

The Journal of Immunology

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This information is current as of December 11, 2021.

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J Immunol 2006; 177:5697-5707; ;
doi: 10.4049/jimmunol.177.8.5697
http://www.jimmunol.org/content/177/8/5697

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The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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

Yucheng Tang,[2]* Hakan Akbulut,* Jonathan Maynard,[2]* Line Petersen,* Xiangming Fang,[†] Wei-Wei Zhang,[†] Xiaoqin Xia,* James Koziol,[‡] Phyllis-Jean Linton,* and Albert Deisseroth[3]*

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

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

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

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

Materials and Methods

Cell lines

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

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

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

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

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0022-1767/06/$02.00

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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 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

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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

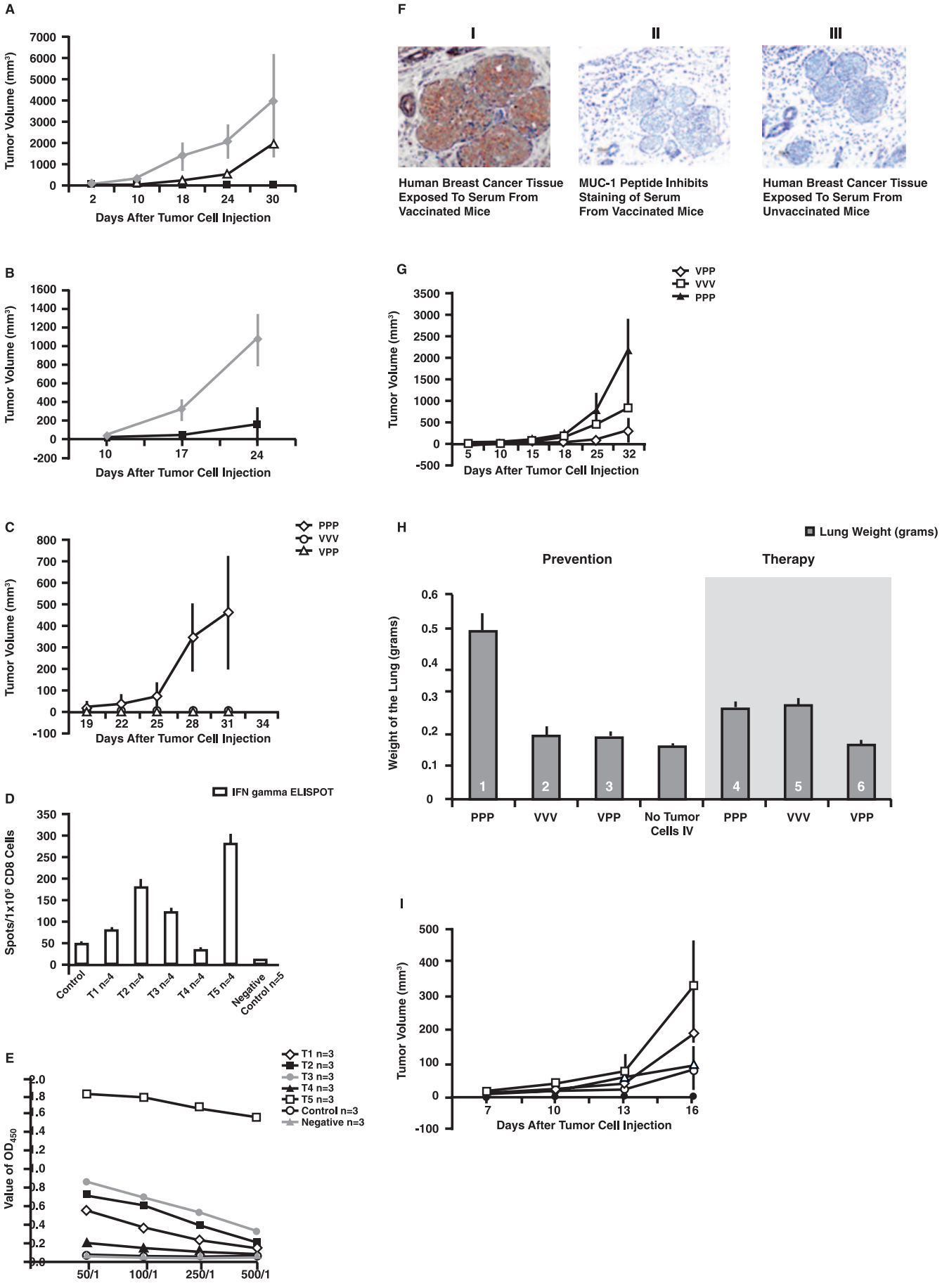
To mimic tumor metastases, we challenged hMUC-1.Tg mice (three mice per group) by tail vein injection of hMUC-1-positive LL2/LL1hMUC-1 tumor cells following completion of the vaccinations. We then weighed the lungs of mice sacrificed 63 days following the initiation of vaccination. As shown in Fig. 1*H* (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.

