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-TAA/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/ecdCD40L vector injections may be of value in treating the many epithelial malignancies in which TAA-like hMUC1 is overexpressed. (Blood. 2004;104:2704-2713)

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disrupting the regulation of anchorage-dependent growth, which leads to metastases.\textsuperscript{2,23} The MUC1 antigen is a self-protein overexpressed in carcinomas of the breast, ovary, lung, prostate, colon, and pancreas, among other carcinomas.\textsuperscript{21} Overexpression in epithelial cancers is thought to disrupt E-cadherin function, leading to anchorage-independent growth and metastases.\textsuperscript{22} Although non-MHC–restricted cytotoxic T-cell responses to MUC1 have been reported in patients with breast cancer,\textsuperscript{23} hMUC1 transgenic mice (MUC1.Tg) have been reported to be unresponsive to stimulation with hMUC1 antigen.\textsuperscript{24}

Our results show that immunizing hMUC1 transgenic mice, which are anergic to the hMUC1 antigen,\textsuperscript{24} with the Ad-sig-hMUC1/ecdCD40L vector induces a CD8\textsuperscript{+} T cell–dependent systemic T-helper 1 (Th1) 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 MHC–restricted cytotoxic 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, 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-C57BL/6/human MUC1\textsuperscript{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 the ecd of CD40L to an octapeptide linker (NDAQAPKS), and the carboxyl terminal end of which was linked to the secretory signal sequence. The resulting fusion protein was expressed from an adenoviral vector and was shown to be capable of inducing 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.

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PCR encoding the mouse immunoglobulin G (IgG) gene 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) \( \kappa \) 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 CCT CTT GCT ACT GGT GAC AAG GTC GAA GAG GAA GTA AAC C-3'; (2) 5'-TG TCG TCTG GTT CCA GCT TCC ACT GGT GAC ATG CAT G-3'; (3) 5'-CTG CTA TGG GAT CTA TGG CTC TGG CCT GCA GTC TC3'; (4) 5'-ACG TATG GAG ACA CAC A TC TCA TGG GTA GTC CTG-3'; (5) 5'-CCG CCC TGT AGA AGC AGA TGG TGA AGC CAA AAG-3'.

The CD40L template is the plasmid pDC406-mCD40L (American Type Culture Collection). PCR conditions are per protocol from the 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 cDNA3-hMUC1/ecdCD40L vector using HindIII-XhoI 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 \( \kappa \) 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 \( \kappa \) chain METDTLLLWVLLL-paraformaldehyde, and observed under a fluorescence microscope. Cells were then washed 3 times with cold medium, fixed with 1% paraformaldehyde, and observed under a fluorescence microscope. The level of murine IL-12 or interferon-\( \gamma \) was measured using ECL (enhanced chemiluminescence) detection kit (Amersham Pharmacia Biotech, Pullman, WA). The band was visualized by exposure to X-ray film. Immunoreactive bands were visualized on membranes by using the ProtoBlot II AP system (Promega).

**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.\(^3\) The coupled in vitro transcription–translation system of reticulocyte lysate (TNT) was used to translate the protein of interest on a TCA-resistant basis. After 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 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 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 transfected into 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 (mCD40L/L; E Bioscience, 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–labeled 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 activation of bone marrow–derived DCs**

DCs were incubated with the supernatant from 293 cells infected by each adenoviral vector described in the preceding sentence at a multiplicity of infection (MOI) of 40. Immunoreactive bands were visualized on membranes by using the ProtoBlot II AP system (Promega).
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 TTC TAA TTC TTC CCT TC-3'; for CCR7 antisense, 5'-AAA CTC ATA GCC AGC ATA GG-3'; for GAPDH sense, 5'-TGG TGA TGG TGT 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 recombiant 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/ecdCD40L 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(49-57) peptide (RA-HYNIVTF) 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/LL.1bMUC1-positive target cells (5 × 10^4) 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 × 10^6 plaque-forming units (pfus) of the Ad-sig-TAA/ecdCD40L, Ad-TAA, Ad-TAA/wtCD40L, Ad-sig-CD40L, Ad-wtCD40L, or Ad-sig-ecdMUC1/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).
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 supernant, whereas exposing DCs to the Ad-E7/ wtCD40L vector supernant 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 supernant 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 supernant from 293 cells infected with nonsecretable CD40L vectors or other control vectors (Figure 2Bi). 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-ecdCD40L (encoding the secretable E7/CD40L protein) compared with the Ad-E7/wtCD40L (encoding the non-secretable 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-ecdCD40L vector or the Ad-E7/wtCD40L vector. 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).
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 2Bi, 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 gradiental gradients of the chemokine ligands CCL19 and CCL21.26 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 × 106 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 transfection 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 nondondraining, 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 × 106 pfu of each vector subcutaneously into each animal. Mice were vaccinated again 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, $5 \times 10^5$ 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 transcriptional 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 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$_{p52}$ 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. 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 ($P \leq 0.05$). The number of splenic T cells producing a Th2 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 Th1 rather than a Th2 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 spleenic 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/ecdCD40L vector overcomes energy 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 vector. 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.](image-url)
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 hMUC1/ecdCD40L transcription unit is needed for complete suppression of the growth of the hMUC1 cell line in the hMUC-1.Tg mice.

