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14. ABSTRACT

Pre-clinical testing has shown that subcutaneous injection of an Ad-sig-TAA/ecdCD40L adenoviral vaccine strategy overcomes anergy to tumor associated antigens (TAA) in mice which are genetically engineered to be tolerant of these antigens. Two injections of the vector confer up to a year's immunity and induce regressions of TAA positive tumor even in old (18mo. Old) mice which are known to be poorly responsive to vaccination. This is important since diminished response to vaccine has been seen clinically in human subjects in the age range of peak prevalence of prostate cancer. We have chosen to use hMUC-1, an antigen which is a marker of poor prognosis in prostate cancer, for the clinical development of this vaccine. We have completed the development of a phase I clinical protocol of the ad-sig-hMUC-1/ecdCD40L adenoviral vector vaccine for individuals diagnosed with localized prostate cancer who have evidence of recurrence of their prostate cancer (rising PSA) following radiation therapy for clinically localized disease. We are in the midst of completing the pharmacology, toxicology, and biodistribution assays, GMP production of the Ad-sig-hMUC-1/ecdCD40L and the quality control testing of the GMP production, all of which are needed for FDA approval of the phase I trial is arranged.

15. SUBJECT TERMS
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A. Introduction.

30% of patients who undergo radiation therapy as front line treatment for their prostate cancer will recur in 5 years. The MUC-1 antigen is present at higher than normal levels in prostate cancer as compared to normal prostate epithelial cells. The over-expression of the human MUC-1 makes these cells more resistant to radiation therapy and promotes metastases. Thus, the patients who already have had spread of the prostate cancer at the time of initial local treatment will have nests of cells spread throughout the body (lymph nodes, bone, etc) from the prostate cancer. These cells will cause recurrence. These cells also will have the MUC-1 marker on their surface. In addition, some of the prostate cancer cells may survive the irradiation therapy. These cells will also contribute to recurrence after radiation therapy. Thus, the development of a vaccine to MUC-1 will reduce the recurrence rate by directing the immune response against these cells.

The individuals who will be chosen for this trial are those who are thought to have localized disease on the basis of clinical staging parameters, but who are thought to have an increased risk of recurrence after radiation therapy on the basis of their PSA and Gleason Score. The patients admitted to this trial will also be required to have overexpression of the MUC-1 antigen on the prostate cancer cells by immunohistochemistry of the biopsy specimen.

The patients that this proposed adjuvant immunotherapy will help are those who are destined to recur following initial local therapy. If this treatment is found to reduce the recurrence rate from prostate cancer following initial radiation therapy, it could also be applied as post surgical adjuvant therapy for prostate cancer. Thus, the proposed therapy is relevant to the mission of the DOD PCRP. In addition, because the MUC-1 antigen is over-expressed on other epithelial cancers (breast, lung, colon, ovary), the vaccine may ultimately be relevant for the reduction of recurrence after local therapy for these patients as well.

B. Body.

B.1. Clinical Protocol Development.

A complete clinical protocol and informed consent was developed for the Phase I trial testing of the Ad-sig-hMUC-1/ecdCD40L vector in prostatic cancer patients who experience biochemical recurrence of prostate cancer (by PSA rise) following initial local radiation therapy. The completed clinical protocol and informed consent are provided in the Appendices.

B.2. Funding Development for Toxicology/Pharmacology/Biodistribution Studies, GMP Manufacturing and Quality Control for the Ad-sig-hMUC-1/ecdCD40L Vector.

Foundation support was obtained for the initial phase I clinical testing of the toxicity of the Ad-sig-hMUC-1/ecdCD40 vaccine vector which is proposed for the clinical protocol in the preceding section. This includes the pre-clinical contract testing for pharmacology, toxicology and biodistribution, GMP manufacturing of the Ad-sig-hMUC-1/ecdCD40L vector and quality control testing by a contract firm (Charles River) of the GMP production log. This will put us ahead in the development of the prostatic vector vaccine clinical development.

B.3. Demonstration that the Ad-sig-TAA/ecdCD40L Vaccine Strategy:

a. Induces an Immune Response in Chronologically Age Advanced Test Subjects in a PreClinical Model.

b. Induces a 10 Fold Increase in the Intratumoral Levels of CD8 Effector T Cells in Chronologically Age Advanced Test Subjects in a PreClinical Model.

c. Induces a 2 Fold Decrease in the Intratumoral Levels of CD4 FOXP3 Negative Regulatory T Cells in Chronologically Age Advanced Test Subjects in a Preclinical Model.

d. Induces an Immune Response Against Tumor Vascular Markers on the Luminal Surface of Tumor Vascular Endothelial Cells.

e. Demonstration that the concomitant administration of chemotherapy and the Ad-sig-TAA/ecdCD40L vaccine expands the immune response induced by the Ad-sig-TAA/ecdCD40L when delivered alone.

B.4. Detailed Research Report on the Advances Summarized Above in B.3.

B.4.a. Levels of hMUC-1 Specific Antibodies with Ad-sig-hMUC-1/ecdCD40L Vector Prime/hMUC-1/ecdCD40L Boost Vaccination.

We first tested for the level of anti-hMUC-1/ecdCD40L antibodies in the serum of the treated mice. 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 below in Figure 1, 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 spaced at a 14 day interval. The increase in the anti ecdhMUC-1/ecdCD40L antibodies following the T5 treatment was 2 times greater than any of the other treatment groups.

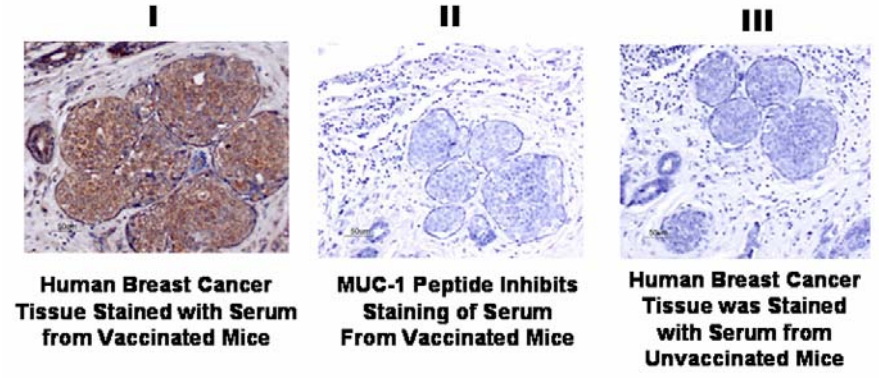
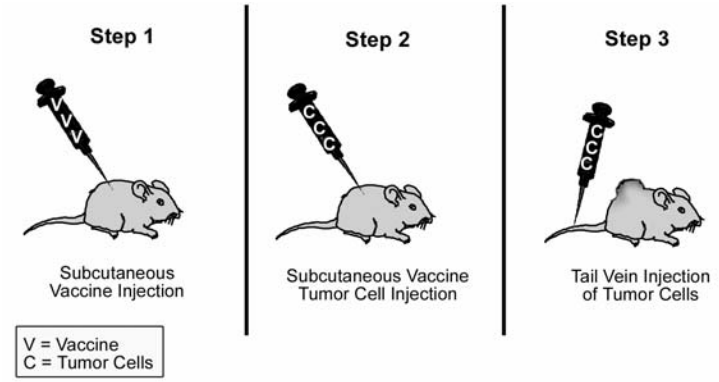


Figure 1

We then tested if the hMUC-1 antibodies induced in the Ad-sig hMUC-1/ecdCD40L vector followed by the hMUC-1/ecdCD40L protein boost in vaccinated mice bind to patient biopsies which contain human (breast) cancer cells. 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 above in Figure 1, Panel I, the FITC conjugated serum from the vaccinated mice bound to the breast epithelial cells in the biopsy specimens of cancerous epithelial cells. There was no binding to the intervening fibroblast or stromal cells in the biopsy specimens indicating that the antibodies specifically recognized breast epithelial cells (see Panel I of Figure 1). As shown in Panel II of Figure 1, 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 1). This suggests that the vector prime/protein boost will induce a hMUC-1 specific immune response in human patients vs the (breast) cancer cells. *Please note: these experiments with breast cancer cells along with experiments conducted with cervical-HPV, lewis lung and avian influenza cells have shown the*

Ad-sig-hMUC-1/ecdCD40L vaccine to be successful in inducing an immune response against hMUC-1. Since the hMUC-1 is the same antigen in cancers of breast, colon, lung and prostate, the results of these experiments have immediate relevance for prostate cancer cell lines. The TAA-hMUC-1is the same for all organ sites.

Figure 2



B.4.b. Treatment of Pre-Existing Cancer and Lung Metastases with Ad-sig-hMUC-1/ecdCD40L Vector Prime/hMUC-1/ecdCD40L Protein Boost. Two types of experiments were carried out, one called "prevention" and one called "treatment". (see Figure 2).

B.4.c. Prevention. Five hundred thousand LL2/LL1hMUC-1 lung cancer cells were injected sc into each of hMUC-1.Tg mice 7 days after the last vaccination. Two weeks following the sc injection of the tumor cells, 500,000 LL2/LL1hMUC-1 tumor cells were injected by tail vein. The size of the sc tumor nodules that developed was measured by caliper at multiple time points to determine the effect of the various vaccine schedules on the growth of the LL2/LL1hMUC-1 cells as sc nodules.

We first measured the growth of the LL2/LL1hMUC-1 cancer cells by measuring the size of the subcutaneous nodule at the site injected subcutaneously with the LL2/LL1hMUC-1 cells on day 35. As shown below in Figure 3, three injections of the ecdhMUC-1/ecdCD40L protein (PPP) without a preceding Ad-sig-ecdhMUC-1/ecdCD40L vector failed to induce complete resistance to the growth of the LL2/LL1hMUC-1 tumor cells as a subcutaneous nodule in the hMUC-1.Tg mice. In contrast, the schedule of three successive vector injections (VVV) or one vector injection followed by two sc protein injections (VPP) both completely suppressed the growth of the LL2/LL1hMUC-1 tumor cell line.

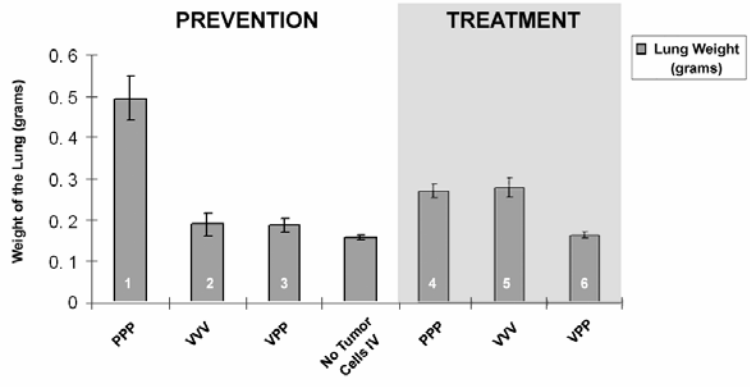
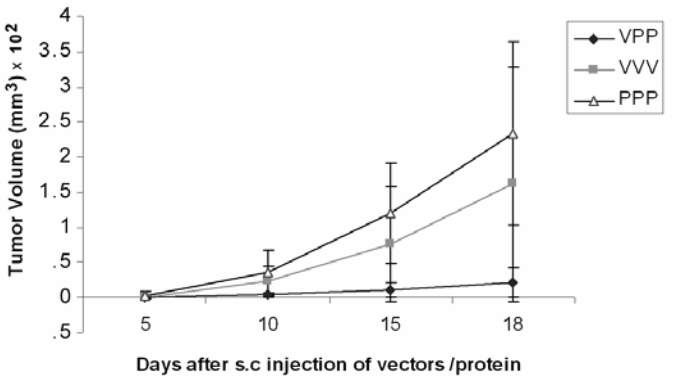


Figure 3

We then weighed the lungs of mice sacrificed 63 days following the initiation of vaccination. As shown to the left in Figure 3, three successive ecdhMUC-1/ecdCD40L protein sc injections (PPP) did not suppress the growth of the LL2/LL1hMUC-1 cancer cells in the lungs of the hMUC-1.Tg mice given the vaccine before the sc and intravenous injection of the LL2/LL2hMUC-1 cancer cells. In contrast, both the three successive Ad-sig-ecdhMUC-1/ecdCD40L vector sc injections (VVV) and the single Ad-sig-ecdhMUC-1/ecdCD40L vector sc injection followed by two successive sc injection of the ecdhMUC-1/ecdCD40L protein at 7 and 21 days (VPP) completely suppressed the engraftment of the lung cancer in the lungs of the vaccinated animals. 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 antibodies in the hMUC-1.Tg mice.

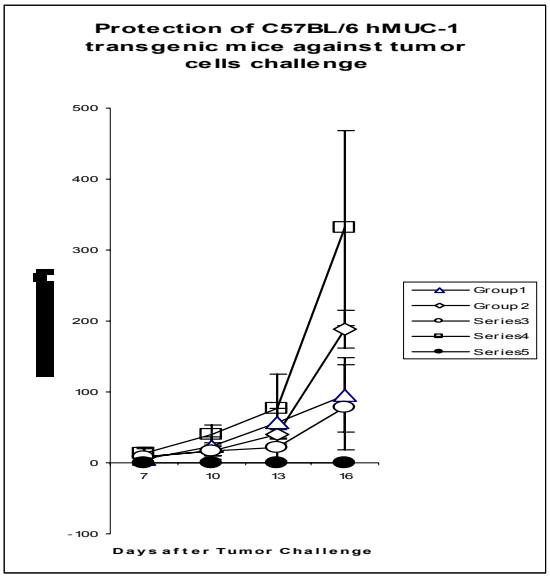
B.4.d. Therapy Experiment. We then studied the effect of the vaccinations on existing subcutaneous nodules of the LL2/LL1hMUC-1 tumor cells. As shown below in Figure 4, vaccinations were carried out starting 5 days following the sc injection of 500,000 of the LL2/LL1hMUC-1 tumor cells. As shown below in Figure 4, the combination of one vector sc injection followed by two protein sc injections (VPP) completely suppressed the growth of established nodules of the hMUC-1 positive cancer cells. Three successive protein sc injections (PPP) did not control the growth of the tumor cells.

Figure 4



Next, we measured the lungs of the animals vaccinated after sc injection of the LL2/LL2hMUC-1 cells (designated "Treatment" in Figure 4 above). Following completion of the sc vaccination, the LL2/LL1hMUC-1 cells were injected intravenously. In this model, the sc tumor cells are already growing by the time of the vaccination. As shown to the right in Figure 4, the only combination of sc vaccination which suppressed the growth of the LL2/LL1hMUC-1 tumor cells in the lung, albeit partially, was the schedule of a single sc injection of the Ad-sig-ecdhMUC-1/ecdCD40L vector followed by two successive sc injections of the ecdhMUC-1/ecdCD40L protein. This was the best schedule in all of the studies throughout our experiments. These data show that the VPP schedule involving two hMUC-1/ecdCD40L protein sc injections after a single initial Ad-sig-ecdhMUC-1/ecdCD40L vector sc injections induced the most robust immune response against the hMUC-1 positive cancer cells in the hMUC-1.Tg mice with irradiated cytokine positive tumor cells.

Figure 5: Testing of Boosting Proteins Composed of hMUC-1 TAA Without CD40L. In order 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 (Solid squares-Series 5): bacterial cell lysate (Open diamonds-Series 2), Keyhole Limpet Hemocyanin (KLH) conjugated hMUC-1 antigen with (Open circles-Series 3) and without (Open triangles-Series 1) incomplete Freund's adjuvant, and PBS (Open squares-Series 4)



B.4.e. Are Both hMUC-1 and CD40L Necessary for Boosting of the Anti-Tumor Immune Response Induced by Ad-sig-hMUC-1/ecdCD40L Vector? In order to test if the hMUC-1 antigen could be used alone without the CD40L to induce immunity against the hMUC-1 tumor cells in the hMUC-1.Tg mice, we compared the suppression of the growth of hMUC-1 positive LL2/LL1 tumor cells in the hMUC-1.Tg mice which had been vaccinated with the Ad-sig-hMUC-1/ecdCD40L vector (Subunit I) followed by a boost of the hMUC-1 Subunit I antigenic peptide linked to the ecdCD40L (hMUC-1/ecdCD40L), or followed by the hMUC-1 Subunit I antigenic peptide linked to the KLH stabilizing molecule (hMUC-1/KLH), or followed by the hMUC-1 Subunit I antigenic peptide linked to the KLH stabilizing molecule (hMUC-1/KLH)

with incomplete Freund's adjuvant, or an extract of the bacterial host strain used to produce the hMUC-1/ecdCD40L as a control. As shown in Figure 5, the hMUC-1/KLH with or without Freunds 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, whereas boosting with the hMUC-1/ecdCD40L protein produced in bacterial cells completely suppressed the growth of the hMUC-1 positive tumor cells. Since the hMUC-1/ecdCD40L protein used for the boost was derived from a bacterial expression vector, we also tested the effect of boost the immune response induced by the Ad-sig-hMUC-1/ecdCD40L vector followed by a boost with a lysate from bacterial cells not containing the hMUC-1/ecdCD40L protein. As shown above in Figure 5, the bacterial cell lysate had little effect on the suppression of the hMUC-1 positive tumor cells over and above that induced by the Ad-sig-hMUC-1/ecdCD40L vector vaccine.

B.4.f. Development of Vaccine for Tumor Vascular Endothelial Cells. Schnitzer and co-workers recently reported that the Annexin 1A (Anx1A) protein is 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 if the subcutaneous injection of the Ad-sig-Anx1A/ecdCD40L vector would suppress the growth of the hMUC-1 positive LL2/LL1hMUC-1 cell line. As shown below in Figure 6A, the sc injection of this vector suppressed the growth of the hMUC-1 positive cells in hMUC-1.Tg mice. In order to test specifically if the immune response generated by the Ad-sig-AnxA1/ecdCD40L vector is directed against the Annexin A1 antigen, serum was taken from a mouse which had been injected sc twice with the Ad-sig-AnxA1/ecdCD40L vector and tested by ELISA assay for the presence of antibodies against the Annexin A1 antigen. As shown in Figure 6B, antibodies which bind Annexin A1 are induced in the serum of the Ad-sig-AnxA1/ecdCD40Lvaccinated hMUC-1.Tg mice.

Figure 6A

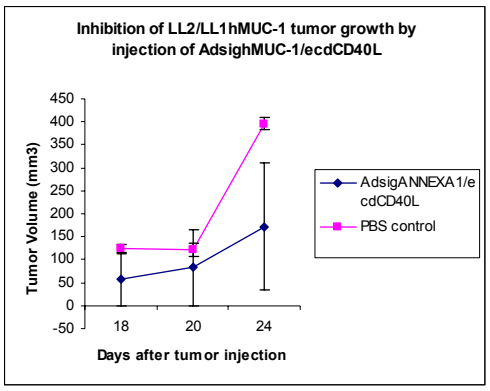


Figure 6B

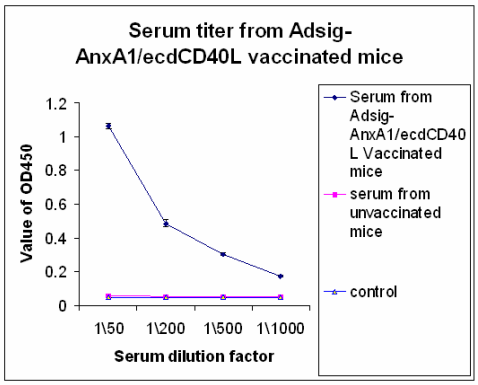
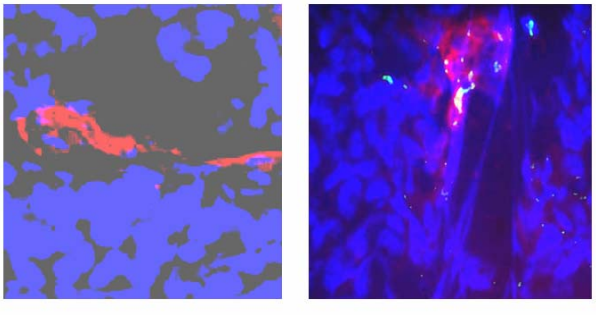


Figure 6C



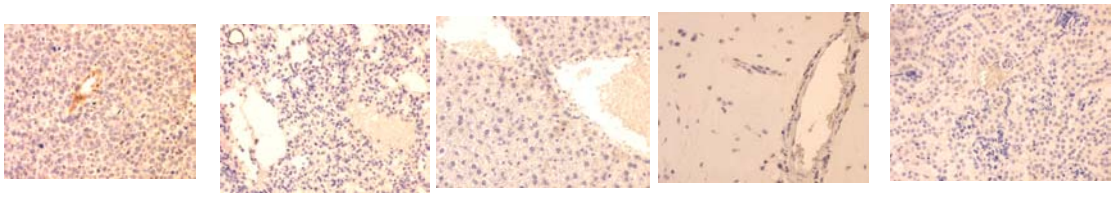
Double staining the tumor tissue with serum from vaccinated mice (green) and PE-labeled Anti-mouse CD31 (red)

In order to directly test if these antibodies are binding to the tumor vasculature, multiparameter fluorescence confocal microscopy was carried out on frozen sections of hMUC-1 positive, Annexin A1 negative tumor tissue. As shown in Figure 6C (left), the binding of the FITC conjugated (green staining) serum antibodies against Annexin A1 generated in the mice injected sc with the Ad-sig-AnxA1/ecdCD40L vector bind to the tumor vasculature as shown by the yellow spots in the right hand panel in Figure 6C. The red color of the anti-CD31 vascular binding antibody

(phycoerythrin 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 hand panel of Figure 6C in which frozen sections of tumor tissue was exposed to FITC conjugated serum from unvaccinated mice and the phycoerythrin conjugated anti-CD31 antibodies. These results suggest that the Ad-sig-TAA/ecdCD40L vaccine strategy can induce an immune response against tumor vascular endothelial cells, thereby suppressing the growth of the tumor tissue which depended on the Anx1A positive tumor vasculature.

B.4.g. Specificity of the Ad-sig-AnxA1/ecdCD40L Vector Vaccination for Tumor Vascular Endothelial Cells. Annexin A1 is a cytosolic protein in normal cilitated tissues, the central nervous system and in 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 (Figure 7A), normal lung-a ciliated tissue (Figure 7B), normal kidney (Figure 7C) normal central nervous system (7D) and normal liver (Figure 7E). As shown below in Figure 7, HRP conjugated secondary anti-mouse antibodies produced positive staining in the vessels of tumor tissue but not in the normal lung, brain, or kidney.