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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 (□).

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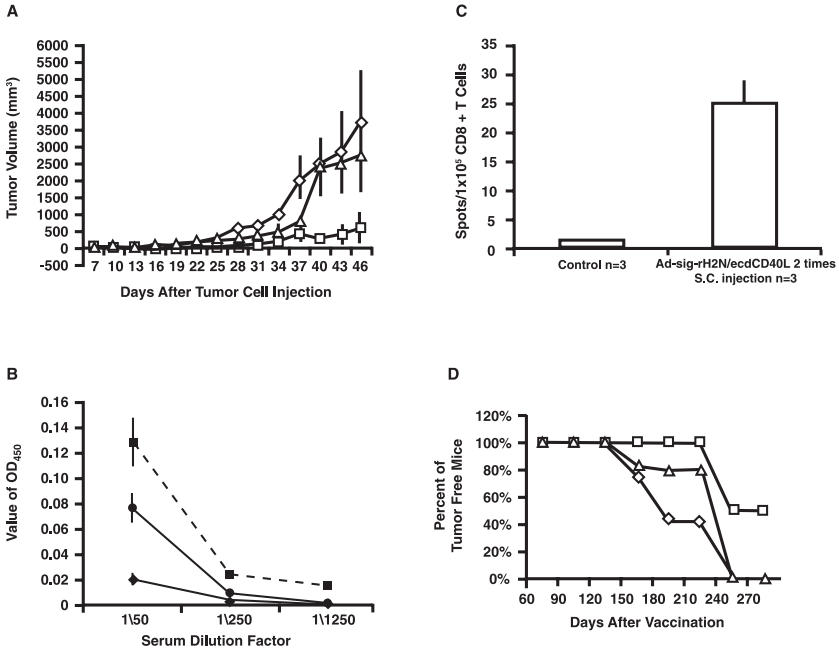


FIGURE 2. *A*, Effect of the Ad-sig-rH2N/ecdCD40L vector on the growth of rH2N-positive NT2 cells. The following vectors were injected s.c. twice at a 7-day interval: no vector (◇); two s.c. injections of the Ad-sig-rH2N/ecdCD40L vector (□), and one s.c. injection of the Ad-sig-rH2N/ecdCD40L vector (△).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

