

Immunogenicity and Therapeutic Efficacy of a Dual-Component Genetic Cancer Vaccine Cotargeting Carcinoembryonic Antigen and HER2/*neu* in Preclinical Models

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Abstract

Several cancer vaccine efforts have been directed to simultaneously cotarget multiple tumor antigens, with the intent to achieve broader immune responses and more effective control of cancer growth. Genetic cancer vaccines based on *in vivo* muscle electro-gene-transfer of plasmid DNA (DNA-EGT) and adenoviral vectors represent promising modalities to elicit powerful immune responses against tumor-associated antigens (TAAs) such as carcinoembryonic antigen (CEA) and human epidermal growth factor receptor-2 (HER2)/*neu*. Combinations of these modalities of immunization (heterologous prime–boost) can induce superior immune reactions as compared with single-modality vaccines. We have generated a dual component–dual target genetic cancer vaccine consisting of a DNA moiety containing equal amounts of two plasmids, one encoding the extracellular and transmembrane domains of HER2 (ECD.TM) and the other encoding CEA fused to the B subunit of *Escherichia coli* heat-labile toxin (LTB), and of an adenoviral subtype 6 dicistronic vector carrying the same two tumor antigens gene constructs. The CEA/HER2 vaccine was tested in two different CEA/HER2 double-transgenic mouse models and in NOD/scid-DR1 mice engrafted with the human immune system. The immune response was measured by enzyme-linked immunospot assay, flow cytometry, and ELISA. The CEA/HER2 vaccine was able to break immune tolerance against both antigens. Induction of a T cell and antibody immune response was detected in immune-tolerant mice. Most importantly, the vaccine was able to slow the growth of HER2/*neu*⁺ and CEA⁺ tumors. A significant T cell response was measured in NOD/scid-DR1 mice engrafted with human cord blood cells. In conclusion, the CEA/HER2 genetic vaccine was immunogenic and able to confer significant therapeutic effects. These data warrant the evaluation of this vaccination strategy in human clinical trials.

Introduction

APPROVAL OF THE FIRST therapeutic cancer vaccine (Sipuleucel-T) by the U.S. Food and Drug Administration represents a breakthrough event in the history of cancer treatment (Cheever and Higano, 2011). The past skepticism toward this type of therapeutic intervention is now replaced by great expectations. The cancer vaccine field is now moving toward the development of alternative vaccination technologies capable of generating stronger, more durable, and effi-

cient immune responses against specific tumor-associated antigens (TAAs) (Aurisicchio *et al.*, 2012). This wave of enthusiasm is rooted in advances in molecular engineering and a better understanding of tumor immunology; new paradigms have emerged, such as the concept of dominant tolerance through T-regulatory and myeloid-derived suppressor cells and the instructive role of the innate immune system in the function of the adaptive immune system (Lindau *et al.*, 2012). This ever-increasing knowledge and the experience gained from earlier clinical studies indicate that efficient therapeutic

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cancer vaccination could be feasible, provided that antigen expression and presentation are increased, and that regulatory circuits are controlled.

Human epidermal growth factor receptor-2 (HER2)/*neu* and carcinoembryonic antigen (CEA) represent ideal TAAs, as they have various biological properties associated with the malignant phenotype of cells. HER2/*neu* is a member of a family of transmembrane receptor tyrosine kinases involved in signal transduction pathways that regulate cell growth and proliferation (Zhou and Hung, 2003). CEA is an immunoglobulin superfamily cell surface glycoprotein that mediates intercellular adhesion through homophilic interactions (Hammarström, 1999). Deregulated overexpression of CEA may contribute to tumorigenesis through the inhibition of cell differentiation and the disruption of tissue architecture (Ilantzis *et al.*, 2002). Both antigens are overexpressed in malignant tissues, as opposed to low-level expression in normal tissues. In particular, HER2/*neu* and CEA are overexpressed in a significant percentage of epithelial tumors (as shown in Supplementary Table S1; supplementary data are available online at www.liebertpub.com/hum). Their expression is present in both primary tumors as well as at metastatic sites. Both antigens are used as indicators of tumor recurrence and of decreased survival (Moertel *et al.*, 1993; Stark *et al.*, 2000). Interestingly, expression of HER2/*neu* appears to further increase with tumor progression, possibly due to gene amplification mechanisms (Slamon *et al.*, 1987). Nonetheless, the sustained expression of these antigens during the course of disease progression suggests that both CEA and HER2/*neu* play a biologically important role in tumor development. Thus, it is less likely that expression of these antigens would be lost without compromising, at least in part, the viability of the tumor cell. The stability of expression of these antigens makes them optimal targets for an immunological approach.

