretable or the nonsecretable vectors. Thus, it appears that cytokine release is greater from bone marrow–derived DCs exposed to the supernatant from 293 cells infected with the Ad-sig-E7/ecdCD40L rather than the Ad-E7/CD40L vector because of the secretable nature of the E7/CD40L protein from the Ad-sig-E7/ecdCD40L-infected cells.

In vitro and in vivo exposure of DCs to the Ad-sig-E7/ecdCD40L vector elevates CC chemokine receptor-7 (CCR-7) expression in mature DCs and induces the migration of DCs to regional lymph nodes

On antigen exposure, DCs become activated, express CCR-7, and migrate in response to differential gradients of the chemokine ligands CCL 19 and CCL 21.[26] Therefore, we investigated the effect of exposing DCs to supernatants from Ad-sig-E7/ecdCD40L-infected 293 cells to determine whether the level of CCR-7 expression increased. As shown in Figure 2C, the level of CCR-7 mRNA in DCs increased significantly when DCs were cultured with supernatants from Ad-sig-E7/ecdCD40L or Ad-sig-E7/ecdCD40L vector–infected 293 cells.[26]

To formally test whether the subcutaneous injection of the Ad-sig-E7/ecdCD40L vector induces migration of the DCs to the regional lymph nodes in vivo,[26] 1×10^6 DCs were loaded with the CFDA SE dye and were exposed to adenoviral vectors at an MOI of 200. Then, the dye-loaded DCs were injected into the left flanks of the C57BL/6 mice. Three days after these injections, the mice were killed, and the regional axillary lymph nodes on the side of the injection were harvested and studied for the presence of the dye-loaded DCs. As shown by the green dots visible in Figure 2Di, CFDA SE–stained DCs are detectably present in the regional lymph nodes after injection of the vector carrying the secretable E7/ecdCD40L transcription unit, whereas no other vector (Figure 2Dii-iv) was associated with detectable fluorescence-labeled DCs in the regional lymph nodes. No CFDA SE–labeled cells were observed in the nondraining, contralateral lymph nodes. One of the sections was stained with PE-labeled CD11C antibody to confirm that the green-stained cells were DCs (data not shown).

Injection of Ad-sig-E7/ecdCD40L suppresses growth of E7-positive cancer cells in syngeneic mice

To assess the effect of subcutaneous injection of the Ad-sig-E7/ecdCD40L vector on the engraftment of the E7-positive TC-1 cell line in C57BL/6 mice, we injected 1×10^8 pfu of each vector subcutaneously into each animal. Mice were vaccinated again 1

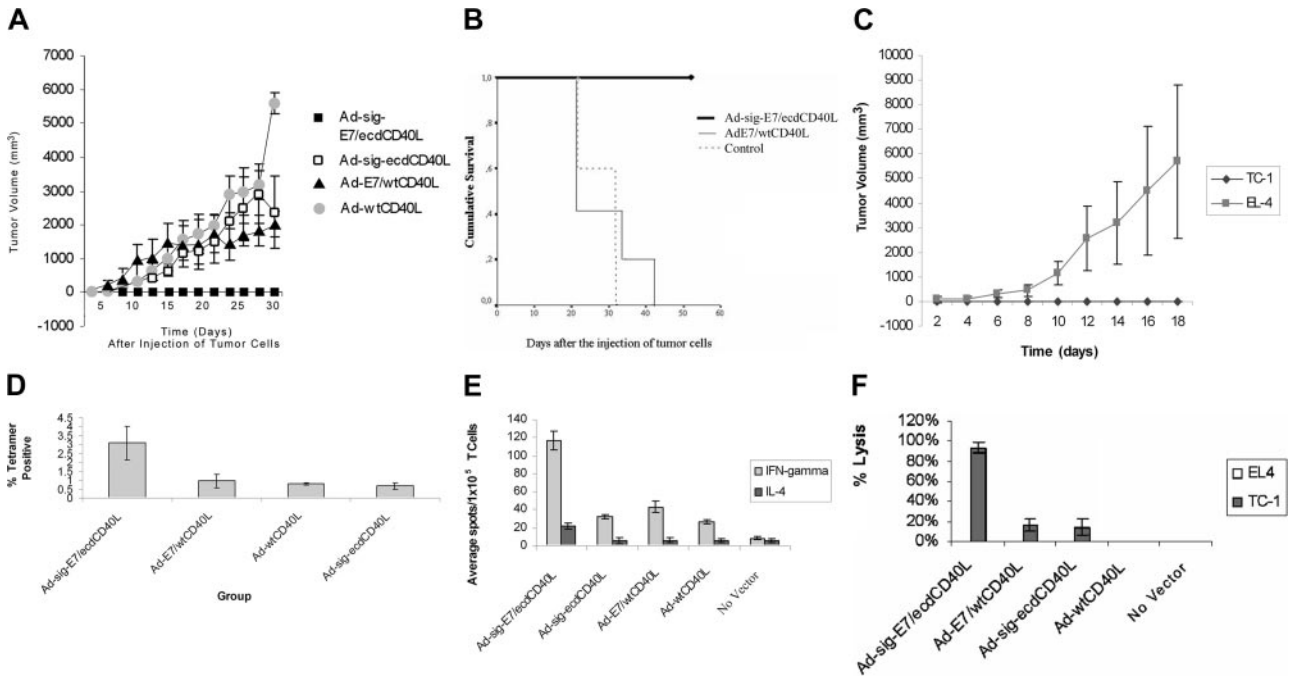

Figure 3. Mechanism of the Ad-sig-E7/ecdCD40L vector–induced suppression of the growth of E7-positive TC-1 tumor cells in C57BL/6 mice. (A) Resistance to the subcutaneous growth of 5×10^5 E7-positive TC-1 cancer cells in mice after 2 injections with 1×10^8 pfu of the Ad-sig-E7/ecdCD40L vector 7 days apart. (■) Ad-sig-E7/ecdCD40L. (□) Ad-sig-ecdCD40L. (▲) Ad-E7/wtCD40L. (●) Ad-wtCD40L. (B) Survival of mice vaccinated with Ad-sig-E7/ecdCD40L vectors. The following vectors were injected into C57BL/6 mice, after which the E7-positive TC-1 cancer cells were injected into the subcutaneous spaces of the mice: bold continuous line, mice treated with 2 subcutaneous injections 7 days apart of 1×10^8 pfu of the Ad-sig-E7/ecdCD40L vector; thin continuous line, mice treated with subcutaneous injections of the Ad-wtCD40L vector; broken thin line, control mice, which were not treated with vector injections. (C) Comparison of the effects of 2 subcutaneous injections of 1×10^8 pfu of the Ad-sig-E7/ecdCD40L vector on the in vivo growth of the E7-positive TC-1 cells (♦) and the E7-negative EL-4 cell line (▨). Sizes of the subcutaneous tumors were estimated by measuring with calipers in 2 separate orthogonal directions and then calculating the volume assuming an elliptical shape. (D) Use of tetramers to measure the level of E7-specific $CD8^+$ T cells in the spleens of Ad-sig-E7/ecd/CD40L vector–immunized C57BL/6 mice. Spleen cells were harvested 10 days after the completion of 2 subcutaneous injections 7 days apart with 1×10^8 pfu of vectors Ad-sig-E7/ecdCD40L, Ad-E7/wtCD40L, Ad-wtCD40L, and Ad-sig-ecdCD40L. T cells were then analyzed for the percentage of $E7_{49\text{-}57}$ peptide-specific $CD8^+$ T-cell lymphocytes by $H\text{-}2D^b$ tetramer staining. (E) ELISPOT assay shows increase in the level of IFN-α–secreting cells in the spleen cells of mice injected subcutaneously twice (7 days apart) with 1×10^8 pfu Ad-sig-E7/ecdCD40 vector. Mice were injected twice with the following vectors: Ad-sig-E7/ecdCD40L, Ad-sig-ecdCD40L, Ad-E7/wtCD40L, and Ad-wtCD40L. Splenic T cells taken from the mice 1 week later were analyzed by ELISPOT assay for the presence of IFN-γ. (F) Increase in the level of E7-specific CTLs in the spleens of Ad-sig-e7/ecdCD40L–injected mice. Mice were injected subcutaneously twice (7 days apart) with 1×10^8 pfu of vectors Ad-sig-E7/ecdCD40L, Ad-E7/wtCD40L, Ad-sig-ecdCD40L, Ad-wtCD40L, and control (no vector injection). T cells were harvested from the spleens of the test mice 1 week after the second adenoviral vector injection and were restimulated in vitro with TC-1. After 7 days, restimulated effector cells (spleen cells exposed to TC-1 cells in vitro) were mixed at varying ratios with TC-1 (E7-positive) and EL-4 (E7-negative) target cells. Then the LDH released from the target cells was measured. No LDH was detectable from any of the mixtures of EL-4 and the restimulated effector cells isolated from the vaccinated mice, whereas significant levels of LDH were released from the TC-1 target cells when they were mixed with the restimulated effector cells isolated from the mice vaccinated with the Ad-sig-E7/ecdCD40L vector.

week later with the same vector. One week after this boost, 5×10^5 E7-positive TC-1 cells were injected subcutaneously on the backs of the C57BL/6 mice at a site different from that of the vector injections. All mice injected with the Ad-sig-E7/ecdCD40L vector remained tumor free throughout the study (up to 18 days after injection), whereas mice injected with all other vectors listed in Figure 3A, including the Ad-E7/wtCD40L vector, which did not carry a secretable TAA/CD40L transcription unit, had measurable tumors within 13 days of tumor challenge (Figure 3A).

As shown in Figure 3B, the survival of the mice injected with the Ad-sig-E7/ecdCD40L vector (bold, unbroken line at the top of the graph) and then injected with the E7-positive TC-1 cells was superior to the survival of mice injected with the Ad-E7/wtCD40L vector (thin, unbroken line), which does not encode a secretable E7/CD40L protein, or injected with no vector (thin, broken line) and then injected with the TC-1 cells.

We then tested whether inducing resistance to engraftment of the E7-positive TC-1 cells was specific for the E7 antigen. As shown in Figure 3C, subcutaneous injection of the Ad-sig-E7/ecdCD40L vector did not protect mice against the engraftment of E7-negative EL-4 cells but did protect against engraftment of the E7-positive TC-1 cells.