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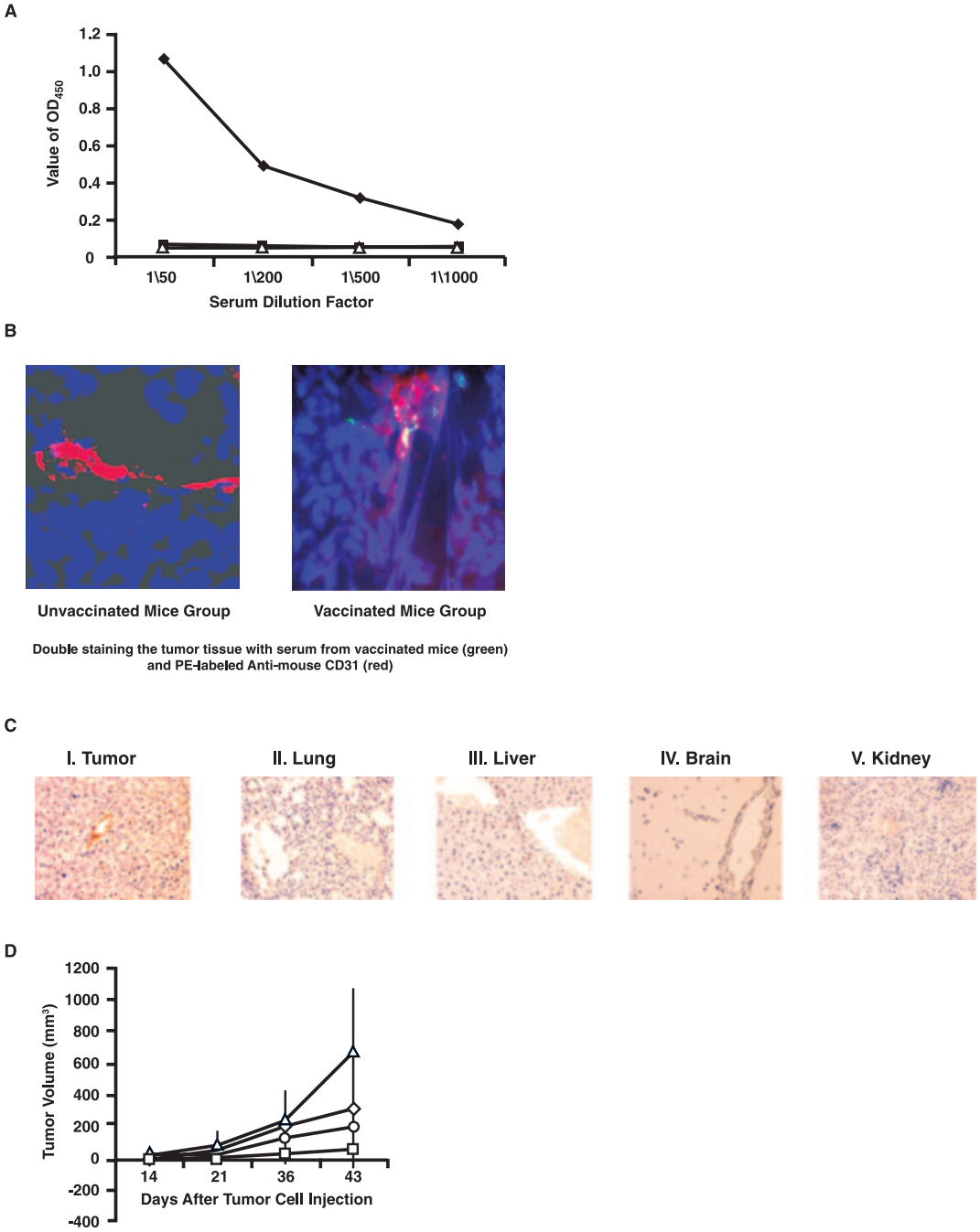


FIGURE 3. *A*, The serum level of Annexin A1-specific Abs before and after vaccination with the Ad-sig-AnxA1/ecdCD40L vector. To test specifically whether the immune response generated by the Ad-sig-AnxA1/ecdCD40L vector is directed against the Annexin A1 Ag, serum was taken from a mouse that had been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

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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 anti-tumor 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.

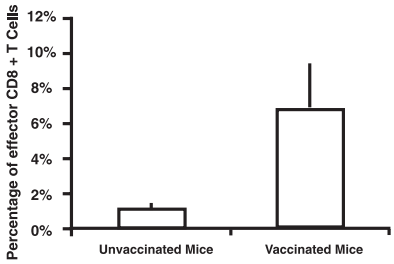


FIGURE 4. Infiltration of CD8 effector cells following Ad-sig-TAA/ecdCD40L vaccination. We minced the s.c. tumor nodules of rH2N.Tg mice before and after two s.c. injections of the Ad-sig-rH2N/ecdCD40L vector. Single-cell suspensions were isolated and treated with 0.03% DNase and 0.14% collagenase I,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