These antigens are naturally immunogenic in patients with cancer (Disis *et al.*, 2000; Nagorsen *et al.*, 2000; Rentzsch *et al.*, 2003). A spontaneous, albeit weak, T cell response against these antigens in patients with metastatic colorectal cancer and breast cancer has been reported. Thus, these proteins are suitable for therapeutic vaccination to induce long-lasting cytotoxic T and B cell responses that may exert therapeutic effects. Several clinical trials for cancer immunotherapy are being targeted against these proteins, among them studies based on the use of peptides, dendritic cells, and recombinant viral vectors have been reported (Turriziani *et al.*, 2012).

Genetic vaccines are emerging among the most promising methodologies for cancer treatment (Aurisicchio *et al.*, 2011a,b, 2012). Intramuscular delivery of DNA plasmids has been hampered by the poor efficiency of gene transfer as a result of the DNA degradation or lack of inefficient transduction of muscle cells. Combining intramuscular injection of plasmid vaccine with intramuscular electro-gene-transfer (DNA-EGT) increases the efficiency of gene expression and improves immune response in animals relative to simple intramuscular injection (Aurisicchio *et al.*, 2011a,b, 2012; Impellizzeri *et al.*, 2012). Adenoviral vectors have been shown to be quite efficient in generating cell-mediated immunity (CMI) against foreign antigens. However, a major liability of the adenoviral vectors is their capacity to rapidly induce a neutralizing antibody response to capsid proteins, which limits their use in revaccination (Dharmapuri *et al.*, 2009).

The majority of cancer vaccine regimens under development consist of repeated injections of the same vaccine. More recently, there has been a growing body of evidence to suggest that heterologous prime–boost immunization regimens are capable of generating higher amplitude and more durable immune responses, and that they exert better prophylactic and therapeutic efficacy in a variety of preclinical disease models (Lu, 2009; Vasconcelos *et al.*, 2012). The heterologous prime–boost approach, particularly when conducted with two different gene-based delivery systems, has been shown to induce a strong CMI response, which is the type of immunity expected to be most involved in the control of cancer cell growth. Data, from both preclinical and clinical vaccination studies against infectious agents, have demonstrated that DNA vectors appear to be the most effective at priming when followed by boosting with a viral vector, being associated with significantly greater immune responses than seen with either agent alone (Woodland, 2004). Hence, combined treatment with DNA-EGT and adenoviral vaccine may give rise to superior immune responses that may result in increased therapeutic efficacy.

In light of these data, we have taken the approach to develop a heterologous cancer vaccine regimen consisting of DNA-EGT injection followed by boosting with an adenoviral vector. An adenovirus subtype 6 (Ad6) vector has been selected because of the substantially lower frequency of pre-existing neutralizing antibodies to Ad6 in the human population as compared with Ad5 (Colloca *et al.*, 2012; and our unpublished data). Furthermore, the immunogenicity of Ad6 is similar to that of Ad5, it usually presents an excellent genetic stability profile, and is amenable to large-scale production.

In this study, we show that a heterologous DNA-EGT/adenovirus vaccine regimen targeting CEA and HER2/*neu* results in an increase in immunogenicity and therapeutic efficacy in two CEA/HER2 double-transgenic mouse models. Moreover, a strong cellular response could be detected in immunocompromised mice engrafted with the human immune system. Our data support the evaluation of a heterologous prime–boost regimen based on DNA-EGT and adenoviral vector targeting CEA and HER2/*neu* in human clinical trials.