**Figure 5. Effect of 2 subcutaneous injections (7 days apart) of 1 x 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 x 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 x 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 x 10^6 pfu Ad-sig-ecdhMUC1/ecdCD40L vector twice at 7-day intervals. One week after the second vector injection, the mice were injected with 5 x 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 x 10^6 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 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 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 hMUC1-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 LL2LL1hMUC1 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 $1 \times 10^8$ pfu Ad-sig-ecdhMUC1/ecdCD40L vector. Five hundred thousand LL2LL1hMUC1 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. (B) No protein injection. (C) Ad-sig-ecdhMUC1/ecdCD40L vector. (D) Two injections of the Ad-sig-ecdMUC1/ecdCD40L protein. One injection of the ecdMUC1/ecdCD40L protein. (B) CD4$^+$-depleted T cells from hMUC1.Tg transgenic mice after 2 subcutaneous injections of $1 \times 10^8$ pfu Ad-sig-ecdhMUC1/ecdCD40L vector secreted increased levels of bgl-1, CD8$^+$ T cells were isolated from hMUC1.Tg mice that had been vaccinated twice with the Ad-sig-ecdMUC1/ecdCD40L vector or with the Ad-sig-ecdMUC1 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 bgl-1 levels. (C) Cytotoxicity of CTLs from hMUC1.Tg transgenic mice after 2 subcutaneous injections (7 days apart) of $1 \times 10^8$ pfu of Ad-sig-ecdMUC1/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-ecdMUC1/ecdCD40L vector. Cells were restimulated in vitro with the LL2/LL1hMUC1 cell line for 5 days (A) or the LL2/LL1 cell line (B). CD8$^+$ T-cell lymphocytes were also isolated from the spleens of hMUC1.Tg mice 1 week after vaccination with the Ad-sig-ecdMUC1 vector, which was then stimulated in vitro with the LL2/LL1hMUC1 cell line (A). 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.Tg mice after 2 subcutaneous injections of $1 \times 10^8$ pfu 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-ecdMUC1/ecdCD40L vector and stimulated in vitro with the Ad-sig-ecdMUC1/ecdCD40L vector–infected DCs.

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–infected mice.

This increase in the TAA-specific CD8$^+$ lymphocytes in the Ad-sig-ecdMUC1/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 Th1 cytokines, is HLA restricted, and is accompanied by an increase in the total phosphorylation of ERK1 and ERK2 pathways in T cells from vector-infected hMUC1.Tg mice when the T cells are exposed to Ad-sig-ecdMUC1/ecdCD40L vector–infected DCs.

In contrast to the subcutaneous injection of the Ad-sig-ecdMUC1/ecdCD40L vector, the subcutaneous injection of the ecdMUC1/ecdCD40L protein does not induce immune protection against the growth of the hMUC1-positive LL2/LL2hMUC1 tumor cells (Figure 6A). This suggests that the danger signal associated with the adenoviral vector carrying the ecdMUC1/ecdCD40L transcription unit is an important part of overcoming the anergy of 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 regional lymph nodes. These events induce increases in the TAA-specific CD8$^+$ cytotoxic T lymphocytes in the spleens of Ad-sig-TAA/ecdCD40L vector–infected mice.

Because the adenoviral vector used in our work (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 injected 7 days earlier with the Ad-sig-ecdhMUC1/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–infected 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.
that subcutaneous injection of the Ad-sig-TAA/ecdCD40L vector is able to overcome the anergy that develops to TAA's, 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 antigens. In vivo activation was an attractive option to study for several reasons. First, the work of Xiang et al. 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. We had many reasons for selecting an in vivo method of vaccination, not ex vivo loading and antigen TAA/CD40L-trimer leads to a homogeneous cell population in vitro. 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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Multistep process through which adenoviral vector vaccine overcomes anergy to tumor-associated antigens

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