Mechanism of suppression of E7-positive tumor cells by Ad-sig-E7/ecdCD40L vector injections

Spleens were harvested 10 days after vector vaccination, and the percentage of $E7_{49\text{-}57}$ peptide-specific $CD8^+$ T cells was determined by H-$2D^b$ tetramer staining. As shown in Figure 3D, the level of E7 peptide–specific T cells in the spleen cells from Ad-sig-E7/ecdCD40L injected animals was increased 3 times compared with the level observed after injection with other vectors, including the Ad-E7/wtCD40L vector.

The frequency of IFN-γ– and IL-4–secreting T cells from the spleens of mice vaccinated with the various vectors was determined by ELISPOT assays.[27] As shown in Figure 3E, mice injected with the Ad-sig-E7/ecdCD40L vector had a greater number of IFN-γ–secreting T cells (117 ± 10.6 spots/1×10^5 spleen cells) than mice injected with the vector carrying the nonsecretable E7/wtCD40L transcriptional unit (26.3 ± 2.4 spots/1×10^5 spleen cells) or any of the other control vectors tested ($P \leq .05$). The number of splenic T cells producing a T_H2 cytokine (IL-4) was only (22.3 ± 3.68 spots/1×10^5 spleen cells). These data indicate that the Ad-sig-E7/ecdCD40L vector vaccination stimulates a T_H1 rather than a T_H2 immune response.

Spleen cells from mice injected with the Ad-sig-E7/ecdCD40L vector were prestimulated in vitro for 7 days with TC-1–positive cells and then mixed in a 100:1 ratio with E7-positive TC-1 cells in a cytotoxicity assay described in "Materials and methods." These studies showed that the splenic T cells from the Ad-sig-E7/ecdCD40L vector–sensitized animals lysed 90% of the TC-1 target cells (Figure 3F). In contrast, spleen cells from uninjected mice or from mice injected with the Ad-E7/wtCD40L vector lysed 0% or 20% of the target cells, respectively.

To test whether the induced cytolytic immune response was mediated through an HLA-restricted process, we added anti-MHC class I antibody or an isotype-matched control antibody to the mixture of effector spleen cells from Ad-sig-E7/ecdCD40L vector–injected mice and E7-positive TC-1 target cancer cells. Adding the anti-HLA antibody suppressed cytotoxicity to the TC-1 target cells to 10.32%, which is significantly lower than the cytotoxicity found with control antibody (76.91%).

Injection of the Ad-sig-ecdhMUC1/ecd/CD40L vector overcomes anergy to hMUC1-positive cells in mice transgenic for the *hMUC1* gene

We first exposed bone marrow–derived DCs to the Ad-sig-ecdhMUC1/ecdCD40L vector or to the Ad-sig-ecdhMUC1 vector. As shown in Figure 4A-B, the ecdhMUC1/ecdCD40L fusion protein can significantly increase the levels of IFN-gamma and IL-12 cytokines secreted from DCs harvested from hMUC1.Tg transgenic mice 48 hours after exposure to the vector. These studies suggest that the ecdhMUC1/ecdCD40L fusion protein can bind to the CD40 receptors on DCs and induce DC activation.

Testing for functional trimers of ecdhMUC1/ecdCD40L proteins induced by the Ad-sig-ecdhMUC1/ecdCD40L vector injections that can activate DCs

To formally test whether trimeric ecdhMUC1/ecdCD40L proteins are released after the infection of cells with Ad-sig-ecdhMUC1/ecdCD40L vector, we purified (using a His Tag purification kit) the ecdhMUC1/ecdCD40L protein from the supernatant of 293 cells exposed to the Ad-sig-ecdhMUC1/ecdCD40L. In this vector, an HSF1 trimer stabilization domain had been placed between the ecdhMUC1 and the ecdCD40L fragments, and a His tag was placed at the carboxyl terminal domain of the ecdCD40L protein. As shown in Figure 4C, the molecular weight of the ecdhMUC1/ecdCD40L protein under nondenaturing conditions was close to 3

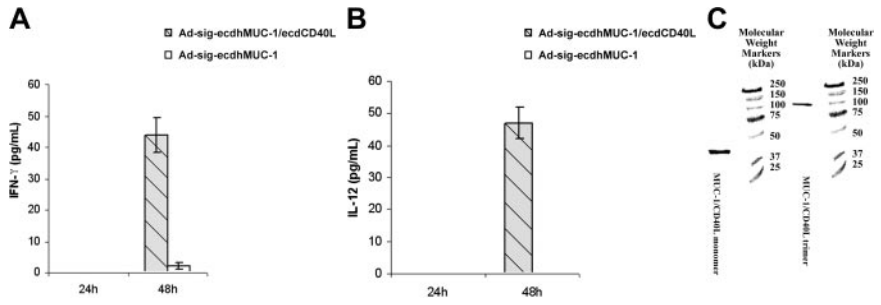

Figure 4. The ecdhMUC1 protein released from Ad-sig-ecdhMUC1/ecdCD40L vector–infected cells forms functional trimers and activates DCs. (A) Induction of IFN-γ secretion from bone marrow–derived DCs induced by exposure to the Ad-sig-ecdhMUC1/ecdCD40L vector. Supernatant medium collected from DCs derived in vitro from hMUC1.Tg mice after exposure to the Ad-sig-ecdhMUC1/ecdCD40L vector or to the Ad-sig-ecdhMUC1 vector and then analyzed for the levels of IFN-γ. (B) Induction of IL-12 secretion from bone marrow–derived DCs induced by exposure to the Ad-sig-ecdhMUC1/ecdCD40L or the Ad-sig-ecdhMUC1 vectors. The same procedure outlined for panel A was carried out, except that the supernatant medium was analyzed for IL-12. (C) Nondenaturing gel analysis of molecular weights of the ecdhMUC1/ecdCD40L protein. A construct was created in which a His tag was placed at the carboxyl terminal end of the CD40L, and an HSF1 trimeric stabilization domain was added between the ecdhMUC1 and ecdCD40L domains. After release from vector-infected cells, the protein was purified using a His tag column, concentrated, and added to a nondenaturing gel. The protein in the lane labeled MUC1/CD40L trimer was added to the nondenaturing gel without treatment. The protein in the lane labeled MUC1/CD40L monomer was first treated with the denaturing conditions before loading on the gel. Molecular weight markers are given in the extreme right lane.

times that seen under denaturing conditions. This experiment showed that trimers could be formed by the ecdhMUC1/ecdCD40L fusion protein.

Subcutaneous injection of the Ad-sig-ecdhMUC1/ecdCD40L vector overcomes anergy for hMUC1 positive cells in mice, which are transgenic for hMUC1

As shown in Figure 5A, mice injected subcutaneously with the Ad-sig-ecdhMUC1/ecdCD40L vector (solid squares) were resistant to engraftment by the hMUC1-positive LL2/LL1hMUC1 mouse cancer cells, whereas mice vaccinated with the Ad-sig-ecdhMUC-1 vector (solid triangles) or the untreated control animals not injected with vector (solid diamonds) were not resistant to the growth of the same cells. These data show that the full chimeric hMUC-1/ecdCD40L transcription unit is needed for complete suppression of the growth of the hMUC-1 cell line in the hMUC-1.Tg mice.

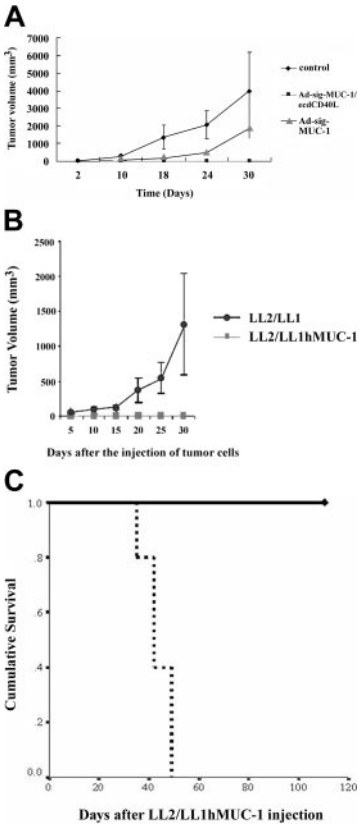

Figure 5. Effect of 2 subcutaneous injections (7 days apart) of 1 × 10^8 pfu of the Ad-sig-ecdhMUC1/ecdCD40L vector on the in vivo growth of the hMUC1-positive LL2/LL1hMUC1 cancer cell line in hMUC1.Tg mice. (A) Two subcutaneous injections (7 days apart) of 1 × 10^8 pfu Ad-sig-ecdhMUC1/ecdCD40L vector suppresses the growth of the human MUC1-positive LL2/LL1hMUC1 cancer cell line. The Ad-sig-ecdhMUC1/ecdCD40L vector or the Ad-sig-ecdhMUC-1 vector was injected twice at 7-day intervals or was not injected with any vector. One week after the second vector injection, the mice were injected with 5 × 10^5 LL2/LL1hMUC1 cancer cells, which were positive for hMUC1, and the growth of these cells was measured with calipers. (B) The Ad-sig-ecdhMUC1/ecdCD40L-induced suppression is specific for the hMUC1 antigen. hMUC1.Tg mice were injected twice subcutaneously (7 days apart) with 1 × 10^8 pfu Ad-sig-ecdhMUC1/ecdCD40L vector twice at 7-day intervals. One week after the second vector injection, the mice were injected with 5 × 10^5 LL2/LL1hMUC1 cells positive for the hMUC1 antigen or the same number of LL2/LL1 cells negative for the hMUC1 antigen. (C) Survival of LL2/LL1hMUC1 cell line–injected hMUC1.Tg mice that were twice (7 days apart) subcutaneously vaccinated or not vaccinated with 1 × 10^8 pfu Ad-sig-ecdhMUC1/ecdCD40L vector. Mice that received the injections outlined in panel A were monitored for survival after injection of the LL2/LL1hMUC1 cells. Continuous bold line indicates mice injected with the Ad-sig-ecdhMUC1/ecdCD40L vector. Broken bold line indicates mice not injected with a vector.