Materials and Methods

Animals and cell lines

Six-week-old BALB/*c*, C57BL/6, and CB6 mice were purchased from Charles River Breeding Laboratories (Calco, Italy). CEA.Tg mice (H-2^b) were provided by J. Primus (Vanderbilt University, Nashville, TN) (Clarke *et al.*, 1998). BALB/NeuT mice (Lucchini *et al.*, 1992) were provided by G. Forni (University of Turin, Turin, Italy) and bred under specific pathogen-free conditions by Charles River Breeding Laboratories. BALB/NeuT and CEA-NeuT (BALB/NeuT × CEA.Tg) were screened for the presence of the transgene by duplex PCR as described (Gallo *et al.*, 2005). B6.HGR mice (Piechocki *et al.*, 2003) carry the full-length wild-type cDNA of human HER2 under the whey acidic protein (WAP) promoter. B6.HGR × CEA.Tg mice (CEA-hHER2) were screened by PCR as previously described (Piechocki *et al.*, 2003; Conforti *et al.*, 2009).

NOD-scid-DR1 mice were bred at Merck Research Laboratories (Whitehouse Station, NJ) by back-crossing the transgenic B10-DR1 mice onto NOD/scid mice from the

Jackson Laboratory. Mice were maintained in the barrier facility. All procedures performed on the animals were approved by the institutional animal care and use committee. At the end of the treatment period and before necropsy, mice were killed by administration of compressed CO₂ gas in cylinder as indicated by the American Veterinary Medical Association (AVMA) Panel on Euthanasia (2013) and according to the guidelines described by the U.K. Coordinating Committee on Cancer Research (1998). The experiments were conducted according to EU Directive EC86/609 on the protection of animals used for experimental and other scientific purposes, which was ratified by Italian legislation (DL no. 116/92) on February 19, 1992.

The MC38-hCEA/hHER2 cell line was generated by transfecting MC38-hCEA cells (Mennuni *et al.*, 2005) with pcDNA3-hHER2/*neu* plasmid followed by clone selection with hygromycin. The JY cell line is an Epstein-Barr virus (EBV)-immortalized B cell lymphoblastoid line and was purchased from the American Type Culture Collection (ATCC, Manassas, VA).

Engraftment of NOD/scid-DR1 mice with human cord blood cells

The procedure followed was as described elsewhere (Carmacho *et al.*, 2007). Cord blood was purchased from the National Disease Research Interchange (NDRI, Philadelphia, PA), and separated by lymphocyte separation medium (ICN Biomedicals, Aurora, OH) density gradient to obtain mononuclear cells (MNCs). Briefly, NOD/scid-DR1 mice were engrafted with HLA-A2 human cord blood MNCs (CBMNCs) at 5 days of age by intrahepatic inoculation with 2–5 million CBMNCs, after light irradiation (60–70 rad).

Plasmid constructs and adenoviral vectors

Plasmids pV1J/CEA-LTBopt and Ad5/CEA-LTBopt carry the codon usage-optimized cDNA of CEA fused to the B subunit of *Escherichia coli* heat-labile toxin (LTB) and has been previously described (Facciabene *et al.*, 2007). pV1J/ratHER2-ECD.TM and Ad5/ratHER2-ECD.TM carry codon usage-optimized cDNA encoding the rat extracellular and transmembrane domains of HER2 (rat ECD.TM) and has been described elsewhere (Gallo *et al.*, 2007; Cipriani *et al.*, 2008). For the construction of pV1J/HER2-ECD.TM, the cDNA corresponding to the human HER2-ECD.TM region was amplified by PCR, using sequence-specific primers from plasmid pV1J/HER2 that contains the entire codon-optimized cDNA of HER2. The forward primer carried an *EcoRV* restriction site at its 5' end, whereas the reverse primer carried an *SalI* restriction site. The amplified DNA fragment was restricted with *EcoRV* and *SalI* and was directionally cloned into the linearized pV1J vector. Key steps involved in the construction of V932 are described in the text that follows.