Fig 7A-Tumor **Fig 7B-Lung** **Fig 7C-Kidney** **Fig 7D-Brain** **Fig 7E-Liver**



B.4.h. Study of the Combination of the Ad-sig-TAA/ecdCD40L and Ad-sig-TVECA/ecdCD40L Vaccines.

In order to test if the combination of the Ad-sig-TAA/ecdCD40L anti-cancer cell vaccine with the Ad-sig-TVECA/ecdCD40L anti-tumor vascular endothelial cell vaccine would produce a tumor suppressive effect that is greater than either vaccine alone, we vaccinated rH2N.Tg mice subcutaneously with the Ad-sig-rH2N/ecdCD40L anti-Her-2-Neu breast cancer cell vaccine and with the Ad-sig-AnxA1/ecdCD40L anti-tumor vascular endothelial cell vaccine. It is noteworthy that the rH2N positive breast cancer cells injected subcutaneously in the vaccinated mice were AnxA1 negative. As shown at left in Figure 8, the effect of the combination of the Ad-sig-rH2N/ecdCD40L and the Ad-sig-AnxA1/ecdCD40L vaccines (open squares) was greater than the effect of either vaccine alone (open diamonds or open circles).

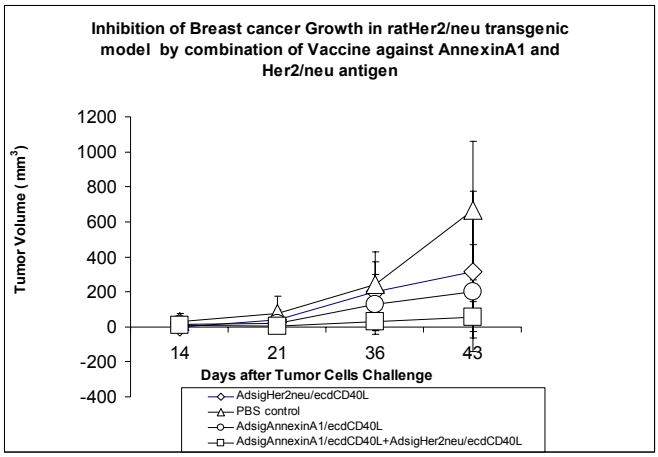
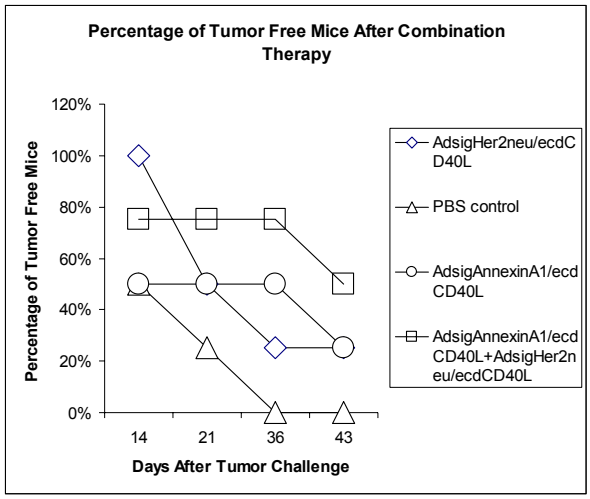


Figure 8

We also followed the vaccinated mice following challenge with the rH2N positive breast cancer syngeneic cell line. As shown below in Figure 9, the mice vaccinated with a combination of the Ad-sig-rH2N/ecdCD40L and the Ad-sig-AnxA1/ecdCD40L tumor vascular targeting vaccine vector showed the highest percentage of mice which remained tumor free.

Figure 9



B.4.i. Levels of Tumor Infiltrating T Cells in Tumor Tissue Following Ad-sig-TAA/ecdCD40L Vaccination. We had shown previously that the sc injection of the Ad-sig-TAA/ecdCD40l vector activated and tumor antigen loaded DCs and promoted their migration to the regional lymph nodes where resulting in an increase in the levels of the TAA specific T cells. 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 which 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 sc tumor nodules of rH2N.Tg mice before and after two sc 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. We found that number of T cells isolated from the tumor tissue after vaccination with the immunophenotype of effector T cells (CD8+, CD44+, LY6C+ and CD62L-) was increased as shown below in Figure 10. This 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.

B.4.j. The Ad-sig-TAA/ecdCD40L Vaccine Decreases the Level of FOXEP3 Negative Regulatory T Cells in the Tumor Tissue While Increasing the Level of Tumor Infiltrating Effector Cells Even In Older Mice. Many researchers have reported that the immune response is diminished both due to quantitative decreases in the absolute level of CD8 and CD4 T cells, and the acquisition of a functional defect in CD4 helper cells. It is clear from recently completed studies of vaccine studies in older individuals that this acquired defect could limit the response of patients with epithelial neoplasms in the age group above 60. If is possible, however, that since the Ad-sig-TAA/ecdCD40L vector prime and TAA/ecdCD40L protein boost vaccine strategy could circumvent the functional defect in CD4 cells that exists in older individuals since the TAA/ecdCD40L vaccine strategy does not depend on CD4 cells (see above).

We therefore tested in older mice whether it was possible to induce an antigen immune response with the Ad-sig-TAA/ecdCD40L strategy. The laboratory of Dr. Phyllis Linton had maintained old (18 months) mice for purposes of testing of the immune response at an advanced chronological age. She had shown that the CD4 acquired defect of the immune response existed in these mice. We vaccinated C57BL/6J mice which were carrying subcutaneous tumors derived from the E7 positive TC-1 syngeneic tumor cells with the Ad-sig-E7/ecdCD40L vector subcutaneous injections. We then measured the level of E7 specific CD8 T cells in the tumor tissue of these mice before and after vaccination. The vaccination increased the level of E7 tetramer positive CD8 T cells in the tumor tissue by 10 fold (Figure 10A). Since the immune response to vaccines is also limited by the level of FOXEP3 negative regulatory CD4 T cells in the tumor tissue, we measured the changes in the level of these negative regulatory T cells in the tumor tissue before and after vaccination. As shown in Figure 10B, the vaccination decreased the level of the FOXEP3 positive negative regulatory CD4 T cells in the tumor tissue. Conclusion and Significance: These data suggest that the vaccine strategy outlined in the above data could have a major impact on the individuals in the age group in which epithelial neoplasms are prevalent. This is the only vaccine strategy to our knowledge

that has been capable of circumventing the acquired defect of advanced chronological age. In addition, the vaccine not only increases the TAA specific CD8 effector T cells in the tumor tissue by 10 fold, but induces a decrease of 2 fold in the intratumoral level of CD4 FOXEP3 positive negative regulatory T cells. Thus, this is a potent vaccine for epithelial neoplasms.

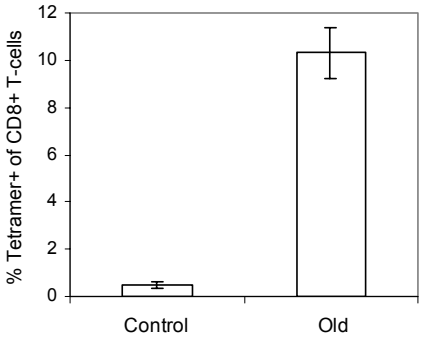


Figure 10A

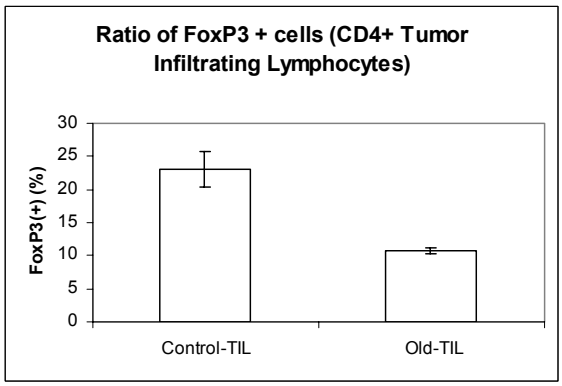


Figure 10B

C. Key Research Accomplishments.

	Task	**Status**
1a.	Design Phase I Clinical Trial	Completed
1b.	Consult w/ Immunology labs, biostatistician, urologists to design/prepare clinical protocol and tests	Completed
1c.	Work w/ SKCC Prostate Cancer Tissue Network and Sharp HealthCare to set up referring urologists for the study	Pending Ph II Funding
1d.	Write clinical protocol (attached as appendix x)	Completed
1e.	Negotiate contract with CRO for Data Management	Pending Ph II Funding
1f.	Negotiate contract with Molecular Medicine Bioservices for Vector Production	Pending Ph II Funding
1g.	Write IND application for FDA	Draft for Ad-sig-hMUC1 vector vaccine completed
1h.	Negotiate contract w/Charles River, Malverne for QC	Pending Ph II Funding
1i.	Write Institutional Biosafety Committee Application	Pending FDA IND
1j.	Respond to NIH RAC questions about vaccine	Completed
1k.	Submission of protocol to Institutional Review Board (IRB)	Completed

The PI and Clinical Coordinator, Joy Hamer, completed a clinical trial design and clinical trial document and IC per the proposed workscope.

The GMP production, which was contingent on additional funding from the DOD for Phase II was never granted. Therefore, the GMP Production could not take place. Without a GMP production, the FDA IND application could not be completed. IBC approval cannot be given until FDA IND is approved.

Funding granted for the first phase was for the development of the protocol document design and submission to the IRB. This has been done. The initiation of the Phase I trial would require a second phase of funding from the DOD which never was approved. Therefore the following could not take place: vector production, IBC, FDA IND, QC, etc.

The funding was provided to develop a design for the Phase I trial, to develop a protocol document and informed consent, to submit it to the IRB. This was done. In addition, the discussions with a urologist (Dr. Chris Amling) were carried out. The infrastructure for the trial was readied. Dr. Amling had access to the requisite patients. Unfortunately the DOD has not yet approved the additional funding which would be required for the actual production of the vaccine and launching the Phase I trial.

Importantly, we have shown that the antibodies that are induced against the hMUC-1 in the hMUC-1.Tg mice by the sc injection of the Ad-sig-hMUC-1/ecdCD40L vector followed by two sc injections of the hMUC-1/ecdCD40L booster protein, react not only with biopsies of human breast cancer, but also with biopsies from human prostate cancer patients. Thus, we feel that we have sufficient data for the Phase I trial in the prostate cancer patients.

D. Reportable Outcomes

1. Completion of Clinical Protocol and Informed Consent for a phase I toxicity trial among prostate cancer patients in whom biochemical recurrence is occurring following radiation therapy.

2. Initiation of pharmacology/toxicology/biodistribution studies for the FDA IND at Molecular Diagnostics, Inc.

3. Initial negotiations with Molecular Medicine Biosciences, Inc. for the GMP manufacturing of the Ad-sig-TAA/ecdCD40L vector for the phase I trial.

4. Initial negotiations with Charles River Malverne for the carrying out of quality control testing of the GMP vaccine vector.

5. Funding for the contract studies for toxicology, GMP manufacturing and contract quality control testing.

6. Demonstration that the Ad-sig-TAA/ecdCD40L vaccine is working in chronically age advanced test subjects in the preclinical models. This is important since the response among older human subjects in vaccine trials is low above the age of 55, the prime age for prostate cancer. Specifically, existing disease in 18 month old mice regresses completely in a fraction of the test subjects.

7. Demonstration that in older mice (18 months) that the Ad-sig-TAA/ecdCD40L vector strategy can induce by 10 fold the levels of antigen specific effector CD8 T cells in the tumor target tissue.

8. Demonstration that in older mice (18 months) that the Ad-sig-TAA/ecddCD40L vector strategy can decrease the levels of CD4 FOXP3 negative regulatory T cells in the target tumor tissue.

E. Conclusions.

1. It is feasible to induce a cellular and humoral immune response with the Ad-sig-TAA/ecdCD40L vector strategy in very old preclinical animal models (18 month old mice which are equivalent to 70 year old human subjects). This makes the proposed clinical trial feasible.
2. It is feasible with the Ad-sig-TAA/ecdCD40L vector strategy to increase the antigen specific effector CD8 T cells in the tumor tissue.
3. It is feasible with the Ad-sig-TAA/ecdCD40L vector strategy to decrease the level of CD4 FOXP3 CD4 cells which are inhibitory to the action of CD8 effector cells in tumor tissue.
4. With the funds developed for the contract toxicology, GMP production and quality control testing, it will be feasible to start an initial phase I testing of the Ad-sig-hMUC-1/ecdCD40L vector which we proposed to use for cancer vaccine therapy of prostate cancer patients who are experiencing biochemical recurrence following initial radiation therapy.

F. References. None.

G. Appendices.

G.1. Tang et al, Blood Paper, Nov 2004

G.2. Tang et al, JNCI, Submitted, 2005

G.3. Clinical Protocol and Informed Consent

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)

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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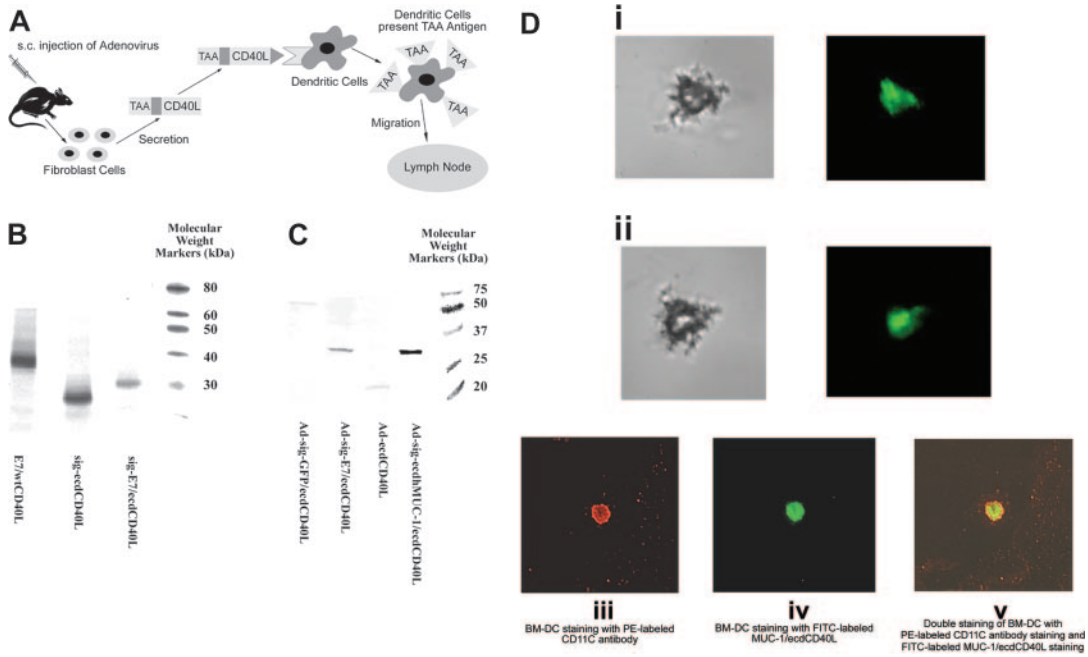

Figure 1. TAA/ecdCD40L protein produced by Ad-sig-TAA/ecdCD40–infected cells binds to DCs. (A) Proposed mechanism for induction of immune response by the Ad-sig-TAA/CD40L vector. Injecting Ad-sig-TAA/ecdCD40L induces in vivo activation and tumor-antigen loading of DCs, migration of the DCs to regional 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

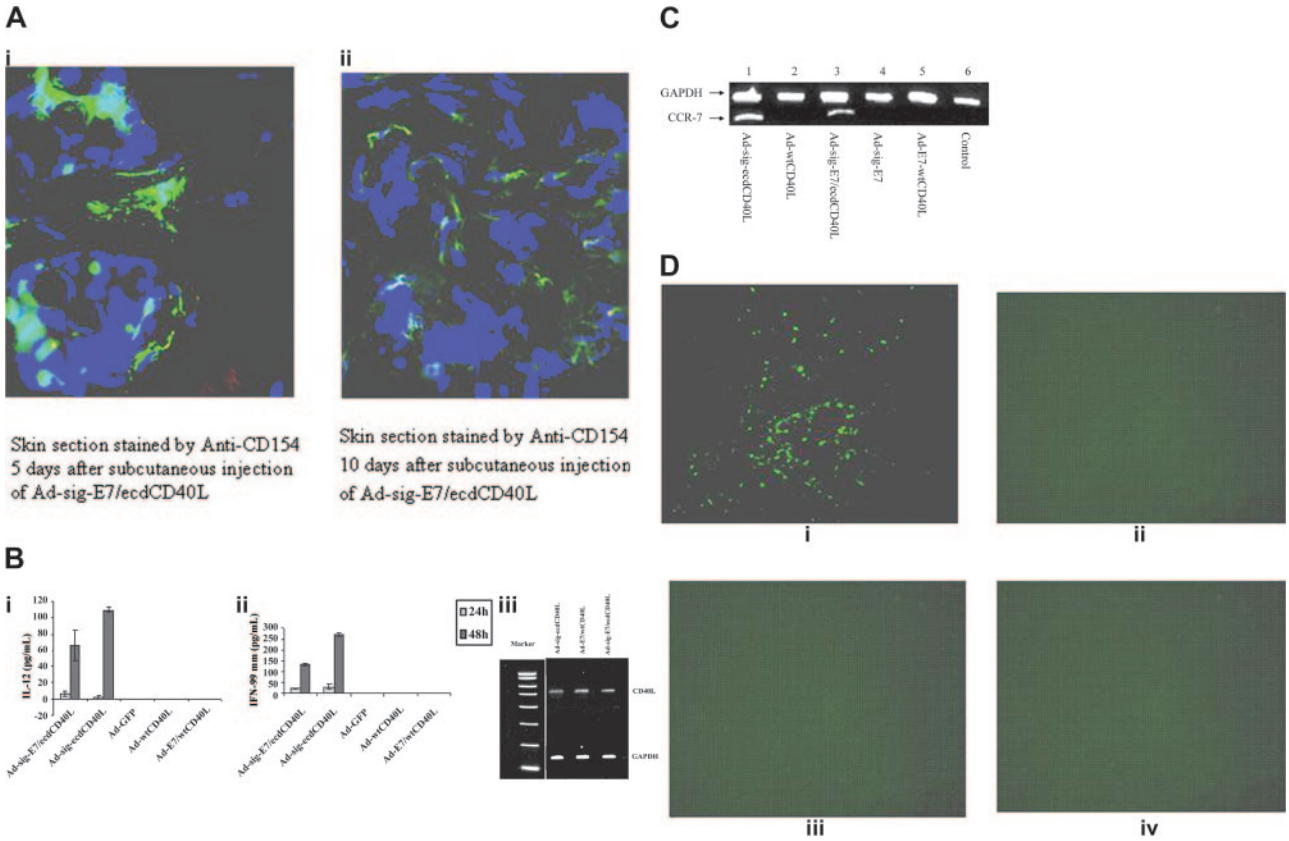

Figure 2. TAA/ecdCD40L protein from Ad-sig-TAA/ecdCD40L vector–infected cells binds to and activates DCs, which induce migration to regional lymphoid tissue. (A) Injection of the Ad-sig-E7/ecdCD40L vector generates the release of the E7/ecdCD40L protein around the vector injection site for up to 10 days. 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

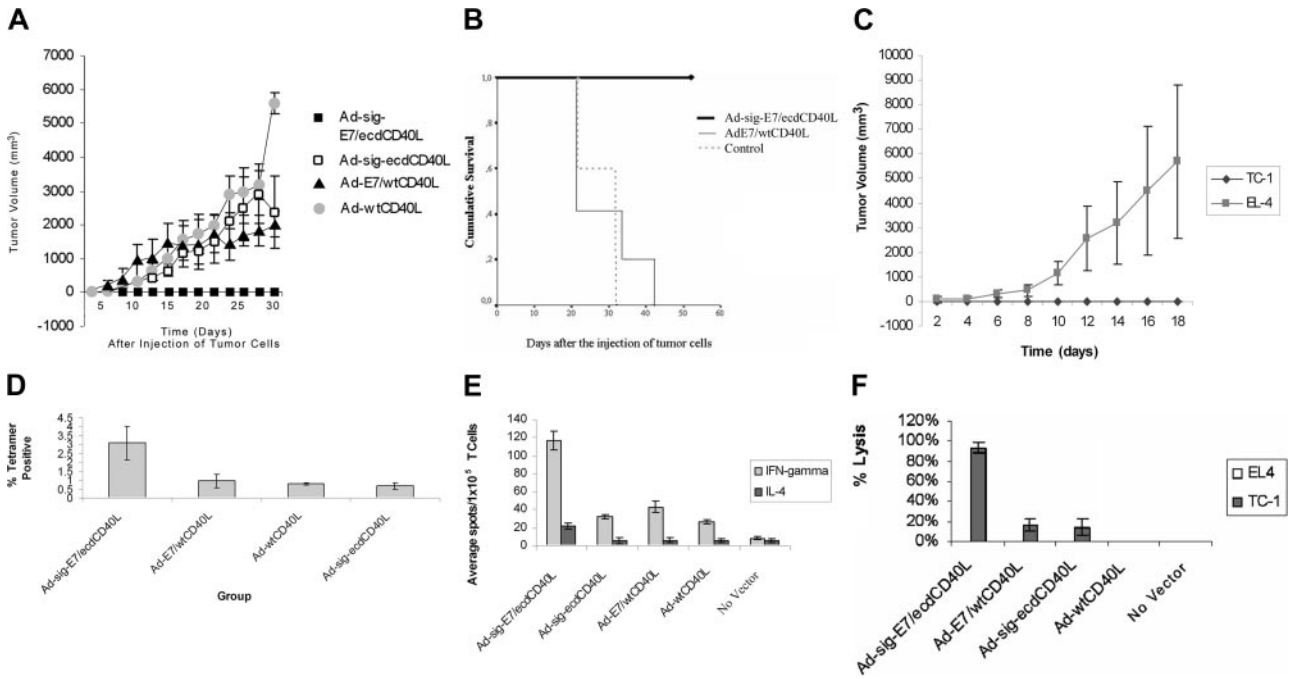

Figure 3. Mechanism of the Ad-sig-E7/ecdCD40L vector–induced suppression of the growth of E7-positive TC-1 tumor cells in C57BL/6 mice. (A) Resistance to the subcutaneous growth of 5×10^5 E7-positive TC-1 cancer cells in mice after 2 injections with 1×10^8 pfu of the Ad-sig-E7/ecdCD40L vector 7 days 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

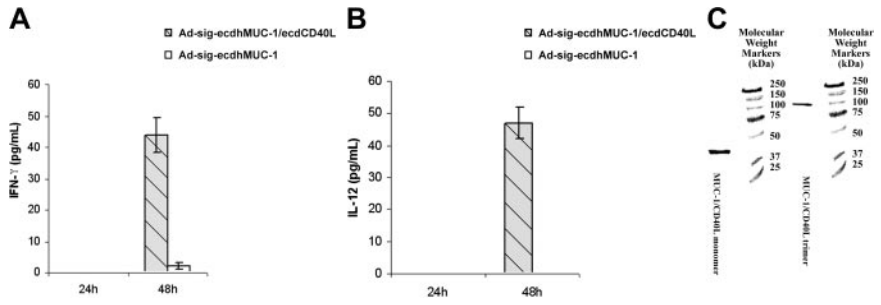

Figure 4. The ecdhMUC1 protein released from Ad-sig-ecdhMUC1/ecdCD40L vector–infected cells forms functional trimers and activates DCs. (A) Induction of IFN-γ secretion from bone marrow–derived DCs induced by exposure to the Ad-sig-ecdhMUC1/ecdCD40L vector. Supernatant medium collected from DCs derived 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.