Mice injected with the Ad-sig-ecdhMUC1/ecdCD40L vector suppressed the growth of the hMUC1 antigen–positive LL2/LL1hMUC1 cell line, whereas this same vector did not suppress the growth of the parental cell line (LL2/LL1), which was not positive for the hMUC1 antigen (Figure 5B).This showed that the immune response was antigen specific.

As shown in Figure 5C, mice injected with the Ad-sig-ecdhMUC1/ecdCD40L vector (solid bold line at the top of Figure 5C) lived longer than did mice injected with a control vector (broken line in Figure 5C) and then injected subcutaneously with the LL2/LL1hMUC1 cell line.

Study of the cellular mechanisms through which Ad-sig-ecdhMUC1/ecdCD40L subcutaneous injections overcome anergy

Will the injection of the ecdhMUC1/ecdCD40L protein overcome anergy in the hMUC1.Tg mouse without the vector danger signal? One question is whether the subcutaneous injection of the ecdhMUC1/ecdCD40L protein would induce the cellular immune response that was seen with the Ad-sig-ecdhMUC1/ecdCD40L vector injections. As shown by the data in Figure 6A, subcutaneous injection of the ecdhMUC1/ecdCD40L protein did not induce an immune response that could protect the hMUC1.Tg mice from the growth of the LL2/LL1hMUC1 cell line. It is possible that the use of the adenoviral vector injections provide the so-called danger signal[2] necessary to induce the immune response in the hMUC1.Tg mice.

Cytokine release from vaccinated compared with nonvaccinated mice. To test whether the Ad-sig-ecdhMUC1/ecdCD40L induction of cellular immunity was mediated by CD8 T cells, the spleen T cells of the Ad-sig-ecdhMUC1/ecdCD40L vector vaccinated hMUC-1.Tg mice or the Ad-sig-ecdhMUC-1 vaccinated mice were depleted of CD4 T-cell lymphocytes with magnetic beads. As shown in Figure 6B, the CD8 T-cell lymphocytes isolated 7 days after injection from the spleens of hMUC1.Tg mice with the Ad-sig-ecdhMUC1/ecdCD40L vector released more than 2500 times the level of IFN-γ as did CD8 T cells taken from control vector–vaccinated MUC1.Tg mice and 50 times the levels of IFN-γ as did mice vaccinated with the Ad-sig-ecdhMUC-1 vector.

Cytotoxicity assay of splenic T cells from Ad-sig-ecdhMUC1/ecdCD40L vector injected mice against LL2/LL1hMUC1 or LL2/LL1 cancer cells. Splenic T cells were collected from hMUC1.Tg mice 7 days after injection with the Ad-sig-ecdhMUC1/ecdCD40L vector or the Ad-sig-ecdhMUC-1 vector and were then exposed to the hMUC1 antigen–positive LL2/LL1hMUC1 cancer cells for 7 days. Stimulated T cells were then mixed in varying ratios with either the hMUC1-positive LL2/LL1hMUC1 cells or the hMUC1-negative LL2/LL1 cancer cells. As shown in Figure 6C, T cells from Ad-sig-ecdhMUC1/ecdCD40L vaccinated mice can specifically kill cancer cells carrying the hMUC1 antigen but not the antigen-negative cells. Moreover, the level of hMUC-1 specific cytotoxic T cells in the Ad-sig-ecdhMUC-1/ecdCD40L mice was 6 times higher than in mice vaccinated with the Ad-sig-ecdhMUC-1 vector.

Ad-sig-ecdhMUC1/ecdCD40L vector injection overcomes resistance to expansion of hMUC1-specific T cells. Although anergic peripheral $CD8^+$ T cells can be induced to lyse target cells in an antigen-specific manner, they have been found to exhibit a block in the activation of the ERK proliferation signal transduction pathway after antigenic stimulation.[28] To determine whether CD8 cells from hMUC1.Tg mice expressed the active form of ERK1/2 on vector immunization, splenic CD8-positive T cells were obtained from noninjected hMUC1.Tg transgenic mice or mice

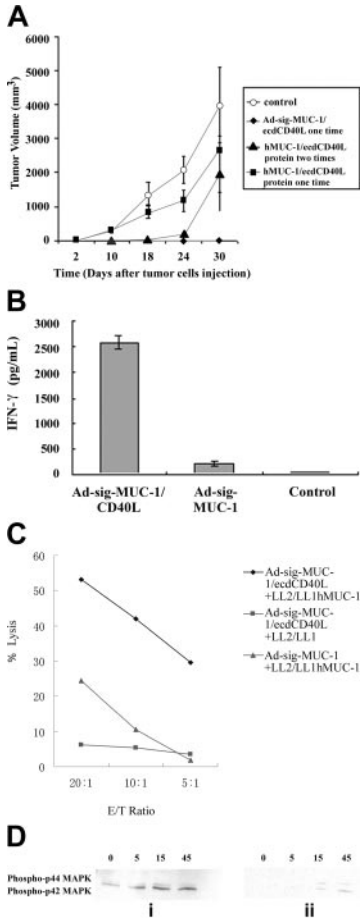

Figure 6. Mechanism of the suppressive effect of the Ad-sig-ecdhMUC1/ecdCD40L vector on induction of the immune suppression of the growth of the LL2/LL1hMUC1 cells in hMUC1.Tg mice. (A) Subcutaneous injection of the ecdhMUC1/ecdCD40L protein does not induce suppression of the growth of hMUC1-positive cells, which is equivalent to that seen with 2 subcutaneous injections of 1×10^8 pfu Ad-sig-ecdhMUC1/ecdCD40L vector. Five hundred thousand LL2/LL1hMUC1 cells were injected subcutaneously into the hMUC1.Tg mice. Two days after injection of the tumor cells, the ecdhMUC1/ecdCD40L protein was injected subcutaneously into hMUC1.Tg mice. (○) No protein injection. (♦) Ad-sig-ecdhMUC1/ecdCD40L vector. (▲) Two injections of the ecdhMUC1/ecdCD40L protein. (■) One injection of the ecdhMUC1/ecdCD40L protein. (B) $CD4^+$-depleted T cells from hMUC1 transgenic mice after 2 subcutaneous injections of 1×10^8 pfu Ad-sig-ecdhMUC1/ecdCD40L vector secrete increased levels of IFN-γ. $CD8^+$ T cells were isolated from hMUC1.Tg mice that had been vaccinated twice with the Ad-sig-ecdhMUC1/ecdCD40L vector or with the Ad-sig-ecdhMUC-1 vector or that had been unvaccinated (labeled as control). Seven days after vaccination, $CD8^+$ cells were harvested from the spleens of the test animals and were incubated for 24 hours. The supernatant medium was analyzed for IFN-γ levels. (C) Cytotoxicity of CTLs from hMUC1.Tg transgenic mice after 2 subcutaneous injections (7 days apart) of 1×10^8 pfu of Ad-sig-ecdhMUC1/ecdCD40L vector against LL2/LL1-MUC1 hMUC1–positive cancer cells or against LL2/LL1 cancer cells negative for the hMUC1 antigen. $CD8^+$ T-cell lymphocytes were isolated from the spleens of hMUC1.Tg mice 1 week after vaccination with the Ad-sig-ecdhMUC1/ecdCD40L vector. Cells were restimulated in vitro with the LL2/LL1hMUC1 cell line for 5 days (♦) or the LL2/LL1 cell line (■). $CD8^+$ T-cell lymphocytes were also isolated from the spleens of hMUC1.Tg mice 1 week after vaccination with the Ad-sig-ecdhMUC1 vector, which was then stimulated in vitro with the LL2/LL1hMUC1 cell line (▲). Different effector/target ratios (20:1, 10:1, and 5:1) were used. The LDH released from each of these cell mixtures (ordinate) was then measured. (D) Phosphorylation of the ERK1/ERK2 proliferation pathway in CD8 T cells from hMUC1 transgenic mice after stimulation with bone marrow–derived DCs infected with the Ad-sig-ecdhMUC1/ecdCD40L vector. CD8 T cells were isolated by CD4 depletion from the spleen cells of hMUC1.Tg mice 1 week after the completion of 2 subcutaneous injections (1 week apart) with the Ad-sig-ecdhMUC1/ecdCD40L vector (i) or from mice that were not vaccinated (ii). DCs that had been infected with the Ad-sig-ecdhMUC1/ecdCD40L vector were then mixed in a 1:1 ratio with the restimulated $CD8^+$ T cells. Proteins were isolated from these mixtures 0, 5, 15, and 45 minutes later and were separated using SDS-PAGE, transferred by Western blot analysis to a filter, and analyzed for phosphorylation of the p44 and p42 mitogen-activated kinase proteins using the New England BioLabs kit for phosphorylated proteins. The blot for the vaccinated mice is shown in panel i, and the blot for the unvaccinated mice is shown in panel ii.

injected 7 days earlier with the Ad-sig-ecdhMCU1/ecdCD40L vector and stimulated in vitro with the Ad-sig-ecdhMUC1/ecdCD40L vector–infected DCs.

CD8 T cells from unvaccinated hMUC1.Tg mice showed delayed kinetics and decreased total phosphorylation of ERK1 and ERK2 proteins (Figure 6Dii) compared with CD8 T cells from Ad-sig-ecdhMUC1/ecdCD40L–vaccinated hMUC1.Tg mice (Figure 6Di). These data suggest that Ad-sig-hMUC1/ecdCD40L vector injection induces an antigen-specific CD8 T-cell immune response to the MUC1 self-antigen through activation of the proliferation induction pathways in CD8 T cells.

Discussion

Our goal was to characterize the steps through which the vaccination of mice with the Ad-sig-TAA/ecdCD40L vector can induce an immune response to TAA-positive cells in anergic animals. Our experimental results suggest that subcutaneous injection of the Ad-sig-TAA/ecdCD40L vector leads to the continuous release of the TAA/ecdCD40L protein for at least a 10-day period. Binding of this protein to DCs induces increased levels of secondary signals of activation (CD80 and CD86) and the CCR-7 chemokine receptor on DCs, which lead to the migration of the TAA-loaded DCs to the regional lymph nodes. These events induce increases in the levels of the TAA-specific $CD8^+$ cytotoxic T lymphocytes in the spleens of Ad-sig-TAA/ecdCD40L vector–injected mice.