Construction of adenoviral shuttle vectors. Shuttle plasmid pNEBA6-CEA-LTB/HER2.ECD.TM was constructed by removing the dicistronic expression cassette from polyMRK-CEA-LTB/HER2ECD.TM SV40 by *SpeI* and *AflII* and inserting it in the same restriction sites of pNEBA6-2HCMVnefMCMVgagpol. The monocistronic shuttle vectors pNEBA6-CEA-LTB and pNEBA6-HER2ECD.TM were constructed by removing the expression cassettes by *SpeI* and

AflII and inserting them in the *Clal* site of the pNEBA6 vector. All the ends were modified by fill-in with T4 DNA polymerase. The genetic structure of pNEBA6-CEA-LTB/HER2ECD.TM was verified by restriction enzyme analysis.

Construction of preadenoviral plasmid. To construct preadenoviral vector pV932, the transgene-containing fragment was liberated from shuttle plasmid pNEBA6-CEA-LTB/HER2ECD.TM by digestion with restriction enzymes *PacI* and *PmeI* and gel purified. The purified transgene fragment was then cotransformed into *E. coli* strain BJ5183 with linearized (*Clal*-digested) adenoviral backbone plasmid pAd6MRK-DE1DE3. Plasmid DNA isolated from BJ5183 transformants was then transformed into competent *E. coli* DH5 α for screening by restriction analysis. The desired plasmid pV932 was verified by restriction enzyme digestion and DNA sequence analysis.

Immunization

Mice were injected with 5–50 μ g of plasmid DNA in a 50- μ l volume in mouse quadriceps muscle followed by electroporation as previously described (Facciabene *et al.*, 2007). Similarly, adenovirus injections (10⁸–10¹⁰ viral particles [VP]/mouse, in a 50- μ l volume) were carried out in quadriceps.

Peptides

Lyophilized CEA and HER2/*neu* peptides were purchased from JPT Peptide Technologies (Berlin, Germany) and resuspended in dimethyl sulfoxide (DMSO) at 40 mg/ml. Pools of peptides consisting of 15 amino acids overlapping by 11 residues were assembled as previously described (Mennuni *et al.*, 2005). Peptides and pools were stored at –80°C.

Antibody detection and titration

Sera for antibody titration were obtained by retro-orbital bleeding. ELISA was performed with human CEA protein as previously described (Mennuni *et al.*, 2005; Cipriani *et al.*, 2008). Anti-TAA serum titers were calculated as the reciprocal limiting dilution of serum producing an absorbance at least 3-fold greater than the absorbance of autologous pre-immune serum at the same dilution.

Cytokine intracellular staining and cytotoxic T lymphocyte assay

The peripheral immune response was measured as previously described (Giannetti *et al.*, 2006). Briefly, peripheral blood mononuclear cells (PBMCs) or scratched splenocytes were treated with ACK lysing buffer (Life Technologies, Carlsbad, CA) for red blood cell lysis and resuspended in 0.6 ml of RPMI-10% fetal calf serum (FCS) and incubated with the indicated pool of peptides (5- μ g/ml final concentration of each peptide) and brefeldin A (1 μ g/ml; BD Biosciences, San Jose, CA) at 37°C for 12–16 hr. Cells were then washed and stained with surface antibodies. After washing, cells were fixed, permeabilized, and incubated with fluorescein isothiocyanate (FITC)-conjugated interferon (IFN)- γ antibodies (BD Biosciences), fixed with formaldehyde (1% in phosphate-buffered saline [PBS]), and analyzed on a FACS-Calibur flow cytometer, using CellQuest software (BD Biosciences). DMSO and staphylococcal enterotoxin B (SEB, cat. no. S-4881; Sigma-Aldrich, St. Louis, MO) at 10 μ g/ml were

used as internal negative and positive controls of the assay, respectively.