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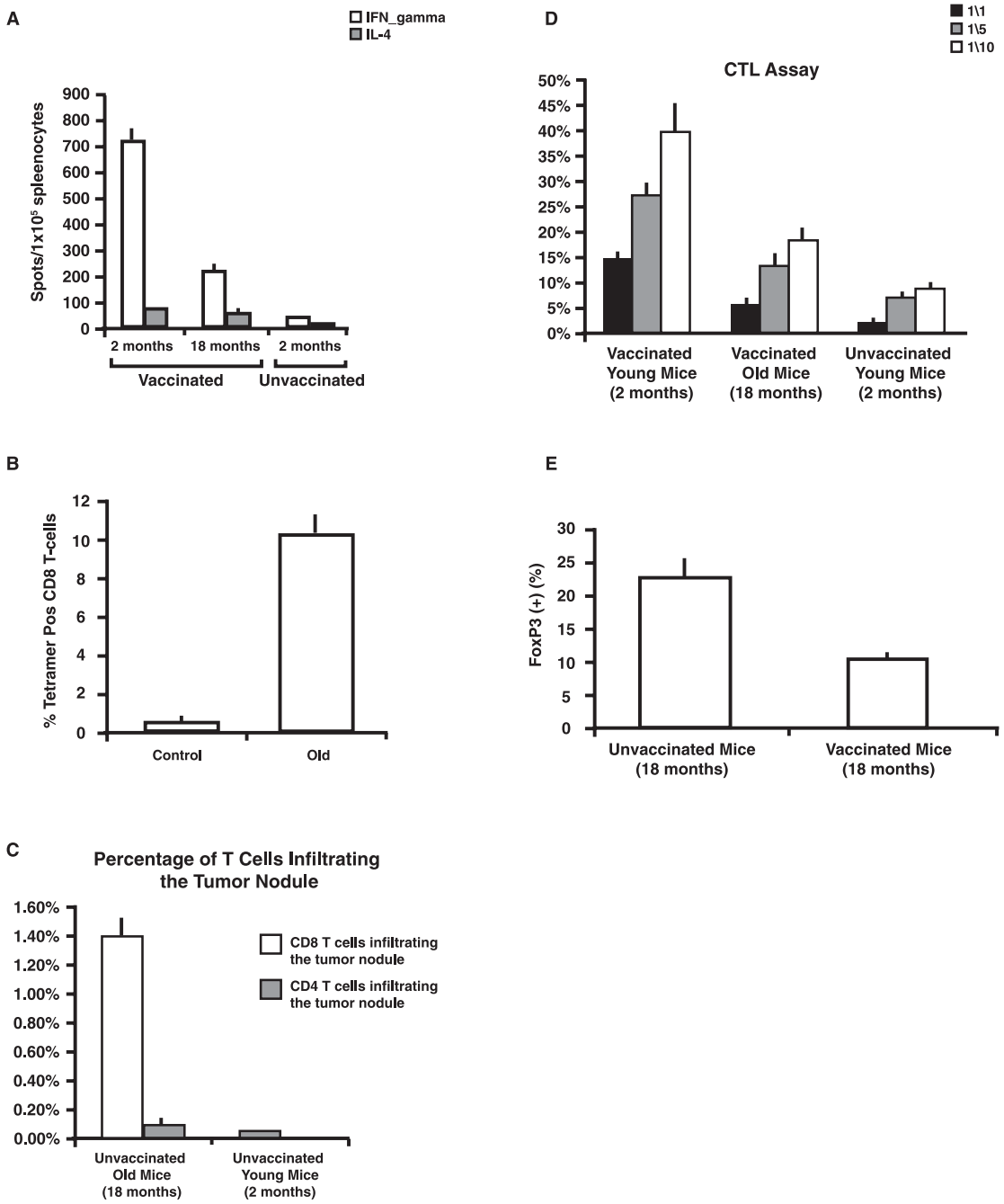


FIGURE 5. *A*, Effect of the Ad-sig-E7/ecdCD40L vector on the induction of E7 specific T cells as measured by the ELISPOT assay in 18- and 2-mo-old mice. The ELISPOT assay was used to measure the level of the IFN-γ- (▨) or IL-4- (■) positive T cells/1 × 10^5 spleen cells following in vitro exposure to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

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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.