This increase in the TAA-specific $CD8^+$ lymphocytes in the Ad-sig-ecdhMUC1/ecdCD40L vector injected mice overcomes the anergy that exists to the hMUC1 antigen in hMUC1.Tg mice, which have expressed the hMUC1 antigen since birth. These experiments further show that inducing immunity is associated with the release of T_H1 cytokines, is HLA restricted, and is accompanied by an increase in the total phosphorylation of ERK1 and ERK2 pathways in T cells from vector-injected hMUC1.Tg mice when the T cells are exposed to Ad-sig-ecdhMUC1/ecdCD40L vector–infected DCs.

In contrast to the subcutaneous injection of the Ad-sig-ecdhMUC1/ecdCD40L vector, the subcutaneous injection of the ecdhMUC1/ecdCD40L protein does not induce immune protection against the growth of the hMUC1-positive LL1/L2hMUC1 tumor cells (Figure 6A). This suggests that the danger signal[2] associated with the adenoviral vector carrying the ecdhMUC1/ecdCD40L transcription unit is an important part of overcoming the anergy to the hMUC1 antigen that exists in the hMUC1.Tg mice.

The oral TAA/CD40L *Salmonella typhimurium* DNA vaccine of Xiang and coworkers[14] had 3 potential limitations: the need for targeted IL-2 in addition to oral DNA bacterial vaccine; the use of a DNA vaccine that, because of its inefficiency of transfection, generated only low levels of expression for a short period of time; and the need to restrict the vaccination to the development of the antigen-loaded and activated DCs to the secondary lymphoid tissue of the gastrointestinal tract. Restriction to the T cells of the secondary lymphoid tissue of the gastrointestinal tract,[29] in accordance with the method of Xiang et al,[14] could be a limitation.

Because the adenoviral vector used in our work ([1]and current results) can be administered to any part of the body, the homing of the T cells to the region of origin could be directed to the secondary lymphoid organs of any tissue by selection of the site of injection. In contrast to Xiang et al,[14] we found no need to follow up the vaccination of mice with targeted IL-2 treatment to break tolerance or to induce resistance to the engraftment of cancer cell lines in 100% of the vaccinated mice in our studies. Finally, we showed

that subcutaneous injection of the Ad-sig-TAA/ecdCD40L vector is able to overcome the anergy that develops to TAAs, which are present from birth.

We had many reasons for selecting an in vivo method of activating and TAA loading DCs. The first is that our goal was to study the steps involved in the in vivo activation and antigen loading of DCs, not to compare in vivo and ex vivo loading of DCs. In vivo activation was an attractive option to study for several reasons. First, the work of Xiang et al[14] with the TAA/CD40L DNA vaccine involved in vivo vaccination, not ex vivo loading and activation. We wanted to determine whether we could improve on the in vivo activation and TAA loading seen when an adenoviral vaccine was used instead of a DNA vaccine. Second, in vivo activation by 1 or 2 subcutaneous injections of a vector could be vastly cheaper and simpler to administer than complex strategies involving ex vivo activation and TAA loading of DCs. Third, the in vitro activation approach was hampered by the limited number of DCs that could be produced, the inability to duplicate an in vivo environment in an in vitro culture system, and the short release as compared to the protracted in vivo TAA/CD40L protein release over a 10- to 14-day period when the ex vivo approach involved just a single injection. Finally, clinical trials involving ex vivo activation or tumor-antigen loading of DCs have proven to be less effective than in vivo methods of vaccination.[13]

A notable finding was that control experiments with vectors encoding TAA alone or CD40L alone were not as effective in activating DCs or inducing a cellular immune response against TAA-positive cancer cells in animal models. The question may be asked why the vaccination with vectors encoding the secretable fusion protein of the TAA/CD40L is more effective in inducing an immune response than vectors containing either TAA alone or CD40L alone. We have shown here that the chimeric TAA/CD40L fusion protein can form functional trimers, a requirement for binding the CD40L end of the fusion protein to the CD40 receptor on the DCs. Once the chimeric protein binds to the DCs, 2 things happen. DCs are activated to be effective at providing CD8 cells with the secondary signals necessary to activate CD8 TAA-specific T cells, and the chimeric TAA/CD40L protein is taken up into the DCs by endocytosis, thereby permitting the TAA to be processed in a way that results in its being available for presentation by MHC class I molecules. The fact that individual DCs are activated and TAA loaded is the advantage of the vectors encoding the TAA/CD40L fusion protein.

The immune response induced by the subcutaneous injection of the Ad-sig-TAA/ecdCD40L vector is antigen specific and is dependent on the activation of the DCs in and around the vector injection site and on the migration of the TAA-loaded and activated DCs to the regional lymph nodes. It is not possible to overcome anergy with subcutaneous injection of the TAA/ecdCD40L protein or the subcutaneous injection of an adenoviral vector that carries a transcription unit encoding a nonsecretable TAA/ecdCD40L protein. These experimental results suggest that this approach to the activation of the immune response against tumor cells merits further study in preclinical and clinical models.

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An adenoviral vector cancer vaccine that delivers a tumor-associated antigen/CD40-ligand fusion protein to dendritic cells

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Communicated by Alan Garen, Yale University, New Haven, CT, August 21, 2003 (received for review February 8, 2003)

To develop a method to overcome the anergy that exists in tumor hosts to cancer, we have designed an adenoviral vector for the *in vivo* activation and tumor antigen loading of dendritic cells. This adenoviral vector encodes a fusion protein composed of an amino-terminal tumor-associated antigen fragment fused to the CD40 ligand (CD40L). Subcutaneous injection of an adenoviral vector encoding a fusion protein of the human papillomavirus E7 foreign antigen linked to the CD40L generates $CD8^+$ T cell-dependent immunoresistance to the growth of the E7-positive syngeneic TC-1 cancer cells in C57BL/6 mice for up to 1 year. We also studied the s.c. injection of a vector carrying the gene for the human MUC-1 (hMUC-1) self-antigen fused to the CD40L. When this vector was injected into hMUC-1.Tg mice, which are transgenic for the hMUC-1 antigen, the growth of syngeneic hMUC-1-positive LL1/LL2hMUC-1 mouse cancer cells was suppressed in 100% of the injected animals. The hMUC-1.Tg mice are anergic to the hMUC-1 antigen before the injection of the vector. These experimental results show that it is possible to use vector injection to activate a long-lasting cellular immune response against self-antigens in anergic animals. The vector-mediated *in vivo* activation, and tumor-associated antigen loading of dendritic cells does not require additional cytokine boosting to induce the immune response against the tumor cells. This vector strategy may therefore be of use in the development of immunotherapy for the many carcinomas in which the hMUC-1 antigen is overexpressed.

E7 viral antigen | MUC-1 antigen | T cells | memory cells | immunotherapy

Xiang *et al*. (1) have used an oral plasmid DNA vaccine to induce immunological resistance to the engraftment of mouse colonic carcinoma cells that are positive for the human carcinoembryonic antigen (hCEA) gene in mice transgenic for hCEA. This plasmid encodes the extracellular domain (ecd) of the hCEA linked to the ecd of the CD40 ligand (CD40L). Oral administration of this plasmid DNA vaccine carried by an attenuated strain of *Salmonella typhimurium* resulted in effective tumor-protective immunity against hCEA-positive mouse colon cancer cells. The induction of immunity in these animals was shown to involve the activation of naïve T cells and dendritic cells (DCs). This vaccine was shown to be capable of activating an immune response against hCEA in animals that were anergic to this antigen and to be 100% effective in the prophylactic setting, but this response required the use of a second treatment, IL-2, which was antibody-targeted to T cells.

To administer the tumor-associated antigen (TAA)/CD40L vaccine in a way that could affect T cells in secondary lymphoid tissue in areas of the body other than the gastrointestinal tract, and to create a therapy that does not require the antibody-targeted IL-2, we constructed replication-incompetent adenoviral vectors encoding chimeric TAA/ecdCD40L transcription units. These transcription units encode either the human papillomavirus (HPV) E7 foreign tumor antigen or the human MUC-1 (hMUC-1) self-antigen fused to the 209-aa ecd of the CD40L. This region of CD40L contains all the sequences necessary for the formation of the CD40L trimer (2). These transcription units resembled the vaccine of Xiang in that they contained a leader sequence for secretion linked to the fusion protein composed of a TAA and the CD40L.

Our vaccine differed from that of Xiang in that it used an adenoviral vector rather than a plasmid in a *Salmonella* bacterial host strain for the delivery of the TAA/ecdCD40L transcription unit. Xiang used a leucine zipper domain in the region between the hCEA antigen and the full-length CD40L, whereas we used an 8-aa linker (NDAQAPKS) between the TAA (E7 or hMUC-1) gene and the ecdCD40L gene. The Xiang construct positions the hCEA antigen at the carboxyl terminus of the full-length CD40L, whereas our vector transcription unit attached the TAA to the amino-terminal end of the ecd of the CD40L. Our arrangement should provide better binding of the CD40L to the CD40 receptor on DCs and better secretion from the cells to the extracellular space. Xiang's method required the administration of a fusion molecule composed of IL-2 linked to a T cell-targeted antibody after the hCEA/CD40L DNA vaccine, whereas our TAA/ecdCD40L adenoviral vaccine was administered without an IL-2 boost. Xiang administered his plasmid DNA vaccine orally, whereas we administered our vector vaccine s.c.

The adenoviral system used in our work has several theoretical advantages over the *Salmonella* delivery system. The expression of the TAA/CD40L gene may be at higher levels and for longer periods of time with the adenoviral delivery than with the DNA vaccine. This adenoviral TAA/ecdCD40L vector injection may thereby induce a more vigorous immune response. Although immune-specific T cells that are elicited after immunization are thought to traffic throughout the body, a propensity still exists for tissue-specific homing by memory T cells to the lymphoid sites draining the natural area of infection (3). Thus, s.c. injection of the adenoviral vector carrying the highly immunogenic TAA/ecdCD40L in the region of the tumor cells may foster the optimal trafficking of sensitized cytotoxic T cells and the generation of memory cells.