Cytolytic activity was assessed by culturing spleen cells isolated from NOD/scid-DR1 mice with the peptides in microtiter wells for 7 days. After washing the cells once, they were incubated with the chromium-51-labeled EBV-transformed cell line JY (HLA-A2⁺), with or without peptides in a 4-hr lytic assay. Cell-free supernatant was collected and the release of chromium by lysed cells was measured with a scintillation counter. Percent lysis was calculated as follows: percent lysis = 100 × (experimental – spontaneous lysis) / (maximum lysis – spontaneous lysis).

Statistical analysis

Log-rank test and a two-tailed Student *t* test were used where indicated. All analyses were performed in JMP version 5.0.1 (SAS Institute, Cary, NC).

Results

Immunogenicity of CEA/rat HER2 vaccine

To verify whether it is possible to simultaneously break tolerance to HER2/*neu* and CEA in an immunologically tolerant mouse model, BALB/NeuT mice (BALB/c background, H-2^d) and CEA.Tg mice (transgenic for human CEA, C57BL/6 background, H-2^b) were crossed and double-transgenic mice from the F₁ progeny (CEA-NeuT) were subjected to repeated immunizations with a mixture of plasmids pV1J/ratHER2-ECD.TM and pV1J/CEA-LTB. CB6 mice (BALB/c × C57BL/6) with the same genetic background were used as the nontolerant control group. Mice were subjected to five weekly injections of 50 μg of each construct; all vaccinations were accompanied by EGT (Fig. 1A). Two weeks after the last DNA-EGT, mice were boosted with the Ad5-ratHER2-ECD.TM and Ad5-CEA-LTB combination,