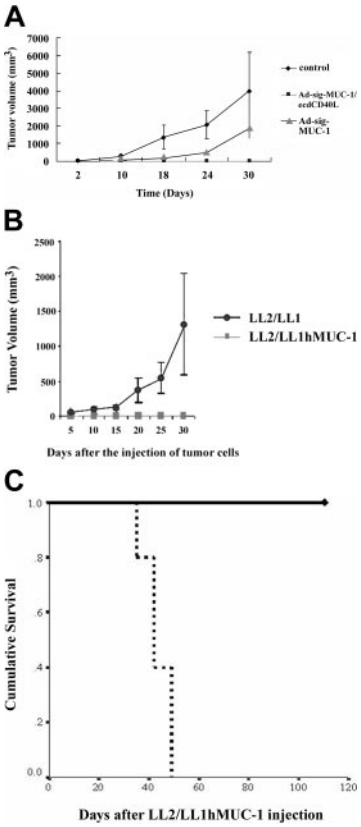

Figure 5. Effect of 2 subcutaneous injections (7 days apart) of 1 × 10^8 pfu of the Ad-sig-ecdhMUC1/ecdCD40L vector on the in vivo growth of the hMUC1-positive LL2/LL1hMUC1 cancer cell line in hMUC1.Tg mice. (A) Two subcutaneous injections (7 days apart) of 1 × 10^8 pfu Ad-sig-ecdhMUC1/ecdCD40L vector 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

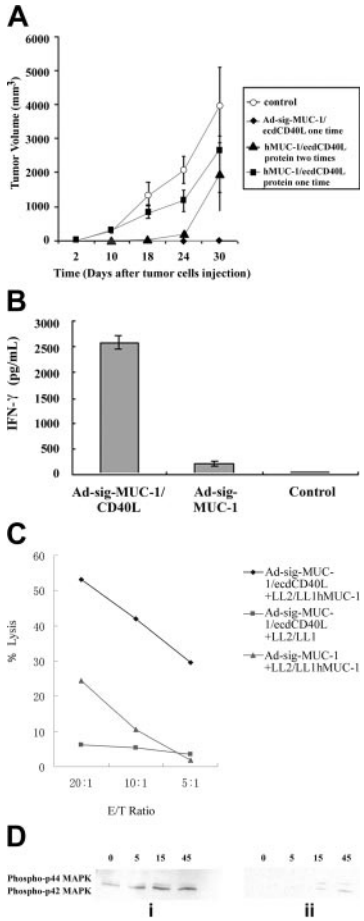

Figure 6. Mechanism of the suppressive effect of the Ad-sig-ecdhMUC1/ecdCD40L vector on induction of the immune suppression of the growth of the LL2/LL1hMUC1 cells in hMUC1.Tg mice. (A) Subcutaneous injection of the ecdhMUC1/ecdCD40L protein does not induce suppression of the growth of hMUC1-positive 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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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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[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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(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.

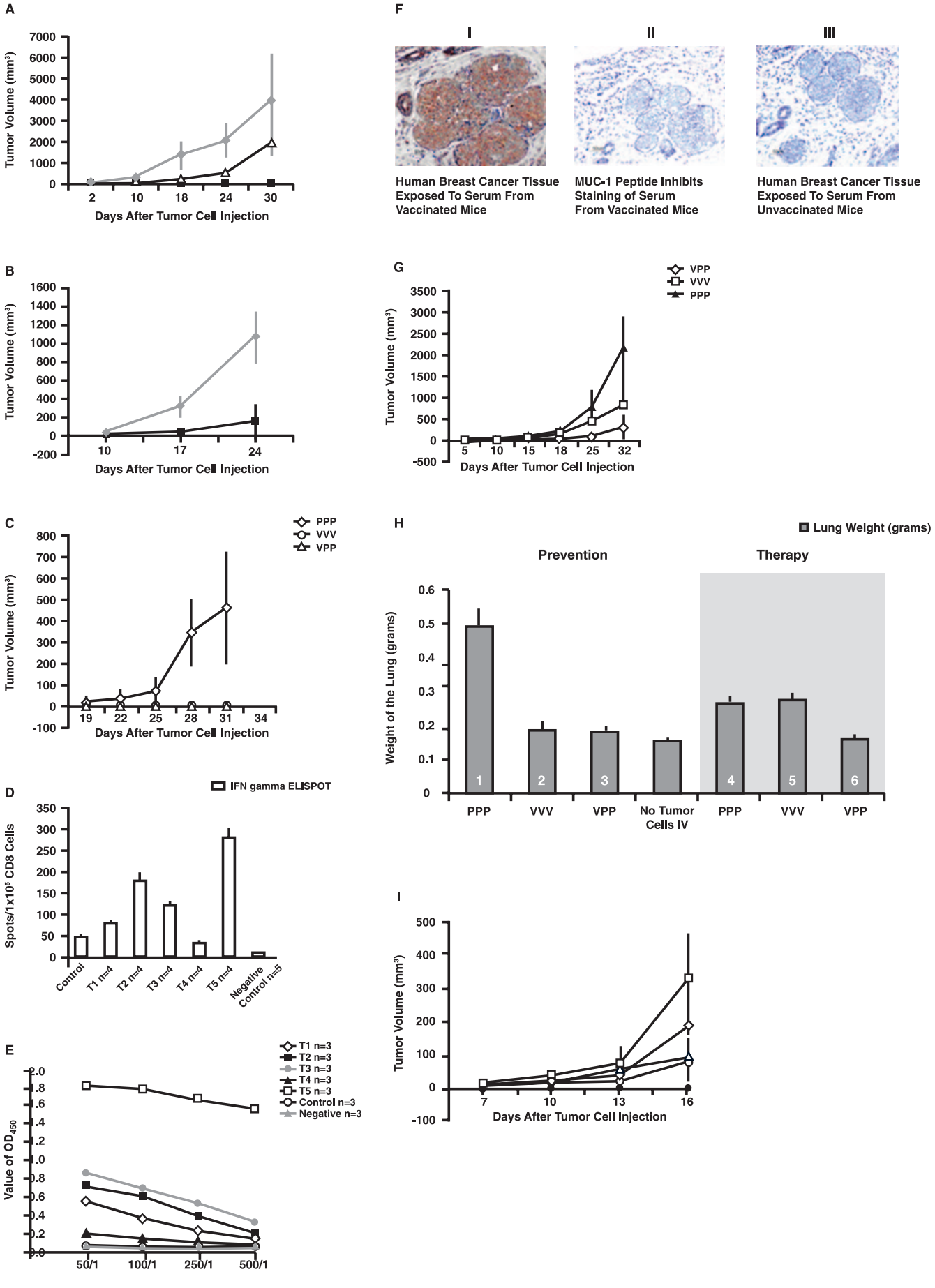
VPP suppresses the growth of i.v. administered MUC-1-positive cancer cells in the lungs of hMUC-1.Tg mice

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 (□).

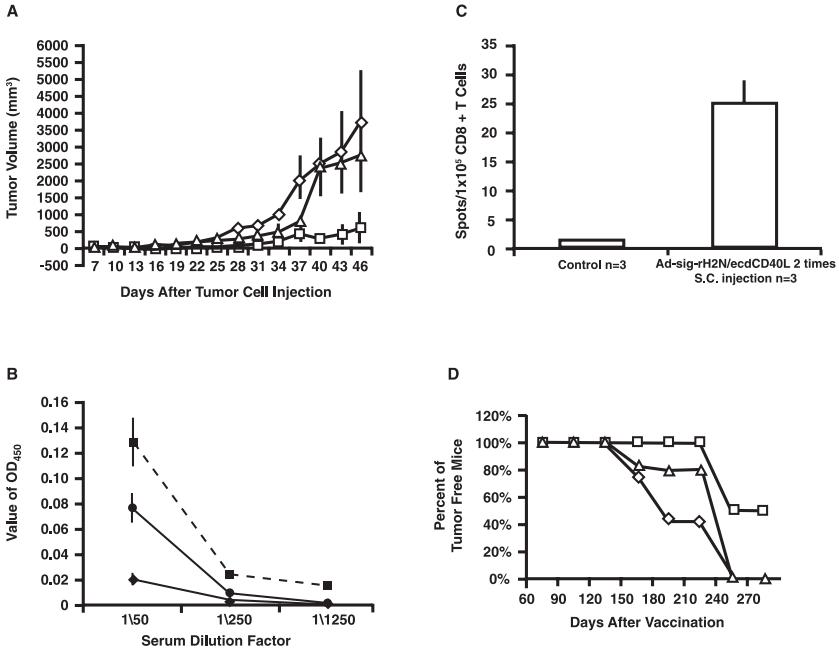


FIGURE 2. *A*, Effect of the Ad-sig-rH2N/ecdCD40L vector on the growth of rH2N-positive NT2 cells. The following vectors were injected s.c. twice at a 7-day interval: no vector (◇); two s.c. injections of the Ad-sig-rH2N/ecdCD40L vector (□), and one s.c. injection of the Ad-sig-rH2N/ecdCD40L vector (△). *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.

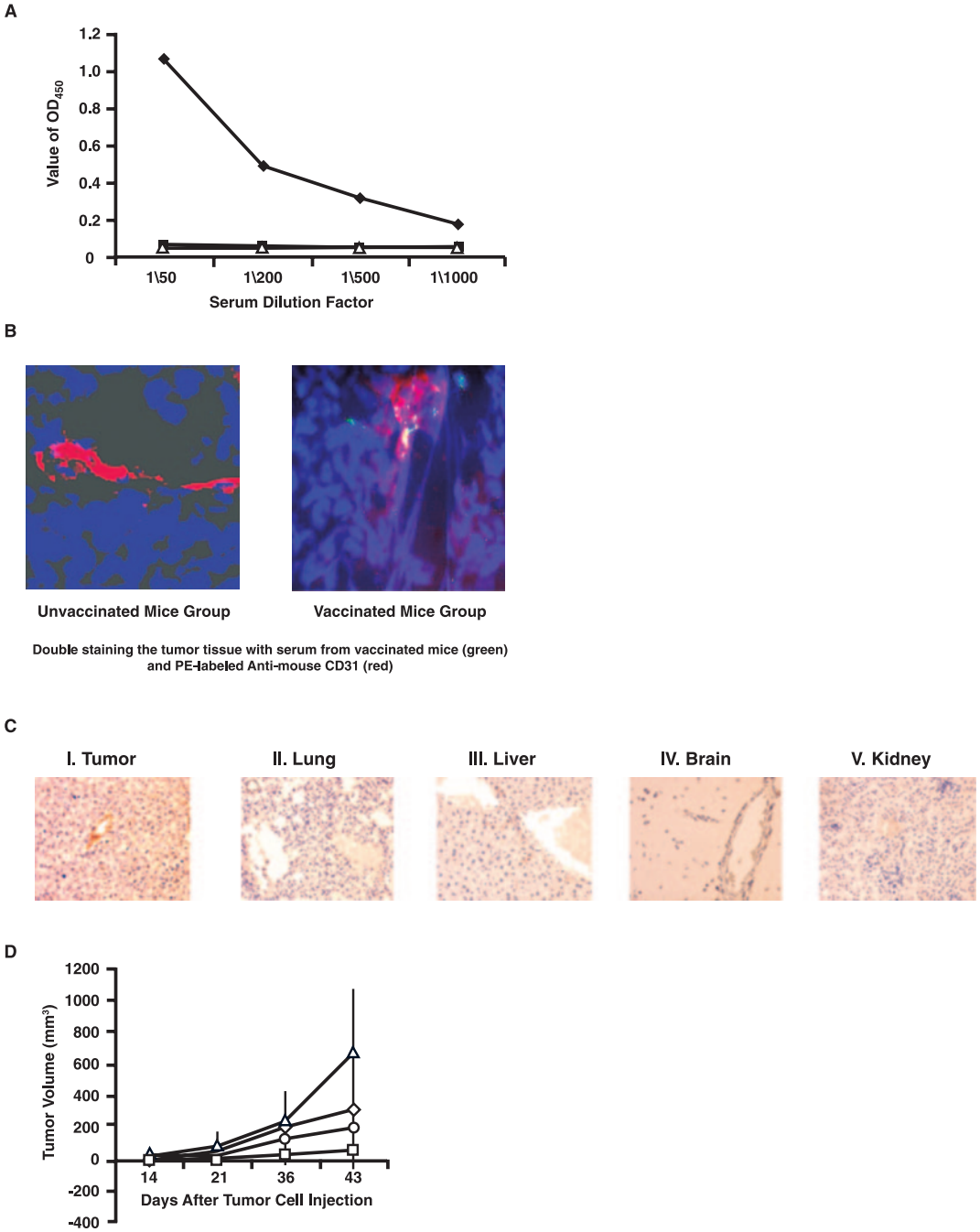


FIGURE 3. *A*, The serum level of Annexin A1-specific Abs before and after vaccination with the Ad-sig-AnxA1/ecdCD40L vector. To test specifically whether the immune response generated by the Ad-sig-AnxA1/ecdCD40L vector is directed against the Annexin A1 Ag, serum was taken from a mouse that had been 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.

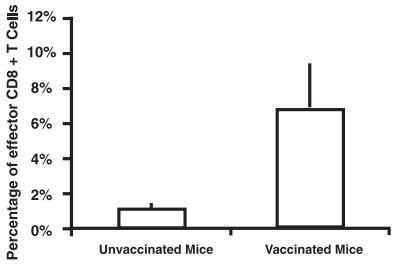


FIGURE 4. Infiltration of CD8 effector cells following Ad-sig-TAA/ecdCD40L vaccination. We minced the s.c. tumor nodules of rH2N.Tg mice before and after two s.c. injections of the Ad-sig-rH2N/ecdCD40L vector. Single-cell suspensions were isolated and treated with 0.03% DNase and 0.14% collagenase I, 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

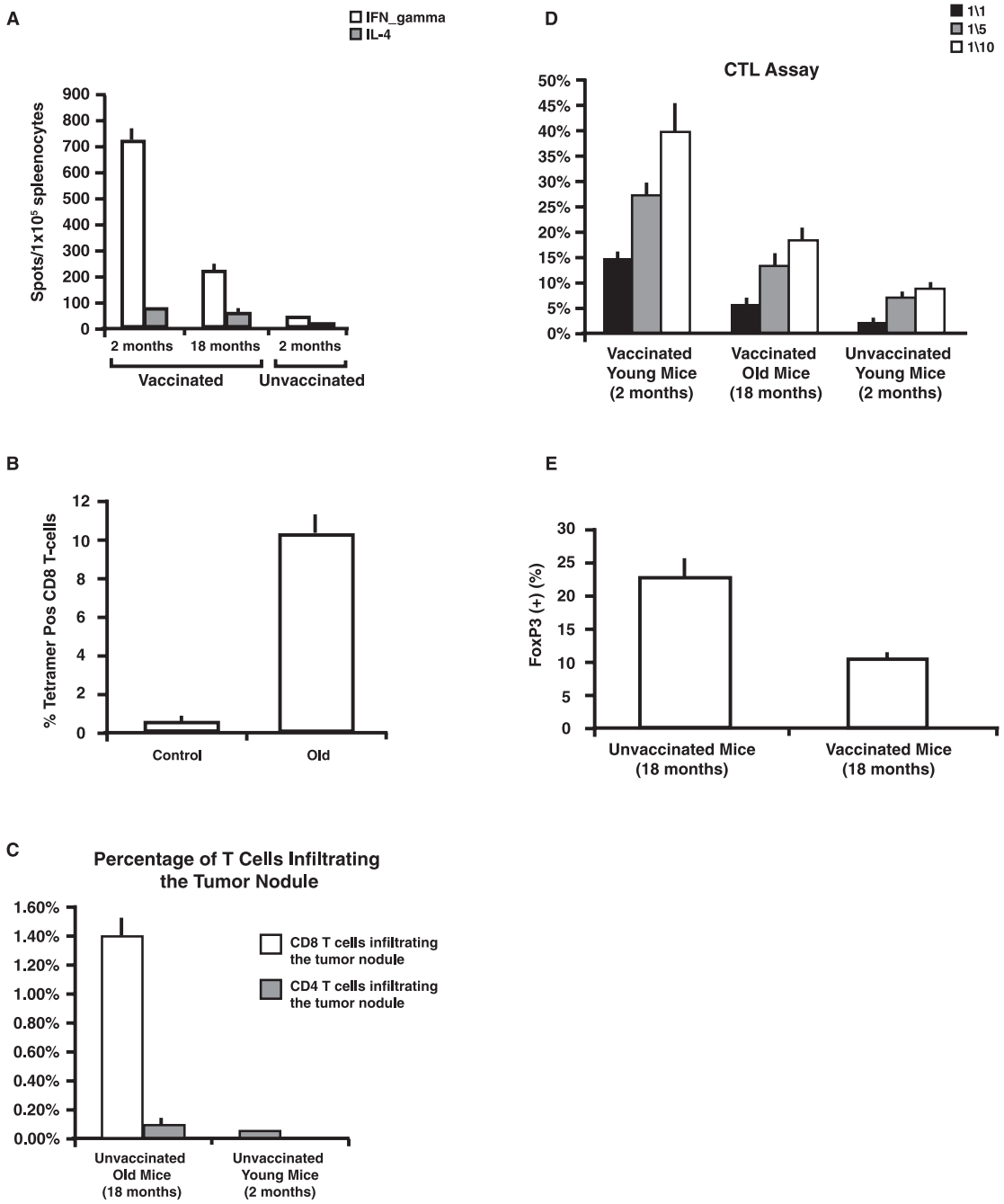


FIGURE 5. *A*, Effect of the Ad-sig-E7/ecdCD40L vector on the induction of E7 specific T cells as measured by the ELISPOT assay in 18- and 2-mo-old mice. The ELISPOT assay was used to measure the level of the IFN-γ- (▨) or IL-4- (■) positive T cells/1 × 10^5 spleen cells following in vitro exposure to 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.

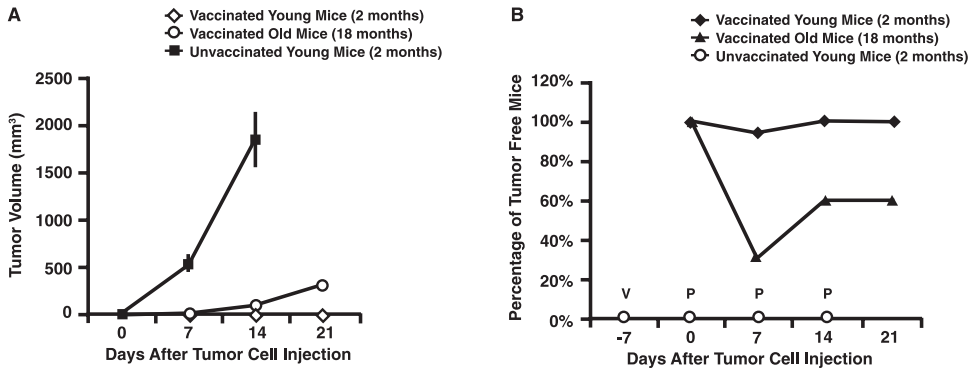


FIGURE 6. Effect of the Ad-sig-E7/ecdCD40L vector prime-E7/ecdCD40L protein (3×) boost (VPPP) vaccine against the E7 viral Ag in old (18 mo) and young (2 mo) mice on growth of E7-positive TC-1 tumor cells. C57BL/6J mice were injected once s.c. with the Ad-sig-E7/ecdCD40L vector and then three times s.c. 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.

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Disclosures

The authors have no financial conflict of interest.