For our experiments, we chose two TAAs. The first is the E7 protein of the most commonly encountered pathogenic (cancer-causing) genotype of the HPV. We selected this antigen because it is expressed in HPV-associated intraepithelial cervical dysplasia and in the primary cells of HPV-associated cervical cancer (4). In addition, several laboratories (5, 6), including the DiMaio laboratory (7) and our own laboratory (8), have shown that the E7 protein

Abbreviations: ecd, extracellular domain; DCs, dendritic cells; sig, a signal or leader sequence for secretion to the extracellular space; HPV, human papillomavirus; CMV, cytomegalovirus; TAA, tumor-associated antigen; hCEA, human carcinoembryonic antigen; pfu, plaque-forming units.

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is required for the maintenance of the malignant phenotype of cervical cancer cells.

The second TAA is the hMUC-1 epithelial antigen. This "self-antigen" is focally expressed from birth in normal epithelial cells, but is diffusely up-regulated on epithelial surfaces in 90% of cancers of the breast, ovary, colon, and lung (9). The overexpression of hMUC-1 has been shown to promote anchorage-independent growth of tumor cells (10). Mice that have been made transgenic for hMUC-1 have been shown to develop tolerance for hMUC-1 antigen-bearing syngeneic mouse cancer cells (11).

The experimental results described in this report show that the s.c. injection of the adenoviral vector carrying the E7/ecdCD40L fusion gene generates immunological resistance to E7-positive cancer cells for at least 1 year. In addition, the s.c. injection of the adenoviral vector carrying the hMUC-1/ecdCD40L fusion gene suppresses the growth of hMUC-1-positive mouse cancer cells in hMUC-1.Tg mice that are transgenic for the hMUC-1 gene. The induction of *in vivo* resistance to the growth of the hMUC-1-positive syngeneic mouse cancer cells was shown to involve a $CD8^+$ T cell immune response against the hMUC-1 self-antigen in hMUC1.Tg transgenic mice. These mice are initially immunologically unresponsive to the hMUC-1-positive mouse cancer cells. Thus, the hMUC-1/CD40L vector injections appeared to overcome anergy. This vaccine may be of use in preventing the recurrence of epithelial malignancies after surgery and for the immunotherapy of advanced epithelial cancers that recur after surgery.

Materials and Methods

Cell Culture and Mice. The hMUC-1.Tg mice were obtained from S. Gendler (Mayo Clinic, Scottsdale, AZ) (11).

Construction of the Adenoviral Vectors. The plasmid pDC406-mCD40L was purchased from ATCC. PCR was used to produce the carboxyl-terminal 209 aa of the ecd of the mouse CD40L (ecdCD40L), which contained neither the transmembrane domain nor the cytoplasmic domain. A spacer (NDAQAPKS) was placed at the 5′ (amino-terminal) end of the transcription unit for the ecdCD40L. This fragment was inserted into the plasmid pShuttle-CMV (ref. 12; CMV, cytomegalovirus) after restriction endonuclease digestion with *Hin*dIII and *Xho*I. This vector is designated pSCMVecdCD40L.

The E7 or ecdhMUC-1 TAA fragments were inserted into the pShuttle between the CMV promoter and the linker at the amino-terminal end of the ecdCD40L transcription unit after digestion of the pSCMVecdCD40L plasmid shuttle vector with *Not*I and *Xho*I. These plasmids were designated pSCMVE7/ecdCD40L and pSCMVecdhMUC-1/ecdCD40L. In a similar fashion, we fused the GFP gene with the ecdCD40L gene and inserted it downstream of the CMV promoter in the pShuttle-CMV vector (12). This plasmid was designated pSCMVGFP/ecdCD40L. We also constructed another set of pShuttle plasmids by inserting the human growth hormone signal sequence (sig) immediately downstream of the CMV promoter in the pSCMVE7/ecdCD40L, pSCMVecdhMUC-1/ecdCD40L, and pSCMVGFP/ecdCD40L plasmids to create the pSCMVsigE7/ecdCD40L, pSCMsigecdhMUC-1/ecdCD40L, and pSCMVsigGFP/ecdCD40L shuttle plasmids. The signal sequence was placed at the amino-terminal limit of the TAA/ecdCD40L protein to promote the release of the TAA/ecdCD40L protein from the vector-infected cells. The secretion is designed to amplify the effect of the vector beyond the infected cells. In addition, for the TAA/ecdCD40L protein to activate DCs and to be taken up by the DCs, the protein must be released from the vector-infected cells.

Ad-sig-E7/ecdCD40L, Ad-sig-ecdhMUC-1/ecdCD40L, and Ad-sig-GFP/ecdCD40L are replication-incompetent adenoviral vectors that are structurally similar, except for the TAA transcription unit between the sig, which is 3′ of the CMV promoter, and at the amino-terminal end of the secretable CD40L transcription unit. All these vectors lack the E1 and E3 adenoviral genes and were assembled by homologous recombination in bacterial host strains by using the AdEasy system (12) with the pSCMVsigE7/ecdCD40L, pSCMVsigecdhMUC-1/ecdCD40L, and pSCMVsigGFP/ecdCD40L shuttle plasmids.

Sig	HPV E7	NDAQAPKS	ecdCD40 ligand (Amino acid 52-261)
	HPV E7	NDAQAPKS	wtCD40 ligand (Amino acid 1-261)
Sig	GFP	NDAQAPKS	ecdCD40 ligand (Amino acid 52-261)

Fig. 1. Organization of the adenoviral transcription units. Elements of the adenoviral vector transcription units include Sig, human growth hormone signal sequence; HPV E7, the HPV E7 gene; NDAQAPKS, a peptide linker between E7 and the ecd of the CD40L gene; ecdCD40L, amino acids 52–261 of the CD40L gene, which contain the ecd of the CD40L without the transmembrane or cytoplasmic domains; wtCD40L, amino acids 1–261 of the full-length (wild-type) CD40L gene, which include the ecd, the transmembrane domain, and the cytoplasmic domain; and the GFP gene.

Tumor Model for Ad-sig-TAA/ecdCD40L Injections. We first conducted studies to evaluate the response of tumors to the Ad-sig-E7/ecdCD40L vector injections in C57BL/6 mice. All mice were treated by using the following protocol unless otherwise described. The Ad-sig-E7/ecdCD40L adenoviral vector [1×10^8 plaque-forming units (pfu)] or control adenoviral vectors (1×10^8 pfu), as shown in Fig. 1, were injected s.c. on days 0 and 7. One week after the last s.c. vector injection, 5×10^5 TC-1 tumor cells (13) were injected s.c. at a different site. For all injections, the vector or the cells were suspended in 100 μl of PBS. Tumor growth was monitored three times each week at the injection site of the tumor cells by caliper measurement and inspection. The mice were killed when their tumors became ulcerated or reached 1.5 cm in diameter. Tumor volumes are calculated by the product of length $\times$ width^2/2. Similar experiments were carried out with the Ad-sig-ecdhMUC-1/ecdCD40L vector, the LL1/LL2hMUC-1 cell line, and the hMUC-1.Tg transgenic mouse strain.

Adoptive Transfer of Lymphocytes from Tumor-Free Mice after Injection with TC-1 Cells, and s.c. Injection of the Ad-sig-E7/ecdCD40L Vector. ***Preparation of the C57BL/6 donor mice.*** C57BL/6 mice were injected s.c. with 5×10^5 TC-1 cells. When a small tumor nodule appeared at the injection site, measurements were conducted with calipers three times a week. Five days after the injection of TC-1 cells, 1×10^8 pfu of the Ad-sig-E7/ecdCD40L vector that expressed the secretable form of the E7/ecdCD40L fusion protein were injected s.c. at a site separate from the tumor nodule in the C57BL/6 mice. This vector injection was repeated in 7 days. Several weeks later, 1×10^7 TC-1 cells were then injected s.c. in the mice that were tumor-free. Of the 10 tumor-free mice that were injected with the vector and the second dose of tumor cells, 8 mice remained tumor-free. Four of the 8 mice that remained free of tumor for 1 year were killed. The splenic T cells were isolated by negative selection by using magnetic bead separation according to StemCell Technologies (Vancouver). The purified T cells were injected i.p. into C57BL/6 *nu/nu* athymic mice that carried s.c. nodules of TC-1 tumor cells (see Fig. 4*A*).

Preparation of the recipient C57BL/6 athymic* nu/nu *mice. C57BL/6 *nu/nu* immunocompromised mice (4- to 6-week-old females) were injected s.c. with 1×10^6 TC-1 cells. One week after the injection of the TC-1 cells, the mice were injected i.p. with spleen cells from the C57BL/6 mice that had been sensitized by s.c. injection of the Ad-sig-E7/ecdCD40L vector.

***In Vivo* Depletion of $CD4^+$ or $CD8^+$ T Lymphocytes.** In a separate experiment, *in vivo* mAb ablation of CD8 (clone 2.43; ATCC TIB 210) or CD4 (clone GK1.5; ATCC TIB 207) T cell subsets was performed by i.p. injection of 0.5 mg of antibody. This antibody was

purified from the culture supernatants of hybridomas. The CD8- or CD4-depleting antibodies were injected i.p. into the immunocompetent C57BL/6 donor mice on days −5, −3, and −1 before the first vector vaccination, and every 6 days thereafter (500 μg of purified antibody per mouse per injection) during vaccination, and also on days 6, 7, 8, 10, 12, and 14 after tumor challenge (14). On day 0 and day 7, 1×10^8 pfu of the Ad-sig-E7/ecdCD40L vector were injected s.c. Seven days later, 5×10^5 E7-positive TC-1 cells were injected s.c. into each mouse. Then, the mice were observed for 3 months.

Antibody suspensions were purified from hybridoma supernatants by passage through protein G columns according to the manufacturer's instructions (Pierce). $CD4^+$ and $CD8^+$ T cell depletion was monitored by flow cytometric analysis of splenocytes isolated from test animals. On the day of tumor challenge, $CD4^+$ and $CD8^+$ cell populations were reduced by 95% and 99%, respectively.