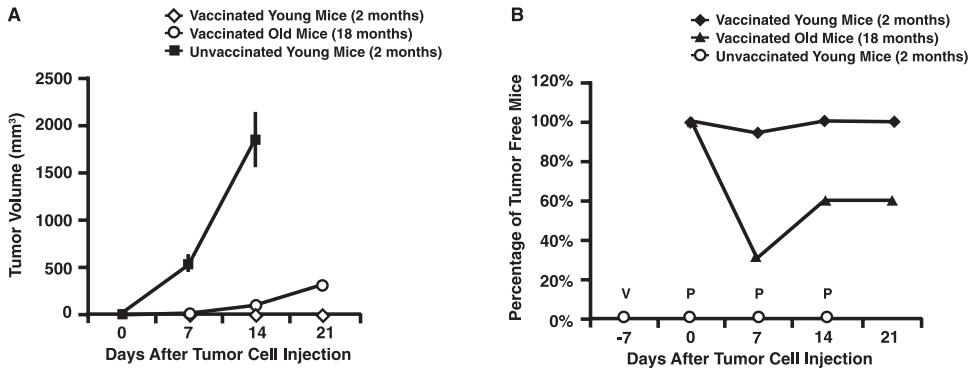


FIGURE 6. Effect of the Ad-sig-E7/ecdCD40L vector prime-E7/ecdCD40L protein (3×) boost (VPPP) vaccine against the E7 viral Ag in old (18 mo) and young (2 mo) mice on growth of E7-positive TC-1 tumor cells. C57BL/6J mice were injected once s.c. with the Ad-sig-E7/ecdCD40L vector and then three times s.c.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

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

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

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

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

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

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

Acknowledgments

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

Disclosures

The authors have no financial conflict of interest.