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

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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)

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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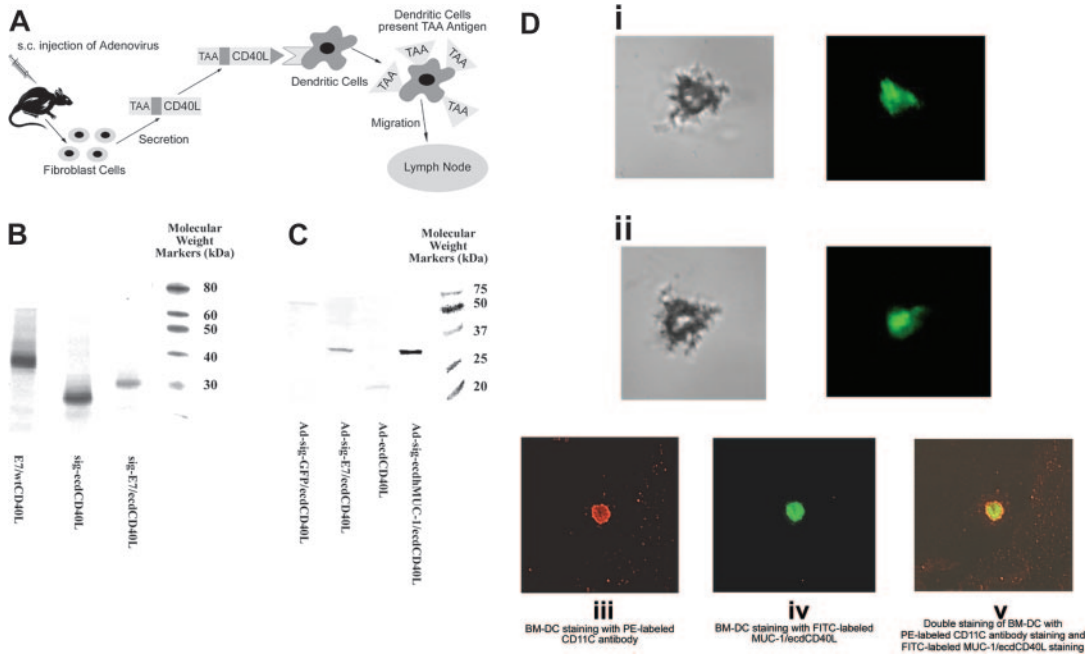

Figure 1. TAA/ecdCD40L protein produced by Ad-sig-TAA/ecdCD40–infected cells binds to DCs. (A) Proposed mechanism for induction of immune response by the Ad-sig-TAA/CD40L vector. Injecting Ad-sig-TAA/ecdCD40L induces in vivo activation and tumor-antigen loading of DCs, migration of the DCs to regional 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

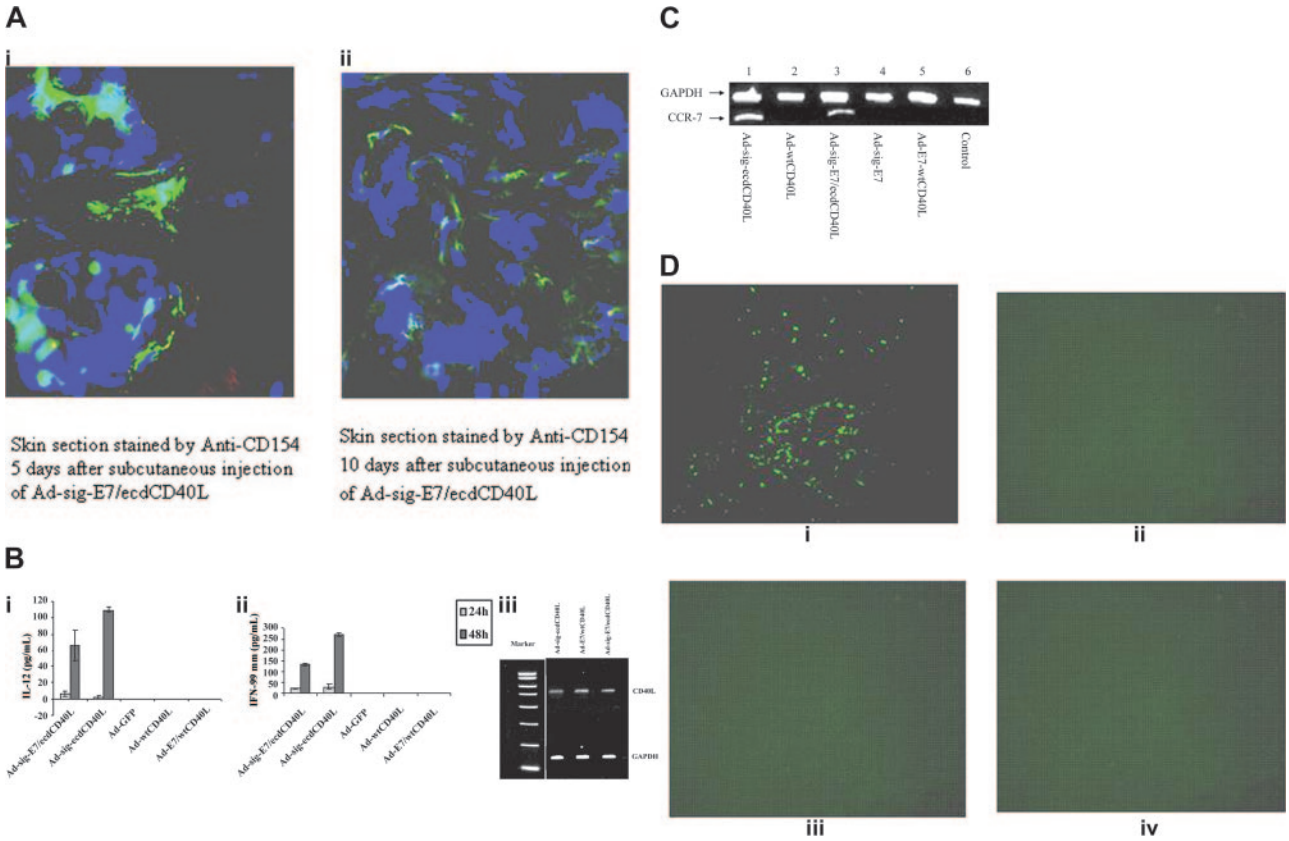

Figure 2. TAA/ecdCD40L protein from Ad-sig-TAA/ecdCD40L vector–infected cells binds to and activates DCs, which induce migration to regional lymphoid tissue. (A) Injection of the Ad-sig-E7/ecdCD40L vector generates the release of the E7/ecdCD40L protein around the vector injection site for up to 10 days. 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

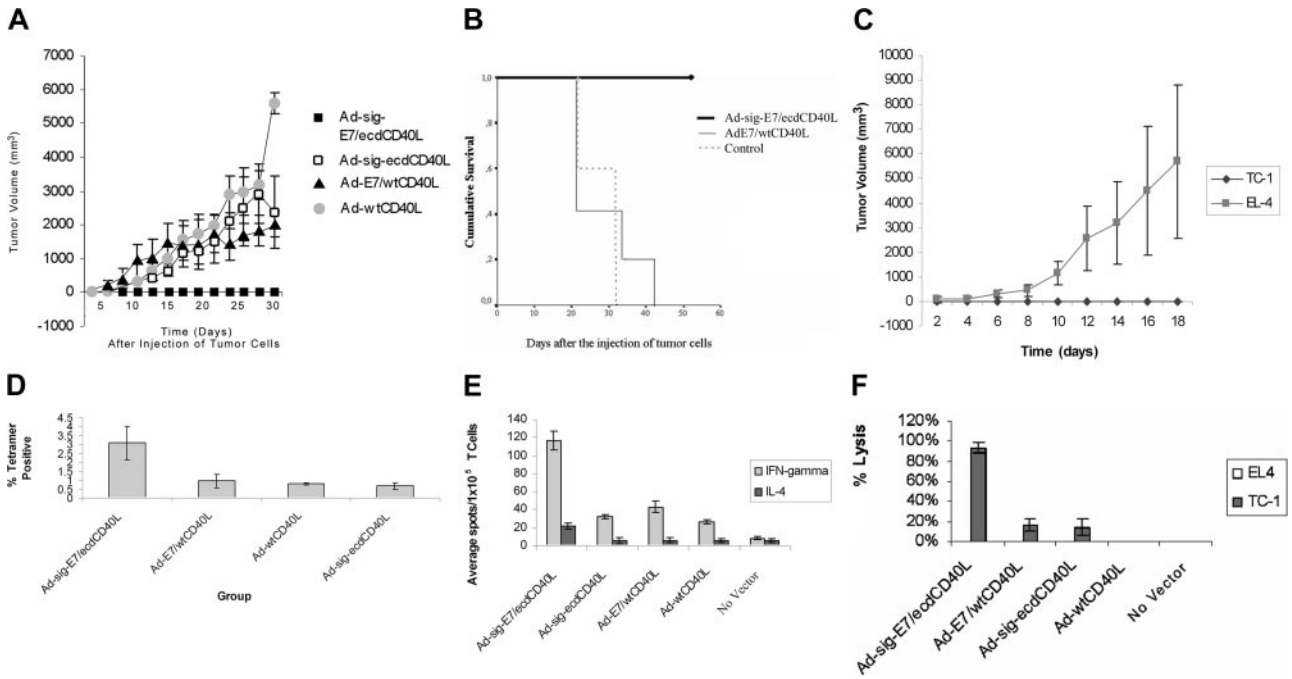

Figure 3. Mechanism of the Ad-sig-E7/ecdCD40L vector–induced suppression of the growth of E7-positive TC-1 tumor cells in C57BL/6 mice. (A) Resistance to the subcutaneous growth of 5×10^5 E7-positive TC-1 cancer cells in mice after 2 injections with 1×10^8 pfu of the Ad-sig-E7/ecdCD40L vector 7 days 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

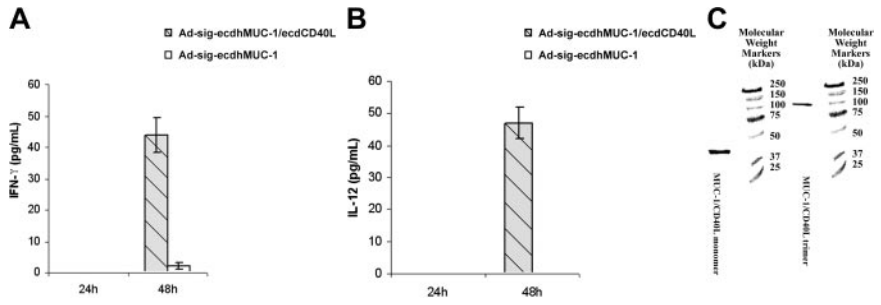

Figure 4. The ecdhMUC1 protein released from Ad-sig-ecdhMUC1/ecdCD40L vector–infected cells forms functional trimers and activates DCs. (A) Induction of IFN-γ secretion from bone marrow–derived DCs induced by exposure to the Ad-sig-ecdhMUC1/ecdCD40L vector. Supernatant medium collected from DCs derived 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.

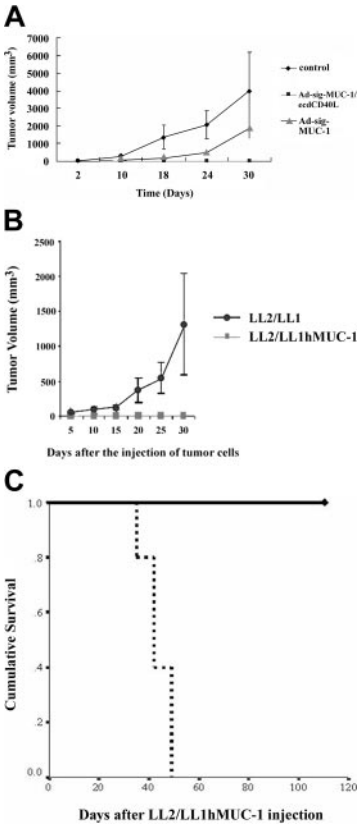

Figure 5. Effect of 2 subcutaneous injections (7 days apart) of 1 × 10^8 pfu of the Ad-sig-ecdhMUC1/ecdCD40L vector on the in vivo growth of the hMUC1-positive LL2/LL1hMUC1 cancer cell line in hMUC1.Tg mice. (A) Two subcutaneous injections (7 days apart) of 1 × 10^8 pfu Ad-sig-ecdhMUC1/ecdCD40L vector 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

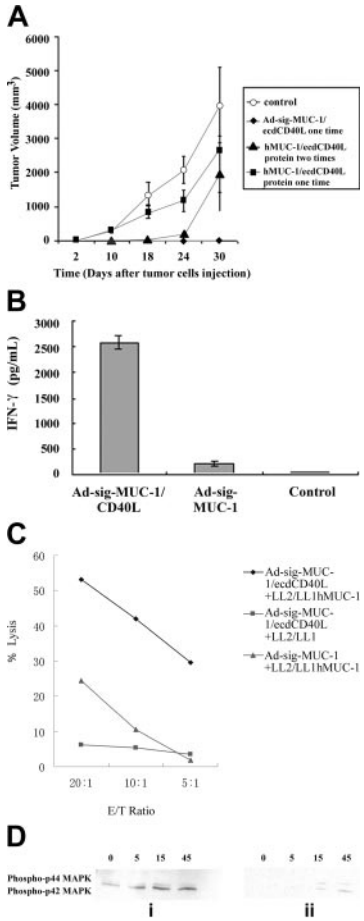

Figure 6. Mechanism of the suppressive effect of the Ad-sig-ecdhMUC1/ecdCD40L vector on induction of the immune suppression of the growth of the LL2/LL1hMUC1 cells in hMUC1.Tg mice. (A) Subcutaneous injection of the ecdhMUC1/ecdCD40L protein does not induce suppression of the growth of hMUC1-positive 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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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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[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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(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.

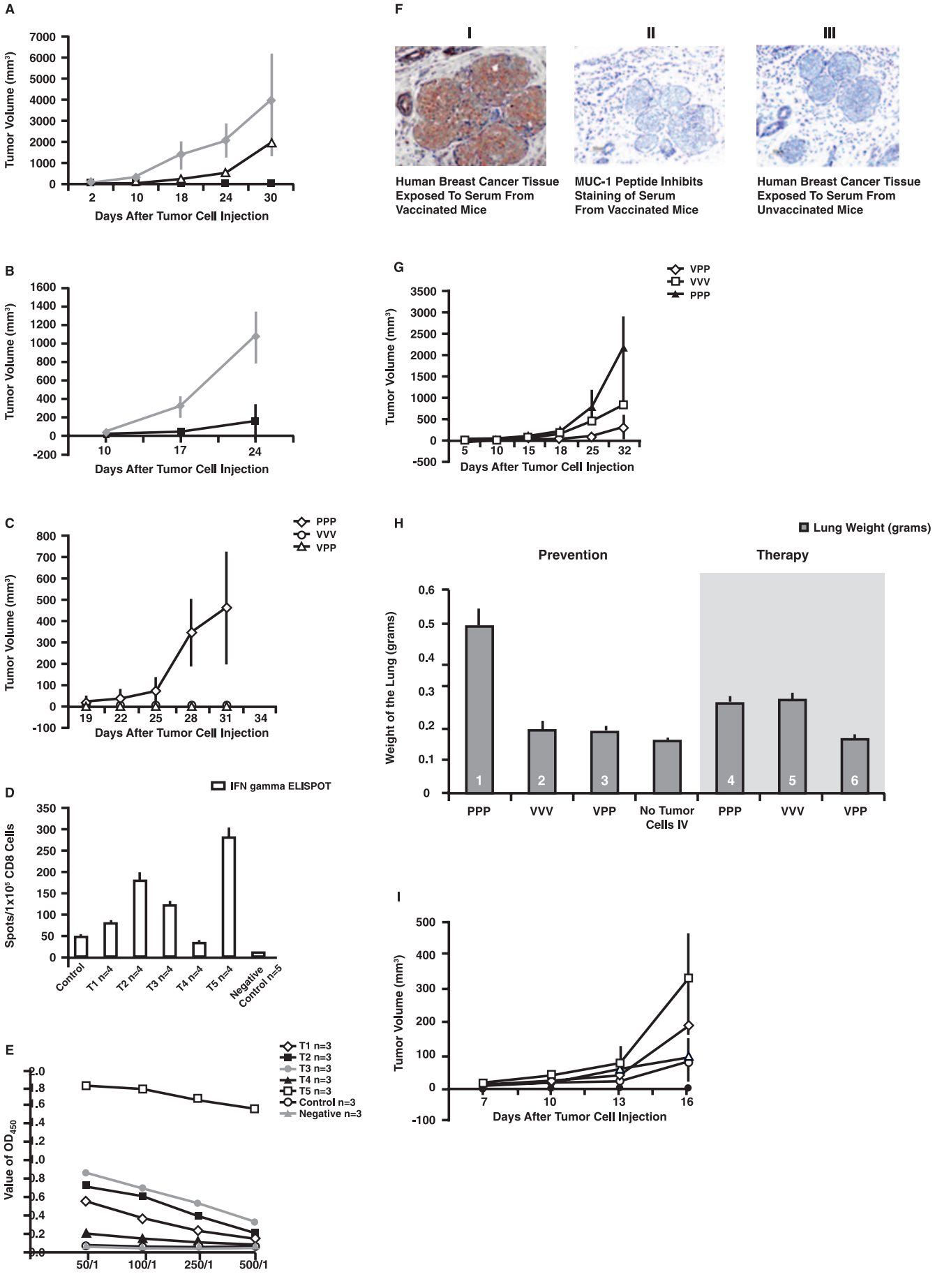
VPP suppresses the growth of i.v. administered MUC-1-positive cancer cells in the lungs of hMUC-1.Tg mice

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 (□).

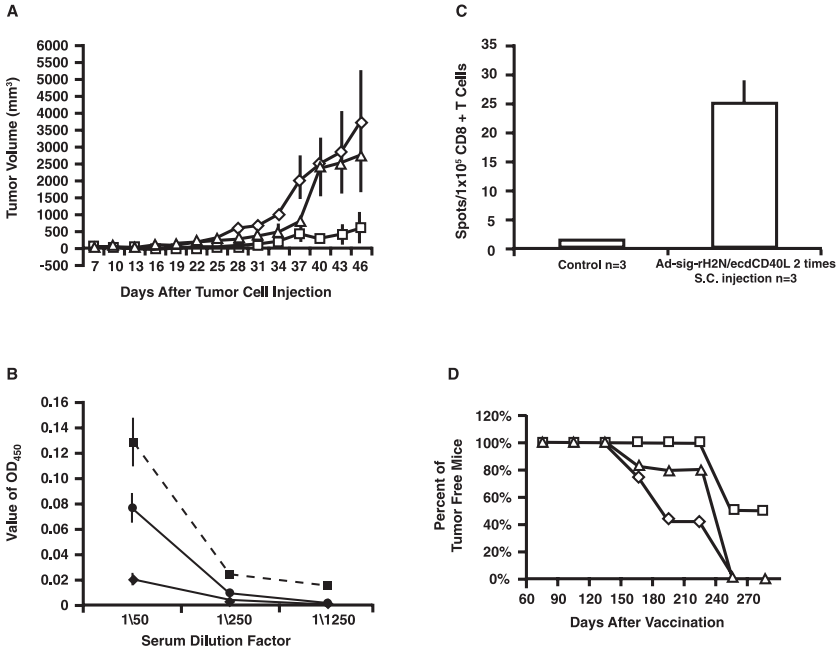


FIGURE 2. *A*, Effect of the Ad-sig-rH2N/ecdCD40L vector on the growth of rH2N-positive NT2 cells. The following vectors were injected s.c. twice at a 7-day interval: no vector (◇); two s.c. injections of the Ad-sig-rH2N/ecdCD40L vector (□), and one s.c. injection of the Ad-sig-rH2N/ecdCD40L vector (△). *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.

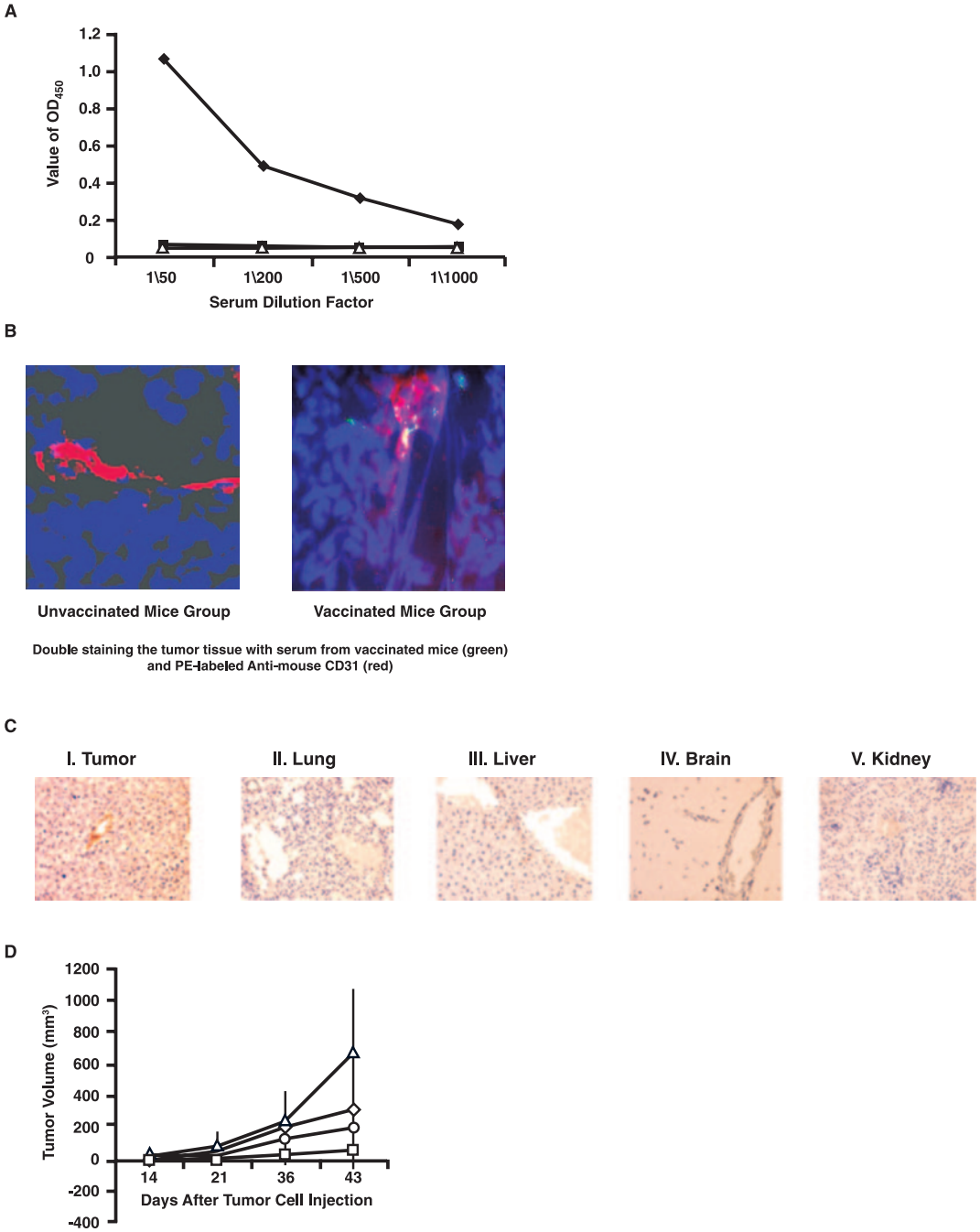


FIGURE 3. *A*, The serum level of Annexin A1-specific Abs before and after vaccination with the Ad-sig-AnxA1/ecdCD40L vector. To test specifically whether the immune response generated by the Ad-sig-AnxA1/ecdCD40L vector is directed against the Annexin A1 Ag, serum was taken from a mouse that had been 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.