Mice were monitored for 3 months after tumor challenge and, at that time, the tumor-free mice were killed. The spleen T cells were then isolated as described above. Five million of the $CD4^+$ T cells from the CD8-depleted, sensitized animals were mixed with five million $CD8^+$ T cells from unsensitized animals. Similarly, five million of the $CD8^+$ T cells from the CD4-depleted, sensitized animals were mixed with five million $CD4^+$ T cells from the unsensitized mice. Ten million of these mixtures of sensitized $CD8^+$ T cells and unsensitized $CD4^+$ T cells, or sensitized $CD4^+$ T cells and unsensitized $CD8^+$ T cells, were injected into C57BL/6 nu/nu mice in which one million TC-1 cells had already been injected 5 days before. The animals were then monitored for survival.

Results

Construction and Analysis of the Ad-sig-E7/ecdCD40L Vector. The organization of the transcription unit of the Ad-sig-E7/ecdCD40L vector, and several control vectors generated for analytical purposes, is shown in Fig. 1. We exposed 293 cells to the following vectors: Ad-sig-E7/ecdCD40L, Ad-sig-GFP/ecdCD40L, Ad-E7, and Ad-wtCD40L (wtCD40L contains the transmembrane and cytoplasmic domains and the ecd of the CD40L). We then subjected the protein cell lysate of the infected cells to SDS/PAGE and Western blotting. The predicted molecular weights for each of the fusion proteins encoded by the CD40L transcription units were observed on the Western blot (data not shown).

Activation of DCs by the E7/ecdCD40L Fusion Protein. To test whether the E7/ecdCD40L fusion protein could bind to the CD40 receptor and activate cytokine release from DCs, we exposed bone marrow-derived DCs (15) to the following adenoviral vectors at a multiplicity of infection of 100: Ad-sig-E7/ecdCD40L, Ad-sig-GFP/ecdCD40L, and Ad-E7. The infected DCs were then inoculated in 24-well plates at 2×10^5 cells per ml. ELISA analyses confirmed that infection of DCs by the Ad-sig-E7/ecdCD40L vector stimulated the DCs to produce 18 pg of IL-12 per 2×10^5 cells per ml in 24 h, and 88 pg of IL-12 per 2×10^5 cells per ml in 48 h. This finding was statistically significantly higher than the level of IL-12 released after exposure of the DCs to the Ad-sig-GFP/ecdCD40L vector, the Ad-E7 vectors, or the PBS control ($P < 0.0001$ as shown in Fig. 2*A*). It is possible that the level of cytokine release in cells exposed to the Ad-sig-GFP/ecdCD40L vector is lower than that seen with the Ad-sig-E7/ecdCD40L vector because of a lower level of expression of the GFP/ecdCD40L, or because of the toxicity of the GFP to cells in which it is expressed at high levels.

Similarly, as shown in Fig. 2*B*, exposure of the DCs to the Ad-sig-E7/ecdCD40L vector also induced secretion of 335 pg of IFN-γ per 2×10^5 cells per ml in 24 h and 769 pg of IFN-γ per 2×10^5 cells per ml in 48 h. This level was statistically significantly higher than the levels induced by the PBS control or the Ad-E7 or Ad-sig-GFP/ecdCD40L vectors ($P < 0.0001$). We also exposed bone marrow-derived DCs to the Ad-sig-E7/ecdCD40L vector and showed that no IL-2 was secreted by the vector-exposed DCs (sensitivity of the assay was 0.3 pg/ml). These data suggest that the Ad-sig-E7/ecdCD40L vector can induce cytokine release from the DCs without the involvement of IL-2.

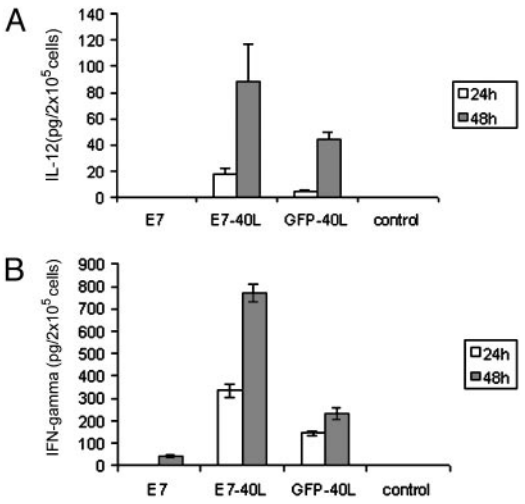

Fig. 2. Study of activation of cytokine release from DCs by exposure to the Ad-sig-E7/ecdCD40L vector. Bone marrow-derived DCs were exposed to the following vectors: E7, Ad-E7; E7–40L, Ad-sig-E7/ecdCD40L; and GFP-40L, Ad-sig-GFP/ecdCD40L. After addition of the vector at a multiplicity of infection of 100, the cells were placed in wells, and an ELISA was used to measure the production of IL-12 (*A*) and IFN-γ (*B*) by the vector-infected DCs during a 24- and a 48-h period.

To test whether the induction of IL-12 and IFN-γ release was caused by the binding of the E7/ecdCD40L protein to the CD40 receptor on the DCs, we added 10 μg of either nonimmune IgG or 10 μg of anti-CD40L antibody to DCs that had been exposed to the Ad-sig-E7/ecdCD40L vector. Addition of this antibody reduced the IL-12 released after exposure to the vector from 27 pg per 200,000 DCs in 24 h to 0 pg per 200,000 DCs in 24 h. The IFN-γ released in the presence of the nonimmune IgG was 366 pg per 200,000 DCs in 24 h, whereas, with the addition of anti-CD40L antibody, the level released was reduced to 71 pg per 200,000 DCs in 24 h. These experiments were carried out in triplicate. These results suggest that the TAA/ecdCD40L fusion protein that is released from Ad-sig-E7/ecdCD40L-exposed DCs can assemble itself into a functional trimer that binds to the CD40 receptor on the DCs, thereby activating the DCs to release IL-12 and IFN-γ.

We were also able to show that the exposure of bone marrow-derived DCs to the Ad-sig-E7/ecdCD40L vector induces an increase in the percentage of DCs that were positive for CD80 and CD86 from 8.4% to 27.7% (for CD80) and from 3.3% to 27.4% (for CD86). Thus, the Ad-sig-E7/ecdCD40L vector induced activation of the DCs.

Subcutaneous Injection of the Ad-sig-E7/ecdCD40L Vector Confers Protection Against Engraftment and Growth of the E7-Positive TC-1 Cancer Cell Line. Female C57BL/6 mice were injected s.c. with 1×10^8 pfu of the Ad-sig-E7/ecdCD40L or Ad-sig-GFP/ecdCD40L vectors once on each of 2 days, 7 days apart. The vector-treated mice were then injected s.c. with 5×10^5 cells from the E7-positive TC-1 cancer cell line (13) 10 days after the last vector injection. As shown in Fig. 3, 0% (0 of 5) of mice injected with the Ad-sig-E7/ecdCD40L vector formed palpable tumors during 2 months of observation after a single injection of TC-1 cells, whereas 100% (5 of 5) of the mice injected with the Ad-sig-GFP/ecdCD40L vector before the injection of the TC-1 cells formed s.c. tumors, which were >500 mm^3 by 15 days after TC-1 tumor-cell injection.

The differences between the sizes of the tumors in mice injected with the Ad-sig-E7/ecdCD40L vector and the Ad-sig-GFP/

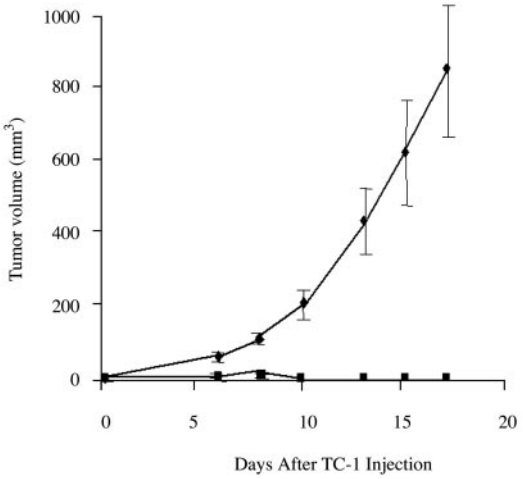

Fig. 3. Analysis of TC-1 E7-positive tumor cell line growth in mice after vector injections. Mice were injected s.c. with 1×10^8 pfu of vectors twice at 7-day intervals. Ten days later, 5×10^5 TC-1 cells were injected s.c. ($n = 5$). The following vectors were injected: ♦, Ad-sig-GFP/ecdCD40L (top line), and ■, Ad-sig-E7/ecdCD40L (bottom line). The growth of the s.c. nodule of TC-1 cells in the Ad-sig-E7/ecdCD40L-injected mice was statistically significantly different from the growth seen in the mice injected with the Ad-sig-GFP/ecdCD40L vector at the $P < 0.001$ level.

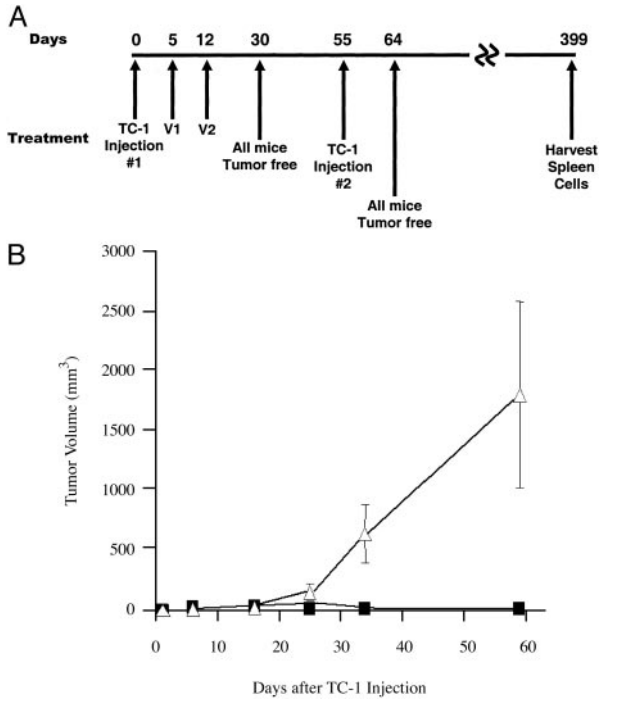

Fig. 4. (*A*) Time course of the events in the experiment presented in *B* and in Fig. 5*A*. TC-1, E7 tumor cell line; V1, first vector injection; V2, second vector injection. (*B*) Analysis of changes in the size of TC-1 E7-positive s.c. tumor nodules in C57BL/6 mice that had been growing for 5 days before injection with the Ad-sig-E7/ecdCD40L vector. As shown in *A*, 5×10^5 TC-1 cells were injected on day 0. Vectors (1×10^8 pfu) were injected on day 5 after the s.c. injection of TC-1 cells when the TC-1 tumor nodule is visible and injected again 7 days later ($n = 5$ per group). ■, Ad-sig-E7/ecdCD40L; △, PBS. The growth of the s.c. nodule of TC-1 cells in the mice injected with the Ad-sig-E7/ecdCD40L vector was statistically significantly different from that in the mice injected with PBS at the $P < 0.0001$ level.

ecdCD40L vector are statistically significantly different at the $P < 0.001$ level. The fact that the Ad-GFP/ecdCD40L vector injection does not protect the mice shows that the protective effect of the Ad-sig-E7/ecdCD40L vector, which prevents the growth of the TC-1 cell line, is not due to the adenoviral infection/transfection process.