References

1. Dong, L., I. More, M. J. Hossain, B. Liu, and Y. Kimura. 2003. An immunostimulatory oligodeoxynucleotide containing a cytodine-guanosine motif protects senescence-accelerated mice from lethal influenza virus by augmenting the T helper type 1 response. *J. Gen. Virol.* 84: 1623–1628.
2. Zhang, L., Y. C. Tang, H. Akbulut, D. Zelterman, P. J. Linton, and A. Deisseroth. 2003. An adenoviral vector cancer vaccine that delivers a tumor-associated antigen/CD40-ligand fusion protein to dendritic cells. *Proc. Natl. Acad. Sci. USA* 100: 15202–15106.
3. Tang, Y., L. Zhang, J. Yuan, H. Akbulut, J. Maynard, P. J. Linton, and A. Deisseroth. 2004. Multistep process through which adenoviral vector vaccine overcomes anergy to tumor-associated antigens. *Blood* 104: 2704–2713.
4. Wurtzen, P. A., M. H. Nissen, and M. H. Claesson. 2001. Maturation of dendritic cells by recombinant human CD40L-trimer leads to a homogeneous cell population with enhanced surface marker expression and increased cytokine production. *Scand. J. Immunol.* 53: 579–587.
5. Fanslow, W. C., S. Srinivasan, R. Paxton, M. G. Gibson, M. K. Spriggs, and R. J. Armitage. 1994. Structural characteristics of CD40 ligand that determine biological function. *Semin. Immunol.* 6: 267–278.
6. Karpuses, M., Y. M. Hsu, J. Wang, J. Thompson, S. Lederman, L. Chess, and D. Thomas. 1995. 2A crystal structure of an extracellular fragment of human CD40 ligand. *Structure* 3: 1031–1039.
7. Taylor-Papdiumitriou, J., J. Burchell, D. W. Miles, and M. Dalziel. 1999. MUC1 and cancer. *Biochim. Biophys. Acta* 1455: 301–313.
8. DiLeo, A., D. Gancberg, D. Larsimont, M. Tanner, T. Jarvinen, G. Rouas, S. Dolci, J. Y. Leroy, M. Paesmans, J. Isola, and M. J. Piccart. 2002. HER-2 amplification and topoisomerase I-α gene aberrations as predictive markers in node-positive breast cancer patients randomly treated either with anthracycline-based therapy or with cyclophosphamide, methotrexate, and 5-fluorouracil. *Clin. Cancer Res.* 8: 1107–1116.
9. Ren, J., N. Agata, D. S. Chen, Y. Li, W. H. Yu, L. Huang, D. Raina, W. Chen, S. Kharbanda, and D. Kufe. 2004. Human MUC1 carcinoma-associated protein confers resistance to genotoxic anticancer agents. *Cancer Cell* 5: 163–175.
10. Rowse, G. J., R. M. Tempero, M. L. VanLith, M. A. Hollingsworth, and S. J. Gendler. 1998. Tolerance and immunity to MUC1 in a human MUC1 transgenic murine model. *Cancer Res.* 58: 315–321.
11. Gut, C. T., M. A.Webster, M. Schaller, T. J. Parsons, R. D. Cardiff, and W. J. Muller. 1992. Expression of the neu protoconcogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc. Natl. Acad. Sci. USA* 89: 10578–10582.
12. Oh, P., Y. Li, J. Yu, E. Durr, K. Krasinska, L. Carver, J. E. Testa, and J. E. Schnitzer. 2004. Subtractive proteomic mapping of the endothelial surface in lung and solid tumours for tissue-specific therapy. *Nature* 429: 629–635.
13. He, T. Z., S. Zhou, L. T. da Costa, J. Yu, K. W. Kinzler, and B. A. Vogelstein. 1998. Simplified system for generating recombinant adenovirus. *Proc. Natl. Acad. Sci. USA* 95: 2509–2514.
14. Tsang, K. Y., C. Palena, J. Gulley, P. Arlen, and J. A. Schlom. 2004. A human cytotoxic T-lymphocyte epitope and its agonist epitope from the nonvariable number of tandem repeat sequence of MUC-1. *Clin. Cancer Res.* 10: 2139–2149.
15. Cole, D. J., P. L. Baron, P. O'Brien, C. E. Reed, J. Schlom, and K. Y. Tsang. 2000. Phase I study of recombinant carcinoembryonic antigen (CEA) vaccinia virus vaccine with post vaccination carcinoembryonic antigen peptide (CAP-1) boost. *Clin. Lung Cancer* 1: 227–229.
16. Gough, M., M. Crittenden, U. Thanarajasingam, L. Sanchez-Perez, J. Thompson, D. Jevremovic, and R. Vile. 2005. Gene therapy to manipulate effector T cell trafficking to tumors for immunotherapy. *J. Immunol.* 174: 5766–5773.
17. Zhang, T., R. Somasundaram, K. Berencsi, L. Caputo, P. Rani, D. Guerry, E. Furth, B. J. Rollins, M. Putt, P. Gimotty, et al. 2005. CXC chemokine ligand 12 (stromal cell-derived factor 1α) and CXCR-4-dependent migration of CTLs toward melanoma cells in organotypic culture. *J. Immunol.* 174: 5856–5863.
18. Provinciali, M., A. Smorlesi, A. Donnini, B. Bartozzi, and A. Amici. 2003. Low effectiveness of DNA vaccination against Her-2/neu in ageing. *Vaccine* 21: 843–848.
19. Haynes, L., S. M. Eaton, E. M. Burns, T. D. Randall, and S. L. Swain. 2003. CD4 T cel memory derived from young naïve cells function well into old age, but memory generated from aged naive cells functions poorly. *Proc. Natl. Acad. Sci. USA* 100: 15053–15058.
20. Jefferson, T., A. Rivetti, M. Rudin, D. Di Pietrantonj, and V. Demicheli. 2005. Efficacy and effectiveness of influenza vaccines in elderly people: a systematic review. *Lancet* 366: 1165–1174.
21. Goodwin, K., C. Viboud, and L. Simonsen. 2006. Antibody response to influenza vaccination in the elderly: a quantitative review. *Vaccine* 24: 1159–1169.
22. Xiang, R., F. J. Primus, J. M. Ruehlmnn, A. G. Niethammer, S. Silletti, H. N. Lode, C. S. Dolman, S. D. Gillies, and R. A. Reisfeld. 2001. Dual function DNA vaccine encoding carcinoembryonic antigen and CD40L trimer induces T cell-mediated protective immunity against colon cancer in carcinoembryonic antigen-transgenic mice. *J. Immunol.* 167: 4560–4565.
23. LaPointe, J., C. Li, J. P. Higgins, M. M. van de Rijn, E. Bair, K. Montgomery, M. Ferrari, L. Egevad, W. Rayford, U. Bergerheim, et al. 2004. Gene expression profiling identifies clinically relevant subtypes of prostate cancer. *Proc. Natl. Acad. Sci. USA* 101: 811–816.
24. Deng, Y, Y. Jing, A. E. Campbell, and S. Gravenstein. 2004. Age-related impaired type 1 T cells responses to influenza. *J. Immunol.* 172: 3437–3446.
25. Eaton, S. M., E. M. Burns, K. Kusser, T. D. Randall, and L. Haynes. 2004. Age-related effects in CD4 T cell cognate helper function lead to reductions in humoral responses. *J. Exp. Med.* 200: 1613–1622.

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