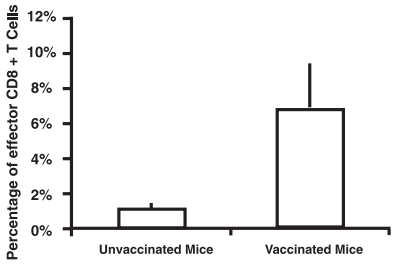


FIGURE 4. Infiltration of CD8 effector cells following Ad-sig-TAA/ecdCD40L vaccination. We minced the s.c. tumor nodules of rH2N.Tg mice before and after two s.c. injections of the Ad-sig-rH2N/ecdCD40L vector. Single-cell suspensions were isolated and treated with 0.03% DNase and 0.14% collagenase I, 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

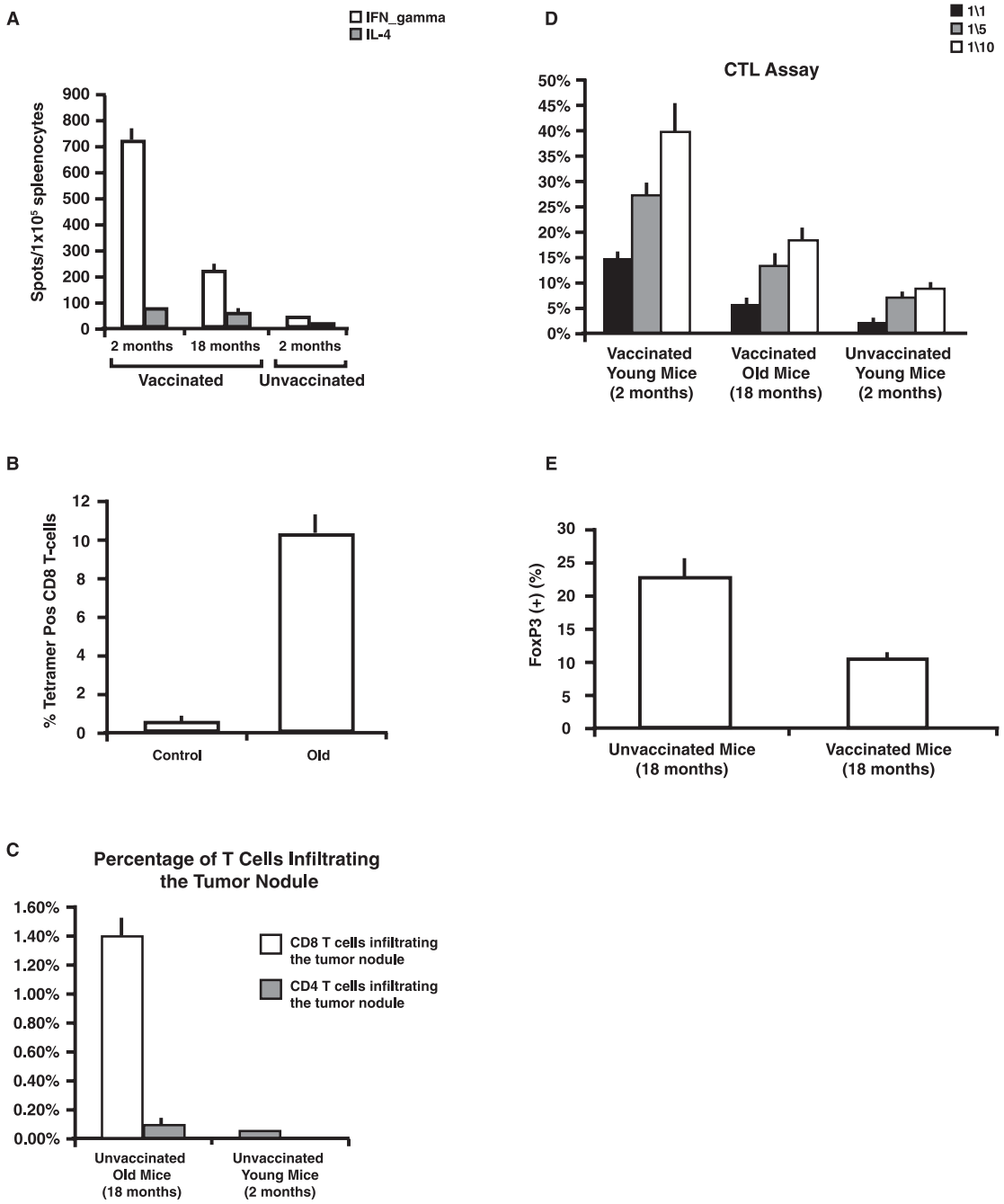


FIGURE 5. *A*, Effect of the Ad-sig-E7/ecdCD40L vector on the induction of E7 specific T cells as measured by the ELISPOT assay in 18- and 2-mo-old mice. The ELISPOT assay was used to measure the level of the IFN-γ- (▨) or IL-4- (■) positive T cells/1 × 10^5 spleen cells following in vitro exposure to 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.

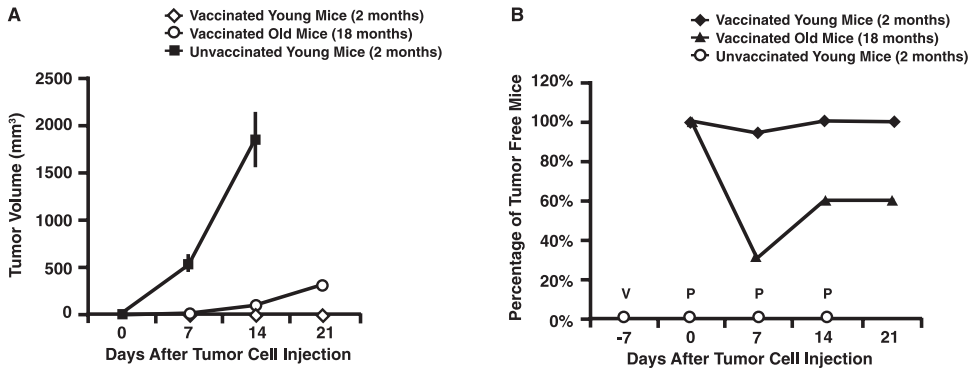


FIGURE 6. Effect of the Ad-sig-E7/ecdCD40L vector prime-E7/ecdCD40L protein (3×) boost (VPPP) vaccine against the E7 viral Ag in old (18 mo) and young (2 mo) mice on growth of E7-positive TC-1 tumor cells. C57BL/6J mice were injected once s.c. with the Ad-sig-E7/ecdCD40L vector and then three times s.c. 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.

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Disclosures

The authors have no financial conflict of interest.

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PROTOCOL NUMBER	**SKCC 109-04-00**
INVESTIGATIONAL AGENT	**Ad-sig-ecdhMUC-1/CD40L Vector**
PROTOCOL TITLE	**A SINGLE ARM OPEN-LABEL PHASE I STUDY OF AN INJECTABLE REPLICATION-INCOMPETENT ADENOVIRAL VECTOR VACCINE WITH A PROTEIN BOOST USED TO PRODUCE AN IMMUNE RESPONSE FOR MUC-1 POSITIVE EPITHELIAL CANCER CELLS IN PROSTATE CANCER PATIENTS POST RADICAL PROSTECTOMY**
IRB REFERENCE NUMBER	**041199**
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PROTOCOL DATE	October 22, 2004

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1. BACKGROUND

1.1 Description and Mechanism of the Ad-sig-ecd hMUC-1/ecdCD40L Vaccine.

The agent used in this clinical trial is an adenoviral vector vaccine against the MUC-1 antigen that is over expressed on most epithelial cancer cells. The cancers in which an over expression of the MUC-1 is known are breast, lung, prostate, colon and ovary.[1]

This is a Phase I 24 patient clinical trial for prostate cancer subjects who are currently experiencing rising PSA levels for three consecutive assays with at least level above 0.2/ng/ml. The clinical trial treatment is one subcutaneous injection of the Ad-sig-ecdhMUC-1/ecdCD40L vector. The last twelve subjects will receive in addition to the vector vaccine injection two subcutaneous booster injections of the sig-ecdhMUC-1/ecdCD40L protein given at one and three weeks after the vector injection. The protein injections start seven days after the vector injection with one subcutaneous protein injection at 7 days and 21 days after the initial Ad-sig-ecdhMUC-1/ecdCD40L vector injection. The prostate cancer patients enrolled in this trial will have had radiation therapy and have experienced a rise in PSA values for 3 consecutive samplings done within the last year with one level above 5/ng/ml.

The clinical trial described herein is based on earlier work published by Xiang et al[2] who had administered a DNA vaccine using a plasmid encoding the CEA/ecdCD40L fusion gene. Although the oral administration of this plasmid in S. Typhimirium induced a unique response in anergic mice. Il-2 conjugated antibody directed to T cells as a boost was required to break tolerance in 100% of the animals.[3-4] The trial proposed is based on preclinical data which shows that the MUC-1 vector vaccine can break tolerance without the need for a cytokine boost and is therefore preferable to the DNA vaccine of Xiang.[2] Prostate cancer is the most common cancer among men in the US with 230,000 new cases predicted in 2004.[1] The incidence and prevalence of prostate cancer will rise as the "baby boomers" age. Because of the prevalence of widespread yearly screening we are identifying prostate cancer earlier with over 80% diagnosed with either local or locally advanced disease. One treatment option for those with locally advanced high Gleason score disease that have a high probability of recurrent disease after local treatment is radiation therapy. By administering the subcutaneous injection of the Ad-sig-ecdhMUC-1/ecdCD40L vector followed by the protein boost injections, the subject may be able reduce his rising PSA values.

In a study of 611 subjects post radical prostatectomy who experienced a recurrence only defined by rising PSA values, the median survival was 4.1 years (range 0.3-11.8 years. The ten year survival rate from prostate cancer with a $\leq$ 3 month PSA doubling time, is less than 40 % [1]. The Ad-sig-ecdhMUC-1/ecdCD40L vector carries a gene encoding the extra cellular domain (ecd) of the CD40L linked to the gene encoding the ecd of the human MUC-1 (hMUC-1) human tumor associated antigen.

In order to administer the 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 which does not require the antibody targeted IL-2 or other cytokine

boosts, we constructed an adenoviral vector encoding a chimeric ecdhMUC-1/ecdCD40L vaccine transcription unit. This transcription unit encodes a portion of the ecd the human MUC-1 (hMUC-1) self antigen fused to the 209 amino acid ecd of the CD40L. The ecd region of CD40L contains all of the sequences necessary for the formation of the CD40L trimer. This transcription unit resembled that of Xiang et al[2] in that they linked a leader sequence for secretion to the fusion protein composed of a TAA and the ecd of the CD40L.

Our vaccine differed from that of Xiang et al[2] in that it utilized an adenoviral vector rather than a plasmid in a Salmonella typhimerium bacterial host strain for the delivery of the ecdhMUC-1/ecdCD40L transcription unit. Xiang et al[2] used a leucine zipper domain in the region between the hCEA antigen and the CD40L, whereas we used an eight amino acid linker (NDAQAPKS) between the ecdhMUC-1 gene and the ecdCD40L gene. Xiang et al[2] construct positions the hCEA antigen at the caboxylterminus of the CD40L whereas our vector transcription unit attached the ecd MUC-1 gene to the aminoterminal end of the ecdCD40L. This arrangement should provide better binding of the CD40L to the CD40 receptor on DCs. The Xiang method required the administration of a fusion molecule composed of IL-2 linked to a T cell targeted antibody following the hCEA/CD40L DNA vaccine, whereas our TAA/ecdCD40L adenoviral vaccine was administered without an IL-2 boost.

The adenoviral system used in our work has several theoretical advantages over the Salmonella delivery system. The expression of the TAA/ecdCD40L gene may be at higher levels and for longer periods of time with the adenoviral delivery than is the case with the DNA vaccine. This adenoviral ecd/CD40L vector injection may thereby induce a more vigorous immune response. Although the 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.[6] Thus, subcutaneous 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 TAA used in this protocol vector 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 [7-18]. The over-expression of hMUC-1 has been shown to promote anchorage independent growth of tumor cells.[7-18]

The MUC-1 gene is over expressed in prostate cancer and there is evidence showing that there are unique patterns of glycosylation that are in expressed in prostate cancer that are not detectable in normal epithelial tissue.[9-13] Mice which have been made transgenic for hMUC-1 have been shown to develop tolerance for hMUC-1 antigen bearing syngeneic mouse cancer cells.[16]

1.2 Prognosis

A National Cancer Institute (NCI) report estimates that about 1 in 6 men in the United States will develop prostate cancer during his lifetime[19]. Prostate cancer is the second most common cause or cancer death in men. With almost 30,000 prostate cancer deaths expected this year, new therapy approaches are needed. Prostate cancer like many other types of cancer does not currently have options that provide a cure once it becomes metastatic.

The pre-clinical results from the Deisseroth laboratory[3-4] show that the subcutaneous injection of the adenoviral vector carrying the TAA/ecdCD40L fusion gene generates immunological resistance to TAA positive cancer cells for at least one year. In addition, the subcutaneous injection of the adenoviral vector carrying the ecdhMUC-1/ecdCD40L fusion gene suppresses the growth of ecdhMUC-1 positive mouse cancer cells in hMUC-1.Tg mice which are transgenic for the ecdhMUC-1 gene.[3-4] 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 lymphocyte 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.[16] Thus, the hMUC-1/CD40L vector injections appeared to overcome immune tolerance. This vaccine may be of use in preventing the recurrence of epithelial malignancies following surgery and for the immunotherapy of advanced epithelial cancers which recur following surgery.

1.3 Summary of Relevant Pre-Clinical Studies from the Deisseroth Laboratory[3-4]

1.3.1 Mouse Experiments with the Ad-sig-E7/ecdCD40L Vector

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 subcutaneously with 1 X 10^8 pfu of the Ad-sig-E7 /ecdCD40L or Ad-sig-GFP/ecdCD40L vectors once on each of two days, seven days apart. The vector treated mice were then injected subcutaneously with 5 X 10^5 cells from the E7 positive TC-1 cancer cell line[5] 10 days after the last vector injection. 0% (0/5) of mice injected with the Ad-sig-E7/ecdCD40L vector formed palpable tumors during a two month period of observation following a single injection of TC-1 cells, whereas 100% (5/5) of the mice injected with the Ad-sig-GFP/ecdCD40L vector prior to the injection of the TC-1 cells formed subcutaneous tumors which were greater than 500 mm^3 by 15 days after TC-1 tumor cell injection.
When the tumor-free animals from the Ad-sig-E7/ecdCD40L treated group were re-challenged with a larger dose of TC-1 cells (1 X 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-TAA/ecdCD40L vector induces tumor regression of established TC-1 tumors. Mice were first injected subcutaneously with 5 X 10^5 TC-1

cells on the hind flank. Five days later, the mice were injected subcutaneously at a different site with the Ad-sig-E7/ecdCD40L vector. Control mice were injected subcutaneously with PBS 5 days following the injection of TC-1 cells. None of the Ad-sig-E7/ecdCD40L vector injected mice developed tumors, whereas 100% of the animals injected with PBS developed tumors at the TC-1 injection site within 14 days.
In order to test the antigen specificity of the immune response induced by the subcutaneous injection of the Ad-sig-TAA/ecdCD40L, we compared the effect of the Ad-sig-TAA/ecdCD40L vector on the growth of the EL-4 cell line which is TAA negative, to its effect on the growth of the TC-1 cell line which is E7 positive. The Ad-sig-TAA/ecdCD40L vector injection induced immune resistance to the growth of the TC-1 cell line but not to the EL-4 cell line in C57BL/6 mice. This experiment showed that the immune response induced by the Ad-sig-TAA/ecdCD40L was antigen specific.

Splenic T cells from Ad sig TAA/ecdCD40L vector injected C57BL/6 mice can passively transfer resistance to TC-1 cells for up to one year following vector injection. We followed the mice from the experiment for up to one year following TC-1 tumor injection, Ad-sig-E7/ecdCD40L vector injection (two injections separated by seven days), and rechallenge with 1 X 10^7 TC-1 cells. Four of the eight animals which remained tumor free for greater than one year were sacrificed and the T lymphocytes were isolated from the spleen by negative selection using antibody and magnetic bead technology.[3] 10 million of these splenic T cells were injected intraperitoneally into C57BL/6 athymic nu/nu mice (n=7), which had been injected subcutaneously five days previously with 5 X 10^5 TC-1 cells. The tumors in the nude mice that were given intraperitoneal injections of T cells from the Ad-sig-E7/ecdCD40L sensitized donor mice grew into palpable subcutaneous nodules for six days and then regressed in all animals to very small tumors. In three of the seven treated mice, the tumors regressed completely. In contrast, none of the subcutaneous tumors in the nude mice injected intraperitoneally with T cells from unsensitized donor mice regressed. All of the mice in the latter group died with progressive tumor growth within three weeks following TC-1 tumor cell injection. The mice treated with the intraperitoneal injection of the splenic T cells from the sensitized donors were followed for three months following the TC-1 challenge and remained tumor free during that time.

1.3.2 Experiments with the Ad-sid-ecdhMUC-1/ecdCD40L Vector and Booster

Subcutaneous injections of the ecdhMUC-1/ecdCD40L Protein. Subcutaneous injection of the Ad-sig-ecdhMUC-1/ecdCD40L vector breaks tolerance for hMUC-1 positive cells in mice which are transgenic for hMUC-1.[3-4] The MUC-1 antigen is over expressed in carcinomas of the prostate, breast, ovary, and pancreas as well as other carcinomas[7]. MUC-1 is also a self antigen which is focally expressed on normal secretary epithelial cell surfaces. The over expression of hMUC-1 in epithelial cancers is thought to disrupt E-cadherin function leading to anchorage independent growth and metastases.[9,13] hMUC-1.Tg mice which are transgenic for the hMUC-1 antigen have been reported to be unresponsive immunologically to the hMUC-1 antigen.[16]
We therefore subcutaneously 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.[16] This experiment would therefore test if the Ad-sig-ecdhMUC-1/ecdCD40L vector injection could produce resistance to the growth of syngeneic mouse cancer cells which were positive for the hMUC-1 antigen. Injection of the hMUC-1 mouse syngeneic 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 subcutaneous tumor. These control animals had to be sacrificed by 25 days after the subcutaneous injection of the tumor cells due to progressive tumor growth.
In contrast, in the hMUC-1.Tg transgenic mice which received subcutaneous injections of the Ad-sig-ecdhMUC-1/ecdCD40L vector, there was complete suppression of the growth of the LL1/LL2hMUC-1 cell line in all of the animals tested. This induction of resistance to LL1/LL2hMUC-1 growth was shown to be dependent on CD8+ T cells from the sensitized animals. Thus, the Ad-sig-ecdhMUC-1/ecdCD40L vector strategy can overcome anergy without the need for additional cytokine booster treatments.
In order to test the effect of boosting the vector vaccination with the subcutaneous protein injections, we studied the effect of various schedules of subcutaneous injections of the ecdhMUC-1/ecdCD40L protein following the subcutaneous injection of the Ad-sig-ecdhMUC-1/ecdCD40L vector on the growth of the LL1/LL2hMUC-1 tumor cell line. When two protein subcutaneous injections of the ecdhMUC-1/ecdCD40L protein, 14 days apart, were given 7 days following the subcutaneous injection of the Ad-sig-ecdhMUC-1/ecdCD40L vector, the level of the induction of the immune response against the hMUC-1 antigen in the hMUC-1.Tg mice was enhanced dramatically.

1.3.3 Discussion of Results of Preclinical Data

The results of the experiments reported above show that the injection of the Ad-sig-E7/ecdCD40L vector into C57BL/6 mice induces immune resistance to the engraftment and growth of E7 positive tumor cells. The Ad-sig-E7/ecdCD40L also induces regression of established subcutaneous 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 over one year following 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. This experiment shows 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 which lasts for over one year.

The study of the effect of subcutaneous injection of the Ad-sig-ecdhMUC-1/ecdCD40L vector into hMUC-1.Tg mice allowed us to test if the Ad-sig-ecdhMUC-1/ecdCD40L vector injection by itself could activate a CD8+ T cell lymphocyte immune response against the hMUC-1 positive mouse cells in animals otherwise anergic to the hMJUC-1 antigen. This proved to be the case.

The Ad-sig-TAA/ecdCD40L vector strategy is unique in several ways. It has been shown to break tolerance in a transgenic mouse model 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 paper induces memory cells. Importantly, the

subcutaneous injections of the ecdhMUC-1/ecdCD40L protein can be used to dramatically enhance the induction of the immune response in the anergic animals.

2. Clinical Trial Objectives

2.1 Ad-sig-ecdhMUC-1/ecdCD40L Vaccine Description

This novel vaccine treatment was developed at the Sidney Kimmel Cancer in the Deisseroth laboratory[3-4]. The hypotheses to be tested in the proposed Phase I trial are as follows.

2.2 Primary Hypotheses

The subcutaneous injection of an adenoviral vector carrying the ecdhMUC-1/ecdCD40L transcription unit in post radiation therapy prostate cancer patients after experiencing 3 consecutive rises in PSA levels with at least 1 level above 5/ng./ml.

2.3 Secondary Hypotheses

The vaccine will reduce and stabilize PSA levels in post radiation therapy prostate cancer patients that before treatment were experiencing a rising PSA levels.

2.4 Primary Objective *Safety Evaluation and Optimal Dose Determination*

The first aim is to conduct a dose-escalation trial with post radical prostectomy prostate cancer patients after they have experienced three consecutive increases in PSA levels with at least one value above 0.2/ng/ml. The treatment is one subcutaneous injection of the Ad-sig-ecdhMUC-1/ecdCD40L vaccine and two subcutaneous injections of the ecdhMUC-1/ecdCD40L protein. The protein injections will act as a boost to the vaccine injection. The trial is designed primarily to assess the safety of this vaccine by determining whether the patients experience Grade III or Grade IV toxicity with vector and protein injections.

Since preclinical data suggests that we may not encounter toxicity and therefore may not be able to define a maximum tolerated dose (MTD), we are carrying out a number of mechanistic assays to determine the optimal biological dose of the vaccine. That is the dose which will be associated with an induction of an immune response as defined by in vitro cellular assays of the immune response on mononuclear cells from participants blood as well as antibody assays on the serum of vaccinated patients.

2.5 Secondary Objective *Efficacy Evaluation*.

The second aim is to assess the efficacy of the vector and protein vaccination, by monitoring the PSA levels of subjects. In the event of PSA increases occurring three assays in a row, the subjects will be considered a treatment failure.

3. TRIAL DESIGN

The subjects in the clinical trial will be monitored to determine whether they experience Grade III or Grade IV toxicity. Early stopping rules are specified in section 4.4.1. The safety of administering multiple intratumoral injections of a nonreplicating adenoviral vector has been reviewed[20-21] documented extensively in clinical tests.[22-23] Since this vector vaccine is injected subcutaneously, the treatment should have minimal toxicity. As discussed above, the focus of this clinical trial is on the safety of the Ad-sig-ecdhMUC-1/ecdCD40L vaccine. The efficacy of the Ad-sig-ecdhMUC-1/ecdCD40L vector also will be evaluated by monitoring changes in the PSA levels.

3.1 Clinical Trial Description

This is a single site 24 patient Phase I non-randomized open label dose escalation trial for men who have undergone radiation therapy for prostate cancer and have experienced 3 consecutive elevations in PSA level with at least one value above 5/ng/ml. The emphasis of the trial is safety with the secondary emphasis on efficacy. The trial will be open to all subjects who meet the eligibility criteria.