When the tumor-free animals from the Ad-sig-E7/ecdCD40L-treated group were rechallenged with a larger dose of TC-1 cells (1×10^7 cells), a period of transient tumor growth was initially seen, which was followed by a decline in the size of the tumor nodule, ultimately leading to complete tumor regression in 100% of the five animals rechallenged with the higher dose of TC-1 cells.

Injection of the Ad-sig-E7/ecdCD40L Vector Induces Tumor Regression of Established TC-1 Tumors. Mice were first injected s.c. with 5×10^5 TC-1 cells on the hind flank. Five days later, the mice were injected s.c. at a different site with the Ad-sig-E7/ecdCD40L vector. This vector injection was repeated 7 days later. Control mice were injected s.c. with PBS 5 days after the injection of TC-1 cells (see Fig. 4*A* for time course of the experiment). As shown in Fig. 4*B*, two s.c. injections of the Ad-sig-E7/ecdCD40L vector resulted in transient growth followed by regression that was complete in 100% of the mice by 25 days after the second Ad-sig-E7/ecdCD40L vector injection. The second challenge of the immunized animals with a 20-fold increase in the number of the TC-1 tumor cells (see Fig. 4*A*) produced a brief transient growth and then complete regression of the tumor nodule in 100% of the mice injected with the Ad-sig-E7/ecdCD40L vector after the injection (data not shown). Although all the Ad-sig-E7/ecdCD40L vector-injected mice showed complete regression of tumors (see Fig. 4*B*), 100% of the animals injected with PBS developed progressive tumor growth at the TC-1 injection site within 14 days.

Splenic T Cells from Ad-sig-E7/ecdCD40L Vector-Injected C57BL/6 Mice Can Passively Transfer T Cell-Mediated Tumor Immunity to TC-1 Cells for up to 1 Year After Vector Injection. We monitored the mice from the experiment shown in Fig. 4*A* (time course of the experiment) for up to 1 year after TC-1 tumor injection, Ad-sig-E7/ecdCD40L vector injection (two injections separated by 7 days), and rechallenge with 1×10^7 TC-1 cells. Four of the eight animals that remained tumor-free for >1 year were killed and the T cells were isolated from the spleen by negative selection by using antibody and magnetic bead technology. Ten million of these splenic T cells were injected i.p. into C57BL/6 athymic nu/nu mice ($n = 7$), which had been injected s.c. 5 days previously with 5×10^5 TC-1 cells. As shown in Fig. 5*A*, the tumors in the nude mice given i.p. injections of the T cells from the Ad-sig-E7/ecdCD40L-sensitized donor mice grew into palpable s.c. nodules for 6 days and then regressed in all animals to very small tumors. The sizes of the s.c. tumor nodules were an average of 114 mm^3 on day 4, 234 mm^3 on day 6, 151 mm^3 on day 8, and 140 mm^3 on day 10 after the i.p. injection of the T cells from the sensitized immunocompetent animals shown in Fig. 4*B*. In three of the seven treated mice, the tumors regressed completely. In contrast, none of the s.c. tumors in the nude mice injected i.p. with T cells from unsensitized donor mice regressed. All the mice in the latter group died with progressive tumor growth within 3 weeks after TC-1 tumor cell injection. The mice treated with the i.p. injection of the splenic T cells from the sensitized donors were monitored for 3 months after the TC-1 challenge and remained tumor-free during that time.

A separate experiment was performed to determine the relative contribution of the $CD8^+$ vs. the $CD4^+$ T cells to the induction of immunoresistance to the TC-1 cells. The C57BL/6 mice were injected with the Ad-sig-E7/ecdCD40L vector twice, with each injection 7 days apart. Seven days after the last injection, 5×10^5 of the TC-1 cells were injected into a separate s.c. site. To deplete CD4 or CD8 T cells, the C57BL/6 donor mice were injected with antibodies against either $CD4^+$ T cells or $CD8^+$ T cells before and during the injection of the Ad-sig-E7/ecdCD40L vector (see *Materials and Methods* for the exact schedule of injections).

The efficiency of such antibody treatment for either the CD4 or

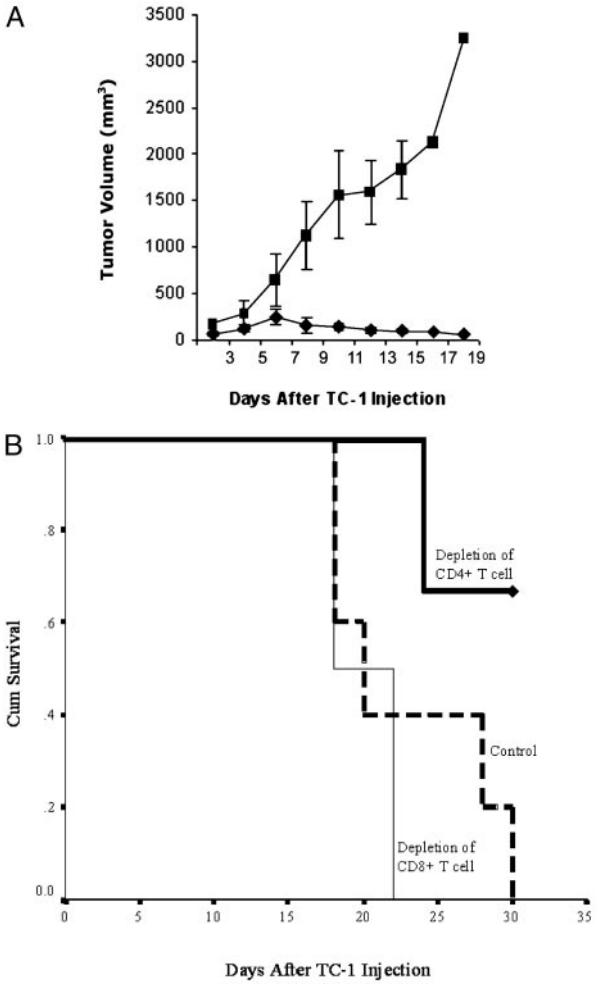

Fig. 5. (*A*) Passive transfer of immune resistance to TC-1 cell growth by using spleen cells collected from sensitized animals 1 year after vector injection. Splenic T lymphocytes were collected 1 year after Ad-sig-E7/ecdCD40L vector vaccination and challenge with TC-1 cells (see Fig. 4*A*). These sensitized spleen cells were then injected i.p. into C57BL/6 nude mice 5 days after injection of 500,000 TC-1 cells. ◆, C57BL/6 nude mice ($n = 7$) were injected s.c. with TC-1 cells and then injected i.p. 5 days later with 10×10^6 splenic lymphocytes from Ad-sig-E7/ecdCD40L-sensitized mice; ■, control animals were injected i.p. with 10×10^6 splenic T cells from unsensitized donor C57BL/6 mice 5 days after injection of TC-1 cells. (*B*) Survival of C57BL/6 nude mice after s.c. injection of TC-1 tumor cells and then i.p. injection of $CD4^+$ (thin unbroken line) or $CD8^+$ (thick unbroken line) T cell lymphocytes from Ad-sig-E7/ecdCD40L-sensitized C57BL/6 donors. Donor C57BL/6 mice were injected s.c. with the Ad-sig-E7/ecdCD40L vector at days 0 and 7. Seven days later, the mice were injected s.c. with 5×10^5 TC-1 cells. The mice were monitored for 3 months. At 5, 3, and 1 days before the vector injection, and every 6 days after the s.c. injection of the Ad-sig-E7/ecdCD40L vector, and also on days 6, 7, 8, 10, 12, and 14 after the injection of the TC-1 cells, the C57BL/6 donor mice were treated *in vivo* with antibodies specific for $CD4^+$ (thick unbroken line) or $CD8^+$ (thin unbroken line) T cell lymphocytes to deplete the respective T cell population. Then the sensitized $CD8^+$ or $CD4^+$ T cell lymphocytes from sensitized (Ad-sig-E7/ecdCD40L-injected) C57BL/6 donors were injected i.p. into C57BL/6 nude mice 7 days after s.c. injection of 5×10^5 TC-1 cells. A third group of C57BL/6 nude mice, which were control mice, did not receive passive transfer of T cells from sensitized mice (thick broken line) 7 days after s.c. injection of TC-1 cells. The mice were then monitored for cumulative survival.

CD8 depletion was determined to be 95% and 99%, respectively. Seven days after the last vector injection, the mice were injected with 5×10^5 TC-1 cells. Splenic $CD8^+$ or $CD4^+$ T cells were collected from the Ad-sig-E7/ecdCD40L vector-treated, $CD4^+$- or $CD8^+$-depleted donor mice, which had remained tumor-free for 3 months.