The duration of the trial is twelve weeks. (See Study Calendar for schedule of procedures and visits). The subjects will be given one subcutaneous vector injection followed by two protein boost subcutaneous injections the first of which starts 7 days after the vector injection and the second and final injection is 14 days later.

The purpose of the clinical trial is to test the safety and measure the adverse events of one subcutaneous injection of the Ad-sig-ecdhMUC-1/ecdCD40L vector followed by two subcutaneous injections of the protein. Secondarily the PSA levels will be evaluated.

3.2 Treatment Plan

The study will enroll 24 evaluable post radiation theray cancer patients which are exhibiting three consecutive elevations in PSA levels with at least one level above 05/ng/ml. The subjects will be divided into 8 cohorts of 3 patients each, with Cohorts 1-4 following a dose-escalation schema for the Ad-sig-ecdhMUC-1/ecdCD40L vector only. Cohorts 4-8 will follow a dose escalation schema for ecdhMUC-1/ecdCD40L for the protein in addition to receiving the highest safe vector dose determined in the first 4 cohorts. The subcutaneous protein injections at the four different doses will follow the vector injection by one week and three weeks. The dose of the vector will be the maximum tolerated or optimal dose as determined in one of the cohorts 1-4. Patients who do not complete all the vaccine and both of the protein boost injections (other than through therapy-related adverse events, disease progression, or death) shall be deemed non-evaluable and will be replaced with a new patient. The replacement patient's treatment must be completed prior to the first patient enrollment in the next cohort.

Prior to the start of treatment subjects will undergo blood tests, an ECG, bone scan, pelvic and chest x-ray.

The treatment consists of one subcutaneous vector injection followed by two protein boost subcutaneous injections one week and three weeks after the vector injection.

3.3 Dosing Schema for Ad-sig-ecdhMUC-1/ecdCD40L Vaccine.

The study will enroll 24 evaluable post radiation therapy prostate cancer patients who have experienced three consecutive elevations in PSA levels with one level above 5ng/ml. The subjects will be divided into eight cohorts of 3 patients each, with Cohorts 1, 2, 3, and 4 being given a dose-escalation treatment schema for the vector vaccine subcutaneous injection and Cohorts 5, 6, 7, and 8 will receive a dose escalation schema for the ecdhMUC-1/ecdCD40L protein boost injections with each cohort receiving a higher dose of protein providing the previous cohort did not have any grade III or IV toxicities. The protein boosting injections are given 7 and 21 days after the vector vaccine injection. The vector vaccine in the last four cohorts will be the dose determined as the highest tolerated or optimal dose in cohorts 1-4.

The trial is designed as a dose escalation study of the Ad-sig-ecdhMUC-1/ecdCD40L vector vaccine subcutaneous injection for the first four cohorts. The last four cohorts will be given the maximum tolerated or optimal dose of the vector vaccine as determined in cohort 1-4 plus 2 more subcutaneous ecdhMUC-1/ecdCD40L protein injections in escalating doses starting 7 days after the vector injection with the last injections given 14 days later.

Table I Cohorts and Doses for Ad-sig-ecdhMUC-1/ecdCD40L Prostate Cancer Study

Cohort and Agent Given	Dose of Subcutaneous Vector Injection of Ad-sig-ecdhMUC-1/ecdCD40L	Dose of Subcutaneous Injections of ecdhMUC-1/ecdCD40L
Cohort 1 Vector only	1×10^9 VP	None
Cohort 2 Vector only	1×10^{10} VP	None
Cohort 3 Vector only	5×10^{10} VP	None
Cohort 4 Vector only	1×10^{11} VP	None
Cohort 5 Vector and Protein	1×10^{11} VP	100 micrograms
Cohort 6 Vector and Protein	1×10^{11} VP	500 micrograms
Cohort 7 Vector and Protein	1×10^{11} VP	1 milligram
Cohort 8 Vector and Protein	1×10^{11} VP	5 milligrams
Vector schedule: Each subject receives 1 vector injection		
Protein schedule: Each subject receives 2 subcutaneous injections. The first injection is given 7 days after the vector injection and the second injection is 14 days after the first protein injection		

The trial begins with the first cohort receiving 1 injection at the lowest planned dose of the vector (1 x 10^9 VP). If none of the patients in the first cohort experience Grade III or IV toxicity, a second cohort will receive 1 injection of 1x10^{10} VP. If none of the patients in the second cohort experience Grade III or IV toxicity, the dose escalation will continue with the third cohort receiving 1 injection of 5 x 10^{10} VP. The patients in the fourth cohort will receive 1 x 10^{11} VP if no Grade III or IV toxicity occurs in the preceding cohort. There will be three patients in each of the cohorts. If grade III toxicity occurs, then 3 additional patients will be added to that dose cohort. However, if Grade IV toxicity is detected in a patient, that patient will not receive any additional injections. If two patients show Grade IV toxicity at any dose level, further injections of all other patients will be done at the previous dose level. If the patient is in first cohort, the dose level will be below the initial starting dose. The last four cohorts in addition to the vector injection, will also receive increasing doses of two subcutaneous injections of ecdhMUC-1/ecdCD40L protein 14 days apart starting 7 days after the vector injection at the following doses: 100 micrograms (5^{th} cohort), 500 micrograms (6^{th} cohort), 1 milligram (7^{th} cohort) and 5 milligrams (8^{th} cohort) in each of the 2 injections, providing that no grade 3 or grade 4 toxicities are seen in the preceding cohorts.

Table 2 Treatment Conditions for Ad-sig-ecdhMUC-1/ecdCD40L Prostate Cancer Study

3.4 Ad-sig-ecdhMUC-1/ecdCD40L Vector Administration

Dose in Viral Particles	Patient Cohorts in Groups of Three Patients Per Cohort
1×10^9 VP*	Cohort 1: If there are no grade III or IV adverse events, cohort 2 will receive the next dose.
1×10^{10} VP *	Cohort 2: If no grade III or IV adverse events occur, cohort 3 will receive the next highest dose.
5×10^{10} VP *	Cohort 3: If none of the 3 patients treated at this dose experience either grade III or IV adverse events; cohort 4 will receive the next dose.
1×10^{11} VP*	Cohort 4: If no Grade III or IV adverse events occur in one patient, the next patient in this cohort will be treated. If none of the 3 patients treated at this dose experience either grade III or IV adverse events, cohort 5 will receive the protein boost in addition to the vaccine.
1×10^{11}VP and 100 micrograms protein *	Cohorts 5: If no patients in prior cohorts experience Grade III or IV adverse events the this cohort will receive this dose of vector and in addition will receive 2 subcutaneous injections of a protein boost 7 days after the vector injection and the second 14 days after the 1st protein boost injection.
1×10^{11}VP and 500 micrograms protein *	Cohort 6: If there are no grade III or IV adverse events, cohort 2 will receive the vector injection and the next higher dose of 2 subcutaneous injections of protein.
1×10^{11}VP and 1 milligram protein *	Cohort 7: If no grade III or IV adverse events occur, cohort 8 will receive the vector injection and the next highest dose of 2 subcutaneous injections of protein.
1×10^{11}VP and 5 milligrams protein	Cohort 8: 1 vector injection and the highest dose of 2 subcutaneous injections of protein.
* SEE STOPPING RULES (SEC 4.4.1)	

The treatment will consist of a single subcutaneous vector injection to be given followed by 2 subcutaneous injections of the ecdhMUC-1/ecdCD40L protein, given 7 and 21 days after last vector injection. These injections will all be given to subjects as outpatients in an outpatient infusion room. A discussion of possible adverse events and potential risks is provided in Sections 1.5, 4.1, 4.2 and 4.3. Appropriate dose modifications for the Ad-sig-ecdhMUC-1/ecdCD40L vector are outlined in Section 3.3. No investigational or commercial agents or therapies other than those described in this protocol may be administered with the intent to treat the patient's malignancy during the treatment period while still on active follow-up. If a patient relapses on or after treatment, they would be able to seek other therapies.

Each treatment will first involve injecting subcutaneously a single dose of the Ad-sig-hMUC-1/ecdCD40L vector vaccine. All patients to be treated on the protocol will be observed for 30 minutes after completing the injection.

The second part of the treatment starts 7 days after the last vector injection. After the subcutaneous injection of the ecdhMUC-1/ecdCD40L protein, the patient will be observed for 30 minutes and may go home if there is not a severe reaction. The evening of the injection, the patient should be instructed to take their temperature at home in the evening and if a fever exists, to take Tylenol.

The subjects will not be premedicated. If the subject runs a fever post injection they may take 625 mg of Tylenol.

3.5 Treatment Monitoring

The vital signs of the patient will be taken as part of the physical exam prior to study entry and prior to all injections. The physician will decide if the injection is to be given. The patient will be observed for 30 minutes after completing the injection for the vaccine and the two protein injections. If vital signs are not stable, or if the patient is complaining of rigors or other side effects such as fever or hypotension (systolic below 90 mm mercury), the patient will be transferred to an emergency room at a hospital, the patient will be transferred to an emergency room at a hospital.

3.6 Studies on Participants in the Clinical Trial

Clinical trial subjects will have the blood tests and clinical monitoring following the schedule below.

Pre study evaluations include blood chemistry tests are as follows: PSA, AST, ALT, alkaline phosphatase, total bilirubin &, serum creatinine, BUN, glucose, total protein, LDH, uric acid, calcium, potassium, inorganic phosphorous, cytokine release tests for interferon γ and IL-12, T cells; levels by flow cytometry of CD8, CD4, and NK cells; hepatitis B and C, HIV, base line PCR for vector, cytotoxicity array of CD8 and T cells from the peripheral blood against hMUC-1 positive cell lines

On study evaluations include serum chemistry tests are as follows: PSA, AST, ALT, LDH, bilirubin, serum creatinine, BUN, LDH, uric acid, alkaline phosphatase.
The blood tests are to be done according to the following schedule; weekly for 4 weeks, week 6 week 8, and week 12. The subject will be considered having failed the study if the PSA rises for 3 consecutive evaluations.

4. PROTECTION OF HUMAN SUBJECTS

4.1 Possible Adverse Events With Vectors

<u>The known or potential risks associated with a nonreplicating adenoviral vectors.</u>
Adenoviral vectors are the most popular delivery system for gene therapy of cancer, having been tested in clinical trials involving more than 400 patients. The clinical data

were obtained mainly for replication-incompetent vectors containing the p53 gene, which were designed to provide a p53 function to patients carrying a mutated p53 gene. Clinical trials[20-31] showed that doses of the adenoviral vector ranging from $9x10^9$ to $7.5x10^{12}$ vector particles could be injected intratumorally into cancer patients repeatedly without causing dose-limiting hypersensitivity or other side effects. Multivariate analysis of vector related parameters, including dose, route of injection, type of transgene and number of injections, indicated that none of these parameters were reliable predictors of adverse events. The conclusion from these studies was that toxicity was related to patient factors rather than vector factors, including the patient's age and underlying symptoms and the inclusion of surgery in the trial.[20-31]

The problem of toxicity associated with adenoviral vectors was reviewed in an NIH RAC meeting on December 8-9, 1999, following the death of a patient undergoing treatment for ornithine transcarbamylase (OTC) deficiency at the University of Pennsylvania. This patient had been injected with $2.5x10^{12}$ replication-incompetent adenoviral vector particles directly into the hepatic artery, resulting in high fevers, disseminated intravascular coagulation, acute respiratory distress syndrome, acute hepatic, cardiac and renal failure, anemia, coma, thrombocytopenia, conjugated hyperbilirubinemia, and finally death within 72 hours. Toxicity was associated with high levels of TNF-alpha, IL6 and IL10 in the systemic circulation. Two factors could have contributed to the toxicity, namely injection of an exceptionally high number of adenovirus particles into the hepatic artery, and an OTC deficiency which results in impairment of the metabolic pathways mediating the introversions of ammonia, a toxic degradation product of protein metabolism, into urea. In an OTC-deficient patient, ammonia accumulates in the serum and tissues, and the increased levels of ammonia can lead to coma and death. The fever and tissue destruction associated with high levels of TNF-alpha, IL6 and IL10 increased dramatically the amount of ammonia being produced in the patient. Similar doses of replication-incompetent adenoviral vectors injected into patients who were not afflicted with OTC deficiency did not result in clinically significant toxicity. Thus, most of the critical parameters in the University of Pennsylvania trial are not relevant to the protocol described in this proposal. Moreover, more than 400 patients have been injected intratumorally with replication-incompetent adenoviral vectors in other trials, and none of the patients developed clinically significant toxicity.[20-31]

4.2 Possible Adverse Events Associated with Protein Only of sig-ecdhMUC-1/ecdCD40L

The possible short term expected adverse events subjects could experience include: rash and pain at the injection site, fever, chills, hypotension, or swelling at the injection site. In addition subjects may also experience nausea, vomiting, pain, headache, dizziness, dyspnea, hyotension, rash and asthenia. The long term adverse event that could occur is autoimmune disease.

4.3 Patient Protection

The Ethical Principles for Medical Research Involving Human Subjects outlined in the World Medical Association Declaration of Helsinki (See Appendix C) will be followed

in the treatment of patients. The study will be carried out applying the principles of Good Clinical Practice. Patients who do not fulfill all of the eligibility requirements for the study will not be enrolled. Subjects who are not able to comply with the requirements of the study will be removed from the study.

Patients with progressive disease will be offered the option for reassignment to other therapies, and will be taken off administration of the vector. All patients that either come off study or those who complete the study will continue to be monitored for Serious Adverse Events for 30 days after removal and until resolution of the SAE.

4.4 Adverse Event Management

4.4.1 Early Stopping Rules

Patients with unacceptable toxicity will be removed from the study on the basis of the following criteria.
The study will enroll 24 evaluable post surgical prostate cancer patients. These patients will be assigned to eight cohorts of 3 patients each, with Cohorts 1, 2, 3, and 4 following a vector dose-escalation schema. Cohorts 5, 6, 7, and 8 will all be treated with the highest tolerated vector dose, as well as the dose escalation schema of the protein ecdhMUC-1/ecdCD40L booster injections. A patient who does not complete the two therapies (other than through therapy-related adverse events, disease progression, or death) shall be deemed non-evaluable and will be replaced with a new patient. The replacement patient's treatment must be completed prior to the first enrollment in the next cohort. Each patient will be injected subcutaneously with a replication-incompetent adenoviral vector carrying the Ad-sig-ecdhMUC-1/ecdCD40L vector and subcutaneous injections of the hMUC-1/ecdCD40L protein.
The clinical trial begins with the first cohort receiving one subcutaneous injection using the initial low dose of 1×10^9 vector vaccine. If none of the patients in the first cohort experience Grade III or IV toxicity, a second cohort will receive an injection of 1×10^{10} VP.

The dose escalations will continue with cohort three receiving 1 injection of 5×10^{10} VP and if cohort does not have grade II or IV toxicity, cohort four will receive 1×10^{11} VP.
If none of the patients experience Grade III or IV toxicity, the remaining 4 cohorts will receive 1 injection of 1×10^{11} VP per injection plus two subcutaneous injections of ecdhMUC-1/ecdCD40L protein given 14 days apart, starting 7 days after the vector injection at the following doses: 100 mcg, 500 mcg, 1 mg, and 5 mg. For all patients who experience a Grade III adverse event the dose will be reduced back to the last level that did not produce a Grade III event. If a Grade III event occurs in the first cohort of either the vector or the protein, a 25% dose reduction will be implemented.
If a Grade IV event occurs, the subject will not be given any more injections. If two grade IV events occur at any dose level, further injections will be done at the previous dose. If these grade IV events occurred at one of the two starting doses, the first does will

be reduced by 25%. If 2 more events occur at this dose a further 25% reduction will be implemented. If three subjects have a grade IV event, the study will be stopped.

4.4.2 Data Safety Monitoring Board

An outside Data Safety Monitoring Board (DSMB) will be established to evaluate the Grade III and IV reports of adverse events and deaths from the clinical trial.
The board will be comprised of at least 3 members with the chairman having prior data monitoring committee experience. The DSMB members shall not be associated with the investigators of the clinical trial. The board members will have expertise in the field of gene therapy trials and safety concerns and will not have a conflict of interest.
The first meeting of the Data Safety Monitoring Board (DSMB) will be held before the start up of the study to discuss the protocol, informed consent, data collection instruments, handling of meeting minutes, definition of a "quorum," stopping rules, and data reporting methods.

The DSMB will have scheduled meetings every six months. Telephone meetings may be held by request of the FDA, Investigator or after receipt of SAE Report or patient death. The DSMB will receive summaries of all Grade III and IV adverse events and patient deaths prior to each formal meeting. The DSMB will receive a summary of SAE, grade III or IV adverse event prior to a telephone meeting. An Investigator prior to DSMB Meeting may invoke the Clinical Trial Stopping Rules. The DSMB will invoke the protocol stopping rules if not already done by the Investigator.
The DSMB will also determine the relationship of an adverse event to the study drug in undecided cases and will assess Risk to Benefit at conclusion of the trial. Having this outside monitoring board provides an additional safeguard for the human subjects participating in the trial.

The principal endpoint of this Phase I pilot study is patient toxicity and mortality. Accrual to the trial will be placed on hold if a patient dies during the 7 day treatment period. The (DSMB) will review the data from the trial to ascertain if the treatment contributed to the patient's death. The DSMB will than determine if the trial should either be temporally or permanently terminated.

5 ELIGIBILITY CRITERIA

5.1 Inclusion Criteria

5.1.1 To be eligible for the trial the following must be true. The patient must be over the age of 18 and be post radiation therapy for prostate cancer and have had three consecutive rises in PSA values with at least one level above 5/ng/ml.

5.1.2 Ability to understand and the willingness to sign a written informed consent document.

5.1.3 Performance status ≤ 2 on the ECOG performance scale (Karnofsky 70%-80% See Appendix A) and life expectancy of greater than 12 months.

5.1.4 Acceptable NYHA Cardiac Function Class I or Class II (See Appendix B) ECG graded a normal. If abnormal an echocardiogram will be to rule out defects such as congestive heart failure.

5.1.5 Acceptable pulmonary function. If questionable a pulmonary function test will be performed.

5.1.6 Negative for hepatitis B, C and HIV.

5.1.7 The following tests must be within the specified limits, red cell mean corpuscular volume at least 80 cu. mm. hemoglobin of 8 g/dl, platelet count greater than 100,000/dl: AST and ALT, LDH $\leq$2 times the upper limit of normal, and serum creatinine of $\leq$1.6.

5.1.8 The effects of the Ad-sig-ecdhMUC-1/ecdCD40L vector on the developing human fetus are unknown. Subjects should wear condoms if they engage in sexual activity.

5.1.9 The following values must be within normal limits: serum bilirubin, serum creatinine, BUN, AST, SGT and uric acid

5.2 Exclusion Criteria

5.2.1 Subjects who have a history of bronchospasm or asthma or any condition that requires the administration of steroids.

5.2.2 Subjects must not have any history of any autoimmune disease such as lupus, rheumatoid arthritis or psoriasis.

5.2.3 Subjects taking steroids, or any immunosuppressive therapy.

5.2.6 Patients who are under age 18, or are unable to undergo repeated clinical evaluations and other diagnostic procedures or unable to sign an informed consent required by the protocol.

5.2.7 Patients with a history of other malignancies except squamous or basal cell carcinomas of the skin.

5.2.8 Any concurrent systemic antimalignancy therapy.

5.2.9 Patients with organ grafts, or allogenic bone marrow transplants.

5.2.10 Patients with a history of ornithine transcarbamylase (OTC) deficiency

5.3 Inclusion of Minorities

Men of all ethnic groups are eligible for this trial.

NOTE: PATIENTS NOT MEETING ALL INCLUSION AND EXCLUSION CRITERIA MAY NOT BE ENROLLED IN THIS STUDY. SHOULD THERE BE ANY QUESTION REGARDING PATIENT ELIGIBILITY, THE FINAL DECISION ON STUDY ENTRY IS TO BE MADE BY DR. DEISSEROTH AND WRITTEN APPROVAL FROM HIM MUST BE OBTAINED.

5.4 Patient Registration and Enrollment

After completing all the prestudy screening requirements and meeting all of the eligibility criteria, the final decision on eligibility is made by the principal investigator Dr. Albert Deisseroth, M.D.,Ph.D.. The patient will then be informed again of all the requirements and risks of the study and asked if she still wants to participate if so and after the patient has signed the informed consent she will then be entered into the study.

5.5 Expense to Subjects

Participants will not be charged for research studies related uniquely to an evaluation of the genetic modification mediated by the adenoviral vector. Patients will be expected to pay for all other costs relating to therapy from indemnity insurance, HMO's, or other reimbursement mechanisms.

The research costs include protocol related physician follow-up visits, treatment with the Ad-sig-hMUC-1/ecdCD40L vaccine, CAT scans, clinical blood tests as well as all the mechanistic research blood assays.

6. DRUG PRODUCTION AND STORAGE

6.1 Vector Production

The Ad-sig-hMUC-1/ecdCD40L and the ecdhMUC-1/ecdCD40L protein will be produced by
Molecular Medicine BioServices
6219 El Camino Real
Carlsbad, CA 92009

Aliquots of the Ad-sig-hMUC-1/ecdCD40L vector stocks will be established as a master vector bank. Samples from each bank as well as the ecdhMUC-1/ecdCD40L protein will be tested for adventitial infections, endotoxin, viruses, and replication-competent adenoviruses.

6.3 Vector Storage and Accountability

The vector and protein preparations for the clinical trial will be labeled and kept at Sidney Kimmel Cancer Center in a minus 70° freezer that has an alarm and temperature monitor. The Ad-sig-ecdhMUC-1/ecdCD40L vector and protein will be packaged in plastic vials.

The vials will be labeled for *Investigational Use Only* and will be numbered and recorded on a perpetual accountability log. Using the prescription order written by the physician administering the vector, the required number of frozen vials will be transported in dry ice to the outpatient infusion unit.

The adenoviral vector will then be thawed at room temperature according to the following SOP. The protein vial will be kept at 4 degrees centigrade and the vial will be opened in a biosafety cabinet under sterile conditions.

SOP for Thawing. The frozen vial will thaw at room temperature under a biosafety cabinet following transportation in a sealed container on dry ice by a biosafety trained individual from Sidney Kimmel Cancer Center to the clinic biosafety cabinet.
The vial will be placed in an upright position in a clean rack.

a) The vial will be sprayed with 70% alcohol and placed in the rack in the biosafety cabinet.
b) After the alcohol is dry, the tube cab will be unscrewed and then contents will be gently aspirated using sterile technique.
c)

Then it will immediately be taken to the infusion room for administration. The physician giving the vector injection and nurse attending will use Biosafety Level Two (BSL2) infectious disease precautions complying with Sharp Health Care policy.
All of the waste such as the syringe protective pads and gloves will be deposited into an biohazard bag. Should a spill occur it will be removed following the SOP below.

SOP for handling a vector vaccine preparation spill.
a) An adsorbent paper will be placed on the puddle of the spill. This will be removed after all of the free liquid has been soaked into the paper. The paper will be placed into a biohazard bag.
b) Then, the surface that was contaminated will be wiped with or 10% bleach solution from the outside in. The papers used for the cleaning will be placed into the biohazard bag.
Should the patient use the bathroom the toilet will be flushed with 10% bleach solution in compliance with clinic policy.

The vial number along with the date and time of administration will be recorded both on the patient record as well as the hospital investigational pharmacy log.
If for some reason any vials of Ad-sig-ecdhMUC-1/ecdCD40L vector are thawed but not administrated to a patient the vial/vials will be destroyed in infectious waste container.

7. STUDY PROCEDURES

7.1 **Prestudy Observations** (to be completed within 4 weeks prior to the start of therapy)

- Signed Informed consent must be obtained before study entry or any study specific tests are performed.
- History and physical exam including performance status, height and weight.
- Evaluation of baseline signs and symptoms.
- Chest x-ray, ECG, bone scan and pelvic CT or MRI.

7.1.1 Prestudy Laboratory Studies

- Hematology: CBC, machine differential, platelets.
- Serum Chemistry: PSA, AST, ALT, total bilirubin, serum creatinine, BUN, glucose, total protein, LDH, uric acid, calcium.
- Hepatitis panel for hepatitis B and C, HIV.
- Immunology panel of NK cells, CD 4 cell and CD 8 cells.
- CA 15-3, 29,27 (MUC-1).

7.1.2 Studies After Enrolling and Prior to Vaccine Injection

- T-Cell cytotoxicity.
- PCR, Blood and urine.
- Antibody level to MUC-1.
- Phosphorylation test for ERK-1, ERK-2.
- Cytokine release-for IL-12, Interferon γ from CD 8 cells
- Immunophenotyping of CD 8 and CD 4 cells by FACS.

7.2 Ad-sig-ecdhMUC-1/ecdCD40L Vaccine Study Calendar (See following Page)

Table 3 Study Calendar for Ad-sig-hMUC-1/ecdCD40L Vector Vaccine Post Radiation Therapy Prostate Cancer Study

Prochedure or Blood Test Prestudy procedures must be done within 4 weeks of study entry.	Prior To Study Entry	Day 0 Vector injection	Day 3 72 hour	Week 1 Protein Injection 1 Day 7	Week 2 Day 14	Week 3 Protein Injection # 2 Day 21	Week 4 Day 28	Week 6 Day 42	Week 8	Week 12 Day 84
Signed Consent Form,	X									
MUC-1, microarray & genetic tests on tumor tissue taken at time of surgery	X									
History	X									
Physical Exam	X	X		X		X				X
Vital signs	X	X		X	X	X				X
Toxicity Evaluation		X		X	X	X	X	X	X	X
CBC, diff, platelets, *Chemistry panel **	X			X		X	X	X		X
Hepatitis, HIV	X									
Immunophenotyping	X			X			X			
PCR blood & urine***		X	X							
Chest x-ray, ECG	X									
Bone Scan, Pelvic CT or MRI	X									
PSA	X				X		X		X	X
CD8, CD4 and NK cells	X				X		X			X
T Cell Cytotoxocity		X			X			X		X
Antibodies to MUC-1		X		X					X	X
Cytokine release assay ELLISPOT from CD 8 T cells Inf γ and IL-12		X		X			X			X
Phosphorylation test For ERK-1,ERK-2		X					X			
CA 15-3, CA 29-27	X				X		X		X	X

*Pre Study Serum Chemistry: AST, ALT, alkaline phosphatase, total bilirubin & (direct and indirect), serum creatinine, BUN, glucose, total protein, LDH, uric acid, calcium, potassium, inorganic phosphorous

**On Study Serum Chemistry: AST, ALT, LDH, bilirubin, serum, creatinine, BUN, LDH, uric acid, alkaline phosphatase.

- The lower shaded area of table contains the assays that will be used to determine the optimal safe dose of the vaccine and the protein as well as Efficacy.

7.3 On Study Evaluations

7.3.1 Disease Evaluations

- **PSA** measurements Week 2, Week 4, Week 8 and Week 12.

7.3.2 Toxicity Evaluations (Adverse Events)

To be done at every patient visit See Study Calendar 7.2

7.3.3 Survival

Patients will not be followed for survival in this phase I study.

7.3.4 Clinical Laboratory Studies

- Within 1 week prior to vector injection and at week 1, 3, 6, 8 and 12
- CBC.
- Serum Chemistry: AST, ALT, LDH, bilirubin (total), serum creatinine, BUN, LDH, glucose, uric acid, alkaline phosphatase.
- PCR for vector and vector protein, Blood and urine ,day 0,day 72 hours or day 3, day

7.3.5 Special Studies on PB Mononuclear Cells.

- ELLISPOT assay for cytokine release using CD 8 cells, weeks , week 4 and week 12.
- Immunology Panel, Week 2, week 4 and week 10.
- Antibodies to MUC-1: weeks 1, 2, 3, 4, 6 and week 10.
- Phosphorylation test for ERD-1, ERK-2, week 4.
- T cell cyotoxicity, days weeks 1, week 8 and week 12.
- CA 15-3, CA 27,29, Prior to study entry, week 2, week 4, week 8 and week 12.

7.3.6 Patient Evaluation

The exam described below is to be done prior to study entry, week 1, week 3 week 12. Physical exam includes all systems listed in the physical exam case report form page, reviewing pertinent history, adverse reactions using the common toxicity criteria version number 3 to grade severity, physical findings including; vital signs, temperature, pulse, respirations, blood pressure and performance status.

8. **Response Criteria** The efficacy of the protocol will be evaluated according to the following response criteria.

Stabilization or reduction of PSA values from baseline pretreatment value. Stabilization of serum CA15-3 and 27,29 if elevated at baseline

Complete Response (CR) Reduction to normal of serum markers for a minimum of 4 weeks.

Incomplete Response An inclusive change in serum markers.

Progressive Disease (PD) 25% increase in serum markers.

8.1 Duration of Overall Response

The duration of overall CR is measured from the time measurement criteria are first met for CR until the first date that recurrent or progressive disease is objectively documented (taking as reference for progressive disease the lowest serum markers recorded since the treatment started). This duration can also be measured in the time it takes for the serum markers to rise above normal from the normal level achieved after treatment. Patients using serum levels will be measured from the time of documentation of the rise of serum markers from the 50% or more reduction to levels prior to treatment.

8.2 Duration of Stable Disease

Stable disease is measured from the start of the treatment until the criteria for progression are met, taking as reference the lowest serum marker measurements recorded since the treatment started.

8.3 Progression-Free Survival

Progression Free Survival is defined as the duration of time from start of treatment to time of progression as defined by a rising PSA level.

9 REGULATORY AND REPORTING REQUIREMENTS

9.1 Adverse Event Reporting

The descriptions and gradin
g scales found in the revised NCI Common Toxicity Criteria (CTC) version 2.0 will be utilized for adverse event reporting. All appropriate treatment areas should have access to a copy of the CTC version 2.0 Appendix D of this protocol. The possible risks to humans are outlined in SEC 4.1 and 4.2. Since this is a Phase I trial and the Ad-sig-ecdhMUC-1/ecdCD40L vector has not been given to humans before there are no known risks associated with this agent. A list showing the possible short and long term adverse events specific for the Ad-sig-ecdhMUC-1/ecdCD40L vector is found in (Section 9.6).

9.2 Written Informed Consent

Before any invasive or study specific procedures are performed, the patient will be given the Institutional Review Board-approved consent form. The investigator will be available to answer any questions the patient might have. Subjects electing to participate in the study must sign the consent form prior to initiation of any procedures relating to this study. The original signed forms will be maintained in the medical

record, with one copy given to the patient and one copy maintained in the investigator's files.

9.3 Retention of Records All protocol records are to be retained as per the FDA code of Federal Regulations 21 section 58.195.

9.4 Adverse Event Reporting

9.4.1 Adverse Event Reporting Guidelines

UNEXPECTED EVENT		EXPECTED EVENT	
GRADES 2 – 3 **Attribution of Possible, Probable or Definite**	**GRADES 4 – 5** **Attribution of Possible, Probable or Definite**	**GRADES 1 – 2** **Regardless of Attribution**	**GRADES 4 – 5** **Regardless of Attribution**
Grade 2 – Record in Case Report Form. Follow to resolution **Grade 3** – Report by phone to Medical Monitor within 24 hrs. Important for Medical Monitor to determine causality since protocol-stopping rules may need be invoked. Medical monitor will notify the DSMB	Report by phone to Medical Monitor Dr. Albert Deisseroth within 24 hrs. Send written report with as much information as possible to the FDA within 7 working days, send follow-up report containing complete information within 8 additional days. Report to Sharp HealthCare IRB, RAC and SKCC IBC within 48 hours of finding out about event.	Record in Adverse Events Pages of Case Report Form If a consistent trend develops report to Medical Monitor. Follow to Resolution	Report to medical Monitor within 24 hours of finding out about event. This includes all deaths within 30 days of the last dose of treatment with an investigational agent regardless of attribution.

9.4.2 Adverse Event Definition

A SERIOUS ADVERSE DRUG EVENT IS ANY ADVERSE DRUG EXPERIENCE OCCURRING AT ANY DOSE THAT RESULTS IN ANY OF THE FOLLOWING OUTCOMES:

- Death
- A life-threatening adverse drug experience
- In-patient hospitalization or prolongation of existing hospitalization
- A persistent or significant disability/incapacity
- A congenital anomaly/birth defect

- Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered a serious adverse drug event when, based upon

appropriate medical judgment, they may jeopardize the patient and may require medical or surgical intervention to prevent one of the outcomes listed above.

9.4.3 Serious Adverse Event Reporting

All serious adverse events will be reported to the Medical Monitor within 24 hours of finding out about the event. A SAE report form should be completed. For a reportable SAE, the Investigator is responsible for submitting to the FDA within 7 working days a preliminary report with a complete report to follow within an additional 8 days for a total of 15 days. The DSMB will also be notified to assist in determining causality to be prepared to invoke stopping rules.

The Serious Adverse Event will also be reported to Sharp HealthCare IRB within 48 hours.

9.5 Adverse Event Definition

An adverse event is any unfavorable, harmful, or unintended change involving function, structure, or chemistry change that occurs during the study, regardless of drug relationship, including any undercurrent illness, injury, toxicity, sensitivity, or sudden death.

A pre-existing condition is one that is present prior to or at the start of the study and is to be reported as part of the patient's medical history. It should be reported as an adverse event if the frequency, intensity, or the character of the condition worsens during study treatment.

Lack of efficacy is a worsening of the disease being studied or lack of desired effect of the study drug. If followed as an efficacy parameter, it should not be recorded as an adverse event.

9.5.1 Reporting Non-SAE Adverse Events

All adverse events that occur at any time during the study, including the 30 day post-treatment period as defined in the protocol after drug administration is discontinued, are to be reported in the patient's Case Report Forms. Any medications given to treat event are recorded in concomitant medications page.

The investigator or study coordinator will evaluate each patient at each visit during the study for any new or continuing symptoms since the previous visit. Any symptoms changing in character or in intensity should be noted. Any clinically significant adverse event reported by the patient or caregiver or noted by the investigator or study coordinator will be recorded on the Adverse Experience Case Report Form. The intensity will be evaluated, relationship to study drug will be determined, and any necessary management will be recorded in the patient's medical record.

Not Related: The event does not meet the criteria above and the event is known to be

associated with a clinical condition or with another medication taken by the patient.

Unknown: Current information about the event and the drug is insufficient to determine that the event is not related to the use of the drug. A definitive response will be required to determine the rehabilitation of the drug to the course of event.

A clinical laboratory abnormality should be reported as an adverse event only if the following conditions are met:

- The laboratory abnormality has been confirmed by at least one repeat test, or the abnormality suggests disease and/or organ toxicity, or
  The abnormality is of a degree that requires active management, e.g., change of dose, discontinuation of drug, more frequent follow-ups, diagnostic investigation.

Health hazard or side effect should also be reported as a Serious Adverse Event.
"Death" should not be reported as an adverse event. The cause of death should be reported as an adverse event. The only exception is "Sudden Death" when the cause is unknown.

9.6 Criteria or Removal From Study

- *Adverse Events:* Should any of the events listed in stopping rules occur and not be resolved. Any other Adverse Event unable to be resolved or that interferes with patient compliance
- *Disease Progression:* Patients will be taken off study if they have progressive disease as defined in Section 8.4.1.
- *Personal Reasons:* As stated in the informed consent, patients may withdraw from the study at any time for any reason.
- *Clinical Judgment of the Investigator:* A patient may be withdrawn from the study if, in the opinion of the investigator, it is not in the patient's best interest to continue (adverse event, concurrent illness, lack of efficacy, etc.).

9.7 Ad-sig-ecdhMUC-1/ecdCD40L Vector Specific Possible Adverse Events List

Short Term Complications: The patient could suffer from reduced blood pressure, elevated temperature, shortness of breath, depressed blood counts, local pain, rash and inflammation of the injection site and possible rigors.
Long Term Complications: Autoimmune disease. Although this is a theoretical possibility, experiments in chimps with MUC-1 vaccines suggest that this will not be a problem.[30-31]

10 Data Analysis and Reporting

10.1 Adverse Event Evaluation

The Common Toxicity Criteria Version 2 located in Appendix D will be used to grade adverse events. Any unexpected or SERIOUS ADVERSE EVENTS must be reported to the Medical Monitor within 24 hours of knowledge of the event.

10.2 Statistical Analysis of Adverse Events

The toxicity data will be analyzed using a separate logistic regression for each different cumulative grade. The dose level (on a logarithmic scale) is the most important covariant in this regression model. The fitted curve can then be used to estimate a safe dosage for subsequent studies. There will be four cohorts of patients treated with 4 different doses of the vector encoding the Ad-sig-ecdhMUC-1/ecdCD40L. It will be possible to test for a correlation between each grade of toxicity and the following parameters: the dose of the vector, the dose of the protein, and the rate of decay of the vector and protein in each patient at each dose level.

Analysis of primary endpoints of toxicity and mortality related to power calculation. The primary outcome of this phase I dose escalation study is toxicity and patient safety. The table below shows that the number of patients enrolled has sufficient power to detect adverse events (AE) rates as low as 7.5%. This is much lower than the usual rate of 1 in 6 or 17% that is the typical endpoint of a phase I trial. The probability of observing at least one AE in the trial is calculated both for the entire group of 24 patients and for the 15 patients to be given the highest dose.

10.3 Data Analysis

10.3.1 Descriptive Statistics

Summaries of the characteristics of the population at baseline will be prepared for both the safety and the evaluable patient cohorts. Data will be summarized using descriptive statistics (numbers of patients, mean, median, standard deviation, quartiles) for continuous variables and frequencies and percentages for discrete variables. Confidence intervals will be emphasized throughout, rather than formal hypothesis testing.

10.3.2 Analysis of Adverse Events

All adverse events will be reported in tabular form indicating the number of patients who reported each type of adverse event by grade. In secondary analyses, correlations between each grade of toxicity and the dose of the vector and the rate of decay of the vector will be examined.

10.3.3 Power Considerations

The table below shows that the number of patients enrolled has sufficient power to detect an adverse event (AE) rates as low as 7.5%, with reasonably high power. The probability of observing at least one AE in the trial is calculated both for the entire projected cohort of 24 patients and for the 15 patients to be given the highest dose.

Table 4.

SAMPLE SIZE ESTIMATES PER GROUP (beta = 0.2, alpha =0.0125)

Control Group	Experimental Group	N
0.7	0.35	50
0.7	0.28	35
0.7	0.21	27
0.8	0.40	37
0.8	0.32	27
0.8	0.24	21
0.9	0.45	27
0.9	0.36	20
0.9	0.27	17
0.95	0.45	17
0.95	0.36	13
0.95	0.27	11
0.99	0.45	21
0.99	0.36	16
0.99	0.27	12

The patients will be treated in Cohorts 1-4 with 3 patients at each of the 4 escalated doses of the vector. Cohorts 5-8 of 3 subjects each will be adding escalating doses of the protein to the highest achieved dose of the vector. The efficacy data from the trial will be analyzed to determine whether a relationship exists between the patient response and the dose of vector particles or dose of protein injected. The definitions of response provided above in section 8.7.1 will be applied to an analysis of the binary valued outcomes using logistic regression as a function of dose of the vector and protein. The rate of change in tumor size will be examined using individual growth curves and longitudinal models with generalized estimating equations. An autoregressive working correlation structure will be assumed for these data. The15 patients receiving the highest dose of the vector will provide sufficient data for an evaluation of the response induced by the treatment within a standard error of less than 16%.

11 REFERENCES:

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APPENDIX A

Performance Status Criteria

ECOG/ZUBROD Performance Status Scale		Karnofsky Performance Scale	
Grade	Description	Percent	Description
0	Normal activity. Fully active, able to carry on all pre-disease performance without restriction.	100	Normal, no complaints, no evidence of disease.
		90	Able to carry on normal activity; minor signs or symptoms of disease.
1	Symptoms, but ambulatory. Restricted in physically strenuous activity, but ambulatory and able to carry out work of a light or sedentary nature (e.g., light housework, office work).	80	Normal activity with effort; some signs or symptoms of disease.
		70	Cares for self, unable to carry on normal activity or to do active work.
2	In bed <50% of the time. Ambulatory and capable of all self-care, but unable to carry out any work activities. Up and about more than 50% of waking hours.	60	Requires occasional assistance, but is able to care for most of his/her needs.
		50	Requires considerable assistance and frequent medical care.
3	In bed >50% of the time. Capable of only limited self-care, confined to bed or chair more than 50% of waking hours.	40	Disabled, requires special care and assistance.
		30	Severely disabled, hospitalization indicated. Death not imminent.
4	100% bedridden. Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair.	20	Very sick, hospitalization indicated. Death not imminent.
		10	Moribund, fatal processes progressing rapidly.
5	Dead.	0	Dead.

APPENDIX B

NEW YORK HEART ASSOCIATION (NYHA) FUNCTIONALCLASSIFICATION FOR CARDIAC PATIENTS

Class	Patient Symptoms
Class I (Mild)	No limitation of physical activity. Ordinary physical activity does not cause undue fatigue, palpitation, or dyspnea (shortness of breath).
Class II (Mild)	Slight limitation of physical activity. Comfortable at rest, but ordinary physical activity results in fatigue, palpitation, or dyspnea (shortness of breath).
Class III (Moderate)	Marked limitation of physical activity. Comfortable at rest, but less than ordinary activity causes fatigue, palpitation, or dyspnea (shortness of breath).
Class IV (Severe)	Unable to carry out any physical activity without discomfort. Symptoms of cardiac insufficiency at rest. If any physical activity is undertaken, discomfort is increased.

APPENDIX C

WORLD MEDICAL ASSOCIATION DECLARATION OF HELSINKI .

Ethical Principles for Medical Research Involving Human Subjects
Adopted by the 18th WMA General Assembly
Helsinki, Finland, June 1964
and amended by the
29th WMA General Assembly, Tokyo, Japan, October 1975
35th WMA General Assembly, Venice, Italy, October 1983
41st WMA General Assembly, Hong Kong, September 1989
48th WMA General Assembly, Somerset West, Republic of South Africa, October 1996
and the
52nd WMA General Assembly, Edinburgh, Scotland, October 2000

INTRODUCTION

1. The World Medical Association has developed the Declaration of Helsinki as a statement of ethical principles to provide guidance to physicians and other participants in medical research involving human subjects. Medical research involving human subjects includes. Research on identifiable human material or identifiable data.
2. It is the duty of the physician to promote and safeguard the health of the people. The physician's knowledge and conscience are dedicated to the fulfillment of this duty.
3. The Declaration of Geneva of the World Medical Association binds the physician with the records, "The health of my patient will be my first consideration," and the International Code of Medical Ethics declares that, "A physician shall act only in the patient's interest when providing medical care which might have the effect of weakening the physical and mental condition of the patient."
4. Medical progress is based on research which ultimately must rest in part on experimentation involving human subjects.
5. In medical research on human subjects, considerations related to the well-being of the human subject should take precedence over the interests of science and society.
6. The primary purpose of medical research involving human subjects is to improve prophylactic, diagnostic and therapeutic procedures and the understanding of the aetiology and pathogenesis of disease. Even the best proven prophylactic, diagnostic, and therapeutic methods must continuously be challenged through research for their effectiveness, efficiency, accessibility and quality.
7. In current medical practice and in medical research, most prophylactic, diagnostic and therapeutic procedures involve risks and burdens.
8. Medical research is subject to ethical standards that promote respect for all human beings and protect their health and rights. Some research populations are vulnerable and need special protection. The particular needs of the economically and medically disadvantaged must be recognized. Special attention is also required for those who cannot give or refuse consent for themselves, for those who may be subject to giving consent under duress, for those who will not benefit personally from the research and for those for whom the research is combined with care.
9. Research Investigators should be aware of the ethical, legal and regulatory requirements for

research on human subjects in their own countries as well as applicable international requirements. No national ethical, legal or regulatory requirement should be allowed to reduce or eliminate any of the protections for human subjects set forth in this Declaration.