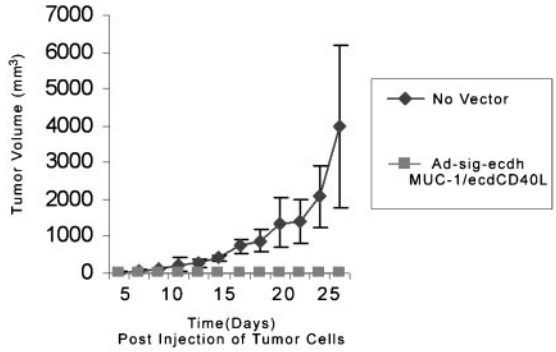

Fig. 6. The effect of the s.c. injection of the Ad-sig-ecdhMUC-1/ecdCD40L vector on the growth of the hMUC-1-positive LL1/LL2hMUC-1 cancer cell line in syngeneic hMUC-1.Tg mice. The Ad-sig-ecdhMUC-1/ecdCD40L vector was injected s.c. twice at 7-day intervals into hMUC-1.Tg mice, which were transgenic for the hMUC-1 gene. One week after the second vector injection, the mice were injected with 5×10^5 LL1/LL2hMUC-1 syngeneic mouse cancer cells that were positive for the hMUC-1 antigen. The growth of the LL1/LL2hMUC-1 cells in mice injected with the Ad-sig-ecdhMUC-1/ecdCD40L vector (■) was significantly different from the growth seen in mice not injected with vector (◆).

Five million of the $CD8^+$ T cells from CD4-depleted sensitized donors were mixed with 5 million of the splenic $CD4^+$ T cells from unsensitized donors. These cells are referred to as $CD8^+$ T cells from sensitized donors. Similarly, 5 million of the splenic $CD4^+$ T cells from CD8-depleted sensitized donors were mixed with 5 million of the splenic $CD8^+$ T cells from unsensitized donors. These cells are referred to as $CD4^+$ T cells from sensitized donors. Five million of each of these populations of cells were injected i.p. into C57BL/6 athymic nude mouse recipients in which TC-1 nodules had already been established.

As shown in Fig. 5*B*, the $CD4^+$ T cells (thin unbroken line) from the sensitized C57BL/6 mice did not protect the C57BL/6 nu/nu mice, whereas the $CD8^+$ cells from the sensitized C57BL/6 mice (thick unbroken line) prolonged the survival of the nude mice after injection of the TC-1 cells. Lymphocytes from unsensitized donor mice (broken thick line) did not protect the mice from TC-1 tumor growth. No statistically significant difference exists between the control- and CD8-depleted groups in Fig. 5 ($P = 0.21$).

Subcutaneous Injection of the Ad-sig-ecdhMUC-1/ecdCD40L Vector Overcomes Anergy for hMUC-1-Positive Cells in Mice That Are Transgenic for hMUC-1. The MUC-1 antigen is overexpressed in carcinomas of the breast, ovary, and pancreas and in other carcinomas (9). MUC-1 is also a self-antigen that is focally expressed on normal secretory epithelial cell apical surfaces. The overexpression of hMUC-1 in epithelial cancers is thought to disrupt E-cadherin function, leading to anchorage-independent growth and metastases (10). hMUC-1.Tg mice, which are transgenic for the hMUC-1 antigen, have been reported to be unresponsive immunologically to the hMUC-1 antigen (11).

We therefore s.c. injected the Ad-sig-ecdhMUC-1/ecdCD40L vector into hMUC-1.Tg mice. The hMUC-1.Tg mice had expressed the hMUC-1 antigen since birth (11). This experiment would therefore test whether the Ad-sig-ecdhMUC-1/ecdCD40L vector injection could produce resistance in anergic mice to the growth of syngeneic mouse cancer cells that were positive for the hMUC-1 antigen. As shown in Fig. 6, injection of the hMUC-1 mouse syngeneic tumor cell line, LL1/LL2hMUC-1, into the hMUC-1.Tg mice, which had not been injected with vector (◆), produced progressive growth of the LL1/LL2hMUC-1 s.c. tumor. These control animals had to be killed by 25 days after the s.c. injection of the tumor cells.

In contrast, in the hMUC-1.Tg transgenic mice that received s.c. injections of the Ad-sig-ecdhMUC-1/ecdCD40L vector, the growth

of the LL1/LL2hMUC-1 cell line was completely suppressed in all the animals tested (see ■, Fig. 6). Thus, the Ad-sig-ecdhMUC-1/ecdCD40L vector strategy can overcome anergy in 100% of the test mice without the need for additional cytokine booster treatments.

Discussion

The results of the experiments reported in this article show that the injection of the Ad-sig-E7/ecdCD40L vector into C57BL/6 mice induces T cell-mediated tumor immunity to the engraftment and growth of E7-positive tumor cells. The Ad-sig-E7/ecdCD40L also induces regression of established s.c. E7-positive tumor nodules in the C57BL/6 mice. Intraperitoneal injection of splenic T cells collected from Ad-sig-E7/ecdCD40L-vaccinated mice, which had remained tumor-free for >1 year after injection of the Ad-sig-E7/ecdCD40L vector and tumor challenge, induced regressions of TC-1 tumors already growing in immunocompromised athymic nude recipient mice (see Fig. 5*A*). This experiment and the experimental results summarized in Fig. 5*B* show that the effect of the Ad-sig-E7/ecdCD40L injections on E7-positive TC-1 cells is mediated by a $CD8^+$ T cell-dependent immune response that lasts for >1 year.

The study of the effect of s.c. injection of the Ad-sig-ecdhMUC-1/ecdCD40L vector into hMUC-1.Tg mice (11) allowed us to test whether the Ad-sig-ecdhMUC-1/ecdCD40L vector injection by itself could activate a $CD8^+$ T cell immune response against the hMUC-1-positive mouse cells in 100% of the animals otherwise anergic to the hMUC-1 antigen. This proved to be the case.

The Ad-sig-TAA/ecdCD40L vector strategy described in this article is unique in several ways. It has been shown to overcome anergy in a transgenic mouse model in 100% of the test mice without the use of cytokine boosting. In addition, it can generate cellular immunity for up to a year, which indicates that the vector strategy outlined in this article induces memory cells.

The Garen laboratory (16) has recently reported that the s.c. injection of 293 cells infected with an adenoviral vector carrying an E7/IgGFc transcription unit can suppress the growth of the TC-1 cell line in a syngeneic mouse model. An interesting parallel exists between the Ad-sig-E7/IgGFc vector of Garen and the Ad-sig-TAA/ecdCD40L vector described in this article: an *in vivo* continuous release strategy is used in both sets of experiments to generate an immune response against a foreign antigen.

The finding about the Ad-TAA/ecdCD40L vector that is different from the findings reported by Garen and his colleagues is the successful activation of an immune response against self-antigens without the need for cytokine booster treatments. The successful induction of T cell-mediated tumor immunity in 100% of the test anergic animals with the Ad-sig-ecdhMUC-1/ecdCD40L vector shows that the adenoviral vector delivery vehicle is superior to the oral DNA vaccine of Xiang (1) that was delivered in *Salmonella*. No need exists to use additional IL-2 treatments after the TAA-ecdCD40L vaccination in the case of the Ad-sig-ecdhMUC-1/ecdCD40L vector, whereas the IL-2 treatments are required to induce an antitumor immune response in 100% of the anergic animals with the *Salmonella* DNA vaccine approach. This experimental result confirms the superiority of the adenoviral vector approach.

The introduction of the secretory sequence at the amino-terminal end of the TAA/ecdCD40L transcription unit and the deletion of the transmembrane domain of the CD40L ensure that this protein will be secreted from the infected cells. Previous reports of the use (17) of Ad-CD40L vectors did not use a secretable CD40L transcription unit because the goal was to display the CD40L on the plasma membrane of the DCs. In this work (17), the effect of the vector was limited to the vector-infected cells and the cells that they directly stimulate. The use of the secretable vector in our work produces an amplification effect beyond the vector infected cells to uninfected DCs.

The statistically significant increase in secretion of IL-12 and IFN-γ at 48 h after exposure of the DCs to the Ad-sig-E7/ecdCD40L vector, as compared with the PBS control (see Fig. 2), shows that the E7/ecdCD40L fusion protein can bind to the CD40 receptor on DCs and stimulate the CD40 receptor sufficiently well to activate the DCs. The fact that the IL-12 and IFN-γ secretion after exposure to the Ad-sig-E7/ecdCD40L vector is statistically significantly greater than the secretion after exposure of the DCs to the Ad-E7 or Ad-sig-GFP/ecdCD40L vectors (see Fig. 2) shows that the increased secretion is not due to the effect of the adenoviral infection/transfection process on the DCs.

One possible complication of inducing an immune response against a self-antigen associated with cancer is that this could generate an autoimmune disease against the normal tissues that normally express that antigen. Several considerations suggest that this will not be a problem with the MUC-1 antigen. First, although the MUC-1 antigen is overexpressed diffusely at very high levels throughout neoplastic epithelial cells, MUC-1 is expressed only very focally and at very low levels in normal epithelial apical structures. Although experiments using a tandem-repeat protein of the MUC-1 antigen with a *Leishmania*-derived protein as adjuvant generated in chimpanzees $CD4^+$ helper and $CD8^+$ cytotoxic responses, no signs of autoimmune disease were detected for up to 1 year after the administration of the vaccine (18). Passive transfer of anti-MUC-12 antibodies does not cause autoimmune disease (19).

The results obtained with the Ad-sig-E7/ecdCD40L vector injections suggest that these vectors may be useful in the prevention of HPV-associated cervical cancer and for the treatment of metastatic cervical cancer. In addition, because hMUC-1 is a self-antigen that is overexpressed in 90% of carcinomas of the breast, ovary, colon, and lung, it is possible that the Ad-sig-ecdhMUC-1/ecdCD40L vector vaccine strategy described in this report could be of use in activating an immune response against a wide range of epithelial neoplasms in human patients.

We thank Sidney Kimmel for supporting our cancer center. This work was also supported by Grant DAMD 17 99 9457 from the U.S. Army, a grant from the Breast Cancer Research Foundation, and support from the Sidney Kimmel Cancer Center Vaccine Fund, the Anthony Dewitt Frost Melanoma Research Fund, the Audrey Demas Melanoma Research Fund, and the George and Barbara Bush Fund for Leukemia Research.

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