B. BASIC PRINCIPLES FOR ALL MEDICAL RESEARCH

10. It is the duty of the physician in medical research to protect the life, health, privacy, and dignity of the human subject.
11.Medical research involving human subjects must conform to generally accepted scientific principles, be based on a thorough knowledge of the scientific literature, other relevant sources of information, and on adequate laboratory and, where appropriate, animal experimentation.
12. Appropriate caution must be exercised in the conduct of research that may affect the environment, and the welfare of animals used for research must be respected.
13. The design and performance of each experimental procedure involving human subjects should be clearly formulated in an experimental protocol. This protocol should be submitted for consideration, comment, guidance, and where appropriate, approval to a specially appointed ethical review committee, which must be independent of the investigator, the sponsor or any other kind of undue influence. This independent committee should be inconformity with the laws and regulations of the country in which the research experiment is performed. The committee has the right to monitor ongoing trials. The researcher has the obligation to provide monitoring information to the committee, especially any serious adverse events. The researcher should also submit to the committee, for review, information regarding funding, sponsors, institutional affiliations, other potential conflicts of interest and incentives for subjects.
14. The research protocol should always contain a statement of the ethical considerations involved and should indicate that there is compliance with the principles enunciated in this Declaration.
15. Medical research involving human subjects should be conducted only by scientifically qualified persons and under the supervision of a clinically competent medical person. The responsibility for the human subject must always rest with a medically qualified person and never rest on the subject of the research, even though the subject has given consent.
16. Every medical research project involving human subjects should be preceded by careful assessment of predictable risks and burdens in comparison with foreseeable benefits to the subject or to others. This does not preclude the participation of healthy volunteers in medical research. The design of all studies should be publicly available.
17. Physicians should abstain from engaging in research projects involving human subjects unless they are confident that the risks involved have been adequately assessed and can be satisfactorily managed. Physicians should cease any investigation if the risks are found to outweigh the potential benefits or if there is conclusive proof of positive and beneficial results.
18. Medical research involving human subjects should only be conducted if the importance of, the objective outweighs the inherent risks and burdens to the subject. This is especially, important when the human subjects are healthy volunteers.
19. Medical research is only justified if there is a reasonable likelihood that the populations in, which the research is carried out stand to benefit from the results of the research.
20. The subjects must be volunteers and informed participants in the research project.
21. The right of research subjects to safeguard their integrity must always be respected. Every precaution should be taken to respect the privacy of the subject, the confidentiality of the patient's information and to minimize the impact of the study on the subject's physical and

mental integrity and on the personality of the subject.
22. In any research on human beings, each potential subject must be adequately informed of the aims, methods, sources of funding, any possible conflicts of interest, institutional affiliations of the researcher, the anticipated benefits and potential risks of the study and the discomfort it may entail. The subject should be informed of the right to abstain from participation in the study or to withdraw consent to participate at any time without reprisal. After ensuring that the subject has understood the information, the physician should then obtain the subject's freely-given informed consent, preferably in writing. If the consent cannot be obtained in writing, the non-written consent must be formally documented and witnessed.
23. When obtaining informed consent for the research project the physician should be particularly cautious if the subject is in a dependent relationship with the physician or may consent under duress. In that case the informed consent should be obtained by a well informed physician who is not engaged in the investigation and who is completely independent of this relationship.
24. For a research subject who is legally incompetent, physically or mentally incapable of giving consent or is a legally incompetent minor, the investigator must obtain informed consent from the legally authorized representative in accordance with applicable law. These groups should not be included in research unless the research is necessary to promote the health of the population represented and this research cannot instead be performed on legally competent persons.
25. When a subject deemed legally incompetent, such as a minor child, is able to give assent to decisions about participation in research, the investigator must obtain that assent in addition to the consent of the legally authorized representative.
26. Research on individuals from whom it is not possible to obtain consent, including proxy or advance consent, should be done only if the physical/mental condition that prevents obtaining informed consent is a necessary characteristic of the research population. The specific reasons for involving research subjects with a condition that renders them unable to give informed consent should be stated in the experimental protocol for consideration and approval of the review committee. The protocol should state that consent to remain in the research should be obtained as soon as possible from the individual or a legally authorized surrogate.
27. Both authors and publishers have ethical obligations. In publication of the results of research, the investigators are obliged to preserve the accuracy of the results. Negative as well as positive results should be published or otherwise publicly available. Sources of funding, institutional affiliations and any possible conflicts of interest should be declared in the publication. Reports of experimentation not in accordance with the principles laid down in this Declaration should not be accepted for publication.

C. ADDITIONAL PRINCIPLES FOR MEDICAL RESEARCH COMBINED WITH MEDICAL CARE

28. The physician may combine medical research with medical care, only to the extent that the research is justified by its potential prophylactic, diagnostic or therapeutic value. When medical research is combined with medical care, additional standards apply to protect the patients who are research subjects.
29. The benefits, risks, burdens and effectiveness of a new method should be tested against those of the best current prophylactic, diagnostic, and therapeutic methods. This does not exclude the

use of placebo, or no treatment, in studies where no proven prophylactic, diagnostic or therapeutic method exists.
To further clarify the WMA position on the use of placebo controlled trials, the WMA Council issued, during
October 2001, a note of clarification on article 29, which is available on this page.
30. At the conclusion of the study, every patient entered into the study should be assured of access to the best proven prophylactic, diagnostic
and therapeutic methods identified by the study. 31. The physician should fully inform the patient which aspects of the care are related to the research. The refusal of a patient to participate in a study must never interfere with the patient-physician relationship.
32. In the treatment of a patient, where proven prophylactic, diagnostic and therapeutic methods do not exist or have been ineffective, the physician, with informed consent from the patient, must be free to use unproven or new prophylactic, diagnostic and therapeutic measures, if in the physician's judgment it offers hope of saving life, re-establishing health or alleviating suffering. Where possible, these measures should be made the object of research, designed to evaluate their safety and efficacy.
In all cases, new information should be recorded and, where appropriate, published. The other relevant guidelines of this Declaration should be followed.

NOTE OF CLARIFICATION ON PARAGRAPH 29 of the WMA DECLARATION OF HELSINKI

The WMA is concerned that paragraph 29 of the revised Declaration of Helsinki (October 2000) has led to diverse interpretations and possible confusion. It hereby reaffirms its position that extreme care must be taken in making use of a placebo-controlled trial and that in general this methodology should only be used in the absence of existing proven therapy. However, a placebo controlled trial may be ethically acceptable, even if proven therapy is available, under the following circumstances:- Where for compelling and scientifically sound methodological reasons its use is necessary to determine the efficacy or safety of a prophylactic, diagnostic or therapeutic method; or where a prophylactic, diagnostic or therapeutic method is being investigated for a minor condition and the patients who receive placebo will not be subject to any additional risk of serious or irreversible harm. All other provisions of the Declaration of Helsinki must be adhered to, especially the need for appropriate ethical and scientific review.

SHARP®

PATIENT INFORMED CONSENT

STUDY TITLE

A SINGLE ARM OPEN-LABEL PHASE I STUDY OF AN INJECTABLE REPLICATION-INCOMPETENT ADENOVIRAL VECTOR VACCINE WITH A PROTEIN BOOST USED TO PRODUCE AN IMMUNE RESPONSE FOR MUC-1 POSITIVE EPITHELIAL CANCER CELLS IN PROSTATE CANCER PATIENTS POST RADIATION THERAPY

SKCC 109-04-00

October 22, 2004

IRB Reference Number 041199

Revised December 08, 2004
Revised August 30, 2005

PRINCIPAL INVESTIGATOR
Albert Deisseroth M.D., PhD.
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Sidney Kimmel Cancer Center
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A single arm open-label phase I study of an injectable replication-incompetent adenoviral vector vaccine with a protein boost used to produce an immune response for muc-1 positive epithelial cancer cells in prostate cancer patients post radiation therapy [SKCC 109-04-00]

Participation in a Research Study

You are being asked to take part in this clinical trial because you have prostate cancer that has been treated. Your physician is using a blood test to measure how your cancer is doing and this test is called a (PSA) test. This test (PSA) in many cases of prostate cancer goes up in number when the cancer is growing. Your PSA test is now going up and you have had a treatment that now may not be working well. Because of this situation, you are now eligible to receive this new treatment that is now being given to humans for the first time.

A clinical trial includes only patients who choose to take part in it. Before entering this trial, your physician will have discussed any other procedures or treatment options that may be available to you.

Please read this consent form. Discuss it with your friends and family. Ask the researcher any questions you may have about the study, and please take your time in making this decision.

Why Is This Study Being Done?

This is a Phase I trial, which means that the drugs or substances that you will receive (a vaccine and a protein) in this study have not been given to humans before. The purpose of this clinical trial is to study the safety of the drugs that are given by injections under the skin.

One of these injections is a vaccine that contains a modified cold virus that carries genetic material that should cause the body to induce an attack by cells of the immune response system on your cancer. This will help kill the cancer cells and prevent them from growing again. The vaccine (also called an adenoviral vector) is a cold virus that has been modified so that it can no longer cause an infection that can spread throughout your body.

You will also be receiving two protein boost injections. This "boost" is made from a protein that may help your body fight against your cancer cells. The combinations of these two preparations should cause an immune response against your metastatic cancer that can suppress the growth of the cancer and determine whether this vaccine will prevent it from coming back.

The purpose of this study is to see how safe it is to give these vaccine and protein injections under your skin by looking to see what affects it will have on your body. It is also of interest to see if these local injections will prevent or delay the progression of your prostate cancer. You will have blood tests to measure the PSA that can help determine the effect of the treatment on your cancer.

This consent form gives you detailed information about this research study and your physician will discuss it with you. Once you understand the study, you will be asked to

sign this form if you wish to take part, and you will be given a copy of this form to keep as a record.

How Many People Will Take Part in the Study?

A total of 24 subjects with prostate cancer that is coming back after radiation treatment and has a PSA level that has risen for three months in a row with at least one of these levels above normal. There may be more patients enrolled if others do not complete the treatment.

What Is Involved in the Study?

Within four weeks of beginning treatment on this study, you will be asked to complete screening procedures to be sure you qualify. It will be necessary for you to sign the consent form before these tests can be done.

- History and physical exam (including height and weight, heart rate, respiratory rate, temperature, and blood pressure) to be done by your doctor. Your study doctor/nurse will ask you about your past medical history and will do an evaluation of your current health problems and the and symptoms you have now from your cancer..
- Chest x-ray
- ECG (checks to see if your heartbeat is okay)
- CAT scan (computer enhanced x-ray) of the pelvis to evaluate your disease.
- Bone Scan (will be testing to see if you have disease in your bones)
- Laboratory blood tests (6 tablespoons) that will be testing for the following things:
  - To see if your immune cells are adequate and to see if you have enough oxygen carrying cells
  - The blood tests will also look at how well your liver is functioning
  - The blood tests will also make sure you do not have hepatitis B and C or HIV
  - We will be doing some baseline tests to compare with later tests for the drug you will be getting.

The first 12 patients will receive 1 vaccine treatment (injections under the skin), and patients 13-24 will receive 3 treatments (1 vaccine and 2 protein). All of the patient treatments will be given at the Sharp Outpatient Infusion Center (3075 Health Center Drive, Suite 102, San Diego, CA 92123). The first 12 patients will receive one vector injection under the skin. The last 12 patients on the trial will receive the one vaccine injection under the skin and in addition will receive 2 protein injections under the skin which will start 7 days after your vector injection under the skin and the last intravenous protein injection will take place 14 days later. You will be asked to stay for 30 minutes after the first injection of each type for observation.

You will be considered to be part of the study as long as your cancer does not get worse. If you have evidence of your cancer getting worse by your PSA continuing to rise or you develop symptoms of your disease in another location, you will be taken off the clinical trial and you or someone close to you will be called occasionally to check up on your condition.

You will be asked to come back to the clinic for blood tests and physical examinations periodically until there are signs that your disease is getting worse. The exam described

in the section above is to be done prior to starting your injections and will be weekly for 4 weeks, on week 6, week 8 and on week 12.

The treatment phase of this study takes a total of 3 weeks. The total duration of the study is one year. Your study doctor may decide to take you off this study if it is in your best medical interest, if the funding for the program is stopped, if there is no longer vaccine available for treatment, if your condition worsens, or if new information becomes available that would change the advisability of your continuing on the study. You may also be asked to leave the study if you cannot complete the required procedures.

What Are the Risks of the Study?

While on the study, you are at risk for the side effects noted below. You should discuss these with the study doctor and/or your regular doctor.

You should be aware that there could be risks and side effects that we do not yet know about that could result from the drugs used in this study.

The treatment of cancer often requires the use of strong medicines that have side effects, some potentially serious. Most drugs do have side effects since the treatment often affects other organs or parts of the body in addition to the cancer cells. The drug (vector vaccine with the genetic material) used in this clinical trial may cause all, some or none of the side effects listed below. In addition, rare or previously unknown side effects, which may be serious, might occur. Most side effects are temporary and manageable but they could lead to other serious or even fatal problems. Other drugs will be given to make side effects less serious and uncomfortable.

Adenoviral Vector Vaccine and Protein (with the specific genetic material used in this study)

Likely Short-term Side Effects (*Note: Because the adenoviral vector with the particular genetic material used in this study has not been given to humans before, these side effects are based on animal data, and the predictions are based on the science that developed the drug*

Less Serious, likely and short term (These effects should go away soon after the injections)

- Fever
- Chills
- Redness or swelling of injection site
- The numbers of some types of blood cells may be diminished.
- Nausea
- Headache
- Dizziness
- Fatigue (tiredness)
- Rash
- Difficulty breathing

Possible and Serious and Potentially Long Term

- Because we are stimulating the immune system there is a possibility of an autoimmune

disorder. This would be a disease like arthritis.

Adenoviral Vector (modified cold virus) (with a different genetic material than used in this study)

You should be aware that a subject in a different gene transfer study using the same kind of modified cold virus (but different genetic material than used in this study) died after receiving a large dose of the adenoviral vector through an injection into the artery that provides blood supply to the liver (rather than an injection under the skin, as done in this study). That subject did not have enough of a substance that is important in handling the breakdown products of protein. This disorder is very rare and is inherited. Even in these individuals, injections of lower doses of the modified cold virus do not cause clinically significant side effects. When very high doses of this adenoviral vector are injected under the skin or other tissues instead of into the bloodstream, there have been no clinically significant complications in over 400 study participants.

Subcutaneous Injections and Blood Work

Some known risks, although rare, associated with giving an injection under the skin. These risks are discomfort, infection, and temporary bruising or swelling. If you have ever felt faint, you should tell the study nurse so that you can lie down while your blood is drawn.

Reproductive Risks

It is not yet known if the adenoviral vector used in this study could cause harm to an unborn child. Because of this, you should use barrier contraception while on this study.

Are There Benefits to Taking Part in the Study?

Although it is hoped that you will benefit from taking part in this study, we cannot guarantee that it will directly benefit you. It is hoped that this research will benefit other patients in the future.

There are no known benefits to humans established at this time. Studies already conducted in mice with human cancer tumors suggest that injection of the vaccine under the skin can prevent the cancer from progressing, but this has never been used in humans. The goal of this clinical trial program in which you are thinking of participating is to test the ***safety*** of these injections in subjects with prostate cancer, and to see if there are any beneficial effects at all on cancer tumors in humans.

What Other Options Are There?

Instead of being in this study you have the following options-chemotherapy surgery or hormonal therapy or a combination of some of the above.

There are many experimental options to this protocol therapy including vaccines, chemotherapy, immunotherapy, radiation, dendritic cell therapy and alternative therapies not involving standard medical treatment (but none of these have been shown to affect the length of time individuals with cancer of the prostate can survive with the disease). Please talk to your physician about these and other options.

What About Confidentiality?

Efforts will be made to keep your personal information confidential. We cannot guarantee absolute confidentiality. Your personal information may be disclosed if required by law.

Organizations or individuals that may inspect and/or copy all or part of your research records for quality assurance and data analysis include groups such as:

- Sharp HealthCare staff and its agents
- Sharp HealthCare Institutional Review Board
- United States Food and Drug Administration (FDA)
- Sidney Kimmel Cancer Center staff
- Other national health and regulatory authorities

You will not be identified by name in any reports or publications resulting from this clinical trial.

You will be asked to sign a document authorizing the use of your health information for specific purposes.

Protected Health Information

As a part of this research study you will be asked to sign a separate document giving your permission to use and disclose your medical records. This document will tell you who will view your records, how they will be used and how long they will be needed. It will also tell you what you can do if you no longer agree to have your medical records used. Your signature will give us permission to use your records. You will receive a copy of the signed document.

What Are the Costs?

Your participation is voluntary and you will not receive any compensation for your participation.

Before you enter the trial your insurance company will be asked if they will pay for the disease related expenses and the expenses that occur if you have a reaction to the medication

Taking part in this study may lead to added costs to you and your insurance company. The sponsor will pay the costs of diagnostic tests that are specific to the study and the study medication or drug. Please ask about any expected added costs **or insurance problems.**

You will not be responsible for the costs related to the study injections.
Neither Sidney Kimmel Cancer Center or Sharp HealthCare will reimburse you for any other medical expenses, nor will these institutions be responsible for payment of your lost wages or other damages or losses resulting from any injury incurred as a result of your participation.

It will be your responsibility to pay any co-pays or meet any deductibles that are required by your insurance. Compensation for medical expenses shall not be deemed an admission

of fault or liability by Sidney Kimmel Cancer Center, Sharp HealthCare, or any other person or institution.

You or your insurance company will be responsible for any tests that are medically indicated to check the status of your disease.

You or your insurance company will be responsible for the costs of any medicines that are administered to treat the side effects of the therapy.

The sponsor will reimburse the study site for time, effort, and oversight by the investigator and professional staff to perform procedures, tasks, and accurately collect and submit data.

Research Related Injury?

In the case of injury or illness resulting from this study, emergency medical treatment will be provided. Sharp HealthCare will not provide any compensation to you in the event you sustain a research related injury while participating in this study. Sidney Kimmel Cancer Center has clinical trial insurance that would provide compensation for a research related injury.

What Are My Rights as a Participant?

Taking part in this study is voluntary.

You will not be paid for taking part in this study.

You may choose not to take part or may leave the study at any time. Leaving the study will not result in any penalty or loss of benefits to which you are entitled. Your decision to not take part in this study will not affect your medical care or your relationship with your doctors. Any new study information regarding your health, welfare or willingness to participate will be made available to you.

A Data Safety and Monitoring Board, an independent group of experts, will be reviewing the data from this research throughout the study. We will tell you about the new information from this or other studies that may affect your health, welfare, or willingness to stay in this study.

You may be asked to participate in the measurement of your prostate cancer after you are off the clinical trial. Clinical data (information) may continue to be collected from your medical records. You will be given information about the experience of previous subjects in the trial and any other information about the trial or previous subjects that might affect your willingness to participate in the trial.

Who Do I Call If I Have Questions or Problems?

If you experience a research-related injury or if you need medical treatment during this study, you can contact your doctor at:

NAME OF YOUR DOCTOR: _____

PHONE NUMBER OF YOUR DOCTOR: _____

In addition, if you have questions about this study, or if you want additional information about this study, you may call the doctor in charge of this study, *Albert Deisseroth, M.D., Ph.D.,* at (858) 450.5990.

For questions about your rights as a research participant, contact the Sharp Healthcare Institutional Review Board (which is a group of people who review the research to protect your rights) at:

Phone: (858-499-4836)

Sharp HealthCare Institutional Review Board
8695 Spectrum Center Blvd.
San Diego, CA 92123

SIGNATURE

Your signature below indicates that you have read the above about (drug name or procedure or device) and have had a chance to ask questions to help you understand what your participation will involve. You agree to participate in the study until you decide otherwise. You are not waiving your legal rights by signing this consent form.

SIGNATURE OF PATIENT *(or of legally authorized representative)*	PRINTED NAME	DATE

SIGNATURE OF WITNESS *(or person obtaining consent)*	PRINTED NAME	DATE

I _____ attest that the requirements for informed consent for
Printed Name of Principal Investigator
the medical research project described in this form have been satisfied – that the participant has been provided with a copy of the Experimental Subject's Bill of Rights, that I have discussed the research project with the participant and explained to him or her in nontechnical terms all of the information contained in this informed consent form, including any risks and adverse reactions that may reasonably be expected to occur. I further certify that I encouraged the participant to ask questions and that all questions asked were answered.

SIGNATURE OF INVESTIGATOR	DATE

SHARP®

California Experimental Subject's Bill of Rights

You have been asked to participate as a subject in an experimental procedure. Before you decide whether you want to participate in the experimental procedure, you have a right to:

1. Be informed of the nature and purpose of the experiment;
2. Be given an explanation of the procedures to be followed in the medical experiment, and any drug or device to be utilized;
3. Be given a description of any discomforts and risks reasonably to be expected from your participation in the experiment;
4. Be given an explanation of any benefits reasonably to be expected from your participation in the experiment;
5. Be given a disclosure of any appropriate alternative procedures, drugs or devices that might be advantageous to you, and their relative risks and benefits;
6. Be informed of the avenues of medical treatment, if any, available to you after the experimental procedure if complications arise;
7. Be given an opportunity to ask any questions concerning the medical experiment or the procedures involved;
8. Be instructed that consent to participate in the experimental procedure may be withdrawn at any time and that you may discontinue participation in the medical experiment without prejudice;
9. Be given a copy of this form and the signed and dated written consent form; and
10. Be given the opportunity to decide to consent or not to consent to the medical experiment without the intervention of any element of force, fraud, deceit, duress, coercion or undue influence on your decision.

I have carefully read the information contained above and I understand fully my rights as a potential subject in a medical experiment involving people as subjects.

_____ _____

Signature of Subject Signature of Witness

_____ _____

Date Date

Authorization to Use your Protected Health Information (PHI)

Protected Health Information: PHI is any personal health information through which you can be identified. We are asking for your permission to use your PHI in this research study. The information we will use includes: your past and present health information, information that can be used to contact you, results of your medical tests.

- Results of blood tests
- Results of physical exams
- Results of CT, MRI, x-rays
- Special lab tests
- Adverse events related to study personnel
- Hospital records, if hospitalized

Who will disclose your PHI?

- Dr. Albert Deisseroth M.D.,Ph.D
  Sidney Kimmel Cancer Center
  10835 Altman Row San Diego, CA 92121
  Phone (858) 450-5990

Who will see your PHI?

- The sponsor of the research study, Dr. Albert Deisseroth
- Sharp HealthCare Institutional Review Board
- United States Food and Drug Administration (FDA)
- Sidney Kimmel Cancer Center clinical staff
- Food and Drug Administration *(FDA)*
- Data Safety Monitoring Board
- Other national health and regulatory authorities
- Sharp HealthCare committees that review research to help protect people who join research studies.

How will Sharp HealthCare use and share my information, and what will it be used for?

The information will be used to complete case report forms required by the study, used to make treatment decisions, to inform data safety monitoring board of adverse events, in reports to the IRB, FDA RAC, and NIH.

- The information will be used during your participation in the study 2 years or more. After you no longer participate in the study publications will not identify patients except

If you decide not to share your information anymore:

You must write to the study doctor and tell him that you no longer want to share your information. Write to the study doctor at

Dr. Albert Deisseroth
Sidney Kimmel Cancer Center
10835 Road to the Cure
San Diego, CA 92121
Phone (858) 450 5990

- You will no longer be a part of the research study.
- You will still get the same medical care that you have always had at Sharp HealthCare.
- The research team can continue to use any of the protected information that they already have.

Do you have the right to see and copy your research information?

You can only see your research information if it is also being used for your health care, or at the end of the study.

If you agree to share your information you must sign this form below. You will be given a copy of this form.

****

_____ _____
Print your name Date

_____ _____ Sign your
name Expiration Date

_____