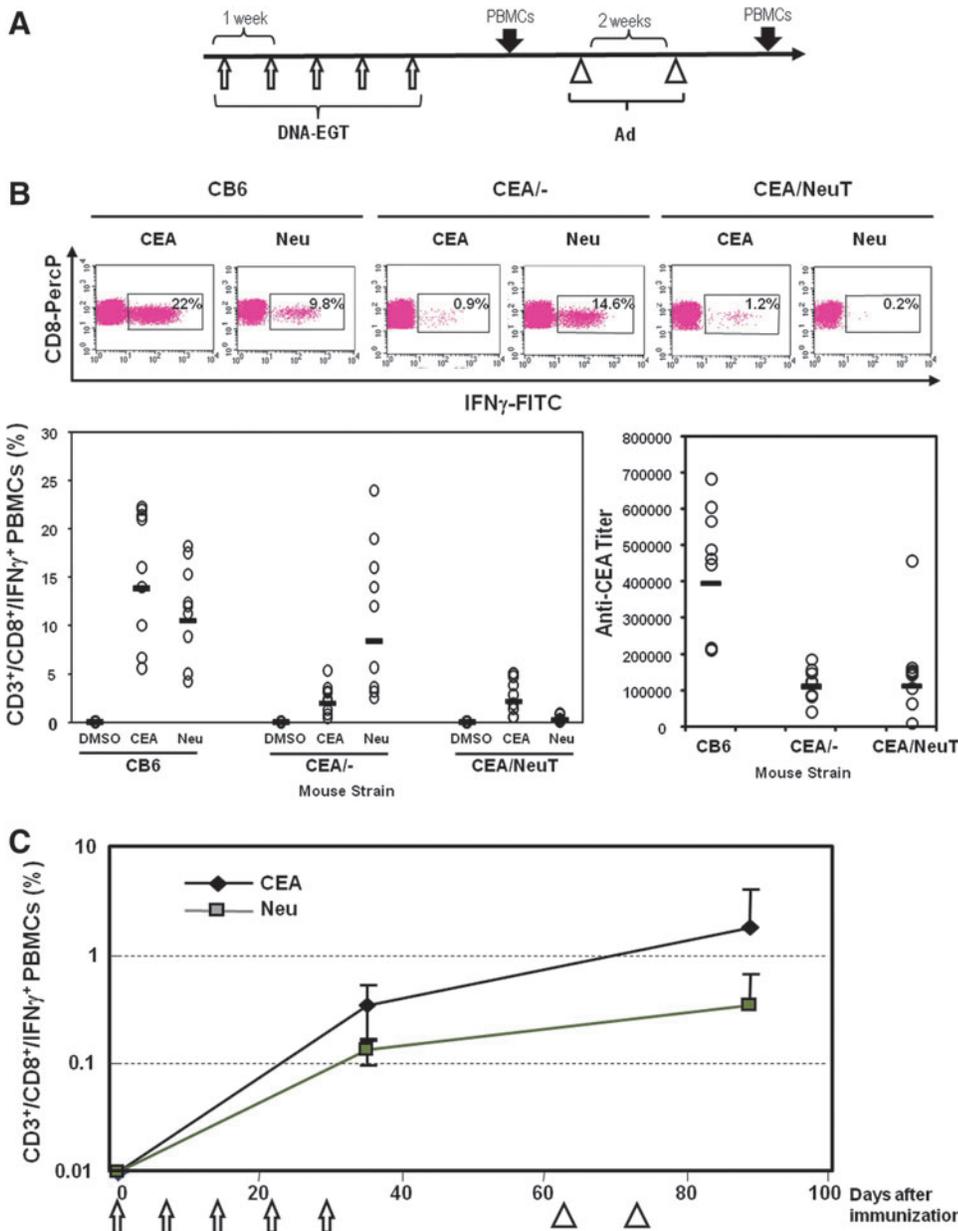


FIG. 1. Electro-gene-transfer of plasmid DNA (DNA-EGT) and adenovirus are immunogenic and can break tolerance simultaneously to human epidermal growth factor receptor-2 (HER2)/*neu* and carcinoembryonic antigen (CEA). (A) Groups of 9 or 10 CB6, CEA^{-/-}, and CEA-NeuT mice were immunized with 5 weekly injections of 50 μg of plasmids pV1J/CEA-LTB and pV1J/ratHER2-ECD.TM (open arrows), followed by 2 administrations of Ad5-ratHER2-ECD.TM and Ad5-CEA-LTB mixture (triangles), 10⁹ VP/each. The CEA and HER2-specific cell-mediated immune (CMI) response was measured by intracellular staining at the indicated time points (PBMCs; solid arrows). (B) A representative dot-plot analysis of CD3⁺/CD8⁺ gated lymphocytes is shown. The areas in rectangles indicate the percentage of CD8⁺/IFN-γ⁺ lymphocytes. Anti-CEA antibodies were measured by ELISA. Bottom left: CD8⁺/IFN-γ⁺ PBMCs. Bottom right: Antibody titer. Each circle represents the value for a single mouse. Each horizontal bar represents the geometric mean of a group. (C) Kinetics of the T cell immune response in CEA-NeuT mice. Each line indicates the group CMI average, and the error bars indicate the standard deviation. The y axis is reported in logarithmic scale. Color images available online at www.liebertpub.com/hum

10^9 VP each. The amplitude of the CEA and rat HER2/*neu*-specific immune responses was analyzed by intracellular staining for IFN- γ . The immune response to CEA was determined with pools of peptides covering amino acids 137–327 and 497–703. The response to rat HER2/*neu* was measured with pool of peptides covering amino acids 1–651. The results in Fig. 1B and C show that the vaccination could induce a strong CMI response to both TAAs in CB6 mice and that immune tolerance significantly affected its amplitude, because the response against CEA and rat HER2/*neu* in CEA-NeuT mice was severely blunted. Similarly, the antibody titer against CEA was significantly lower in CEA-NeuT mice. CEA/- littermates were used as control, showing tolerance only to CEA antigen. As shown in other models, the adenovirus boost induced a steady increase in the immune response ($p=0.002$; Fig. 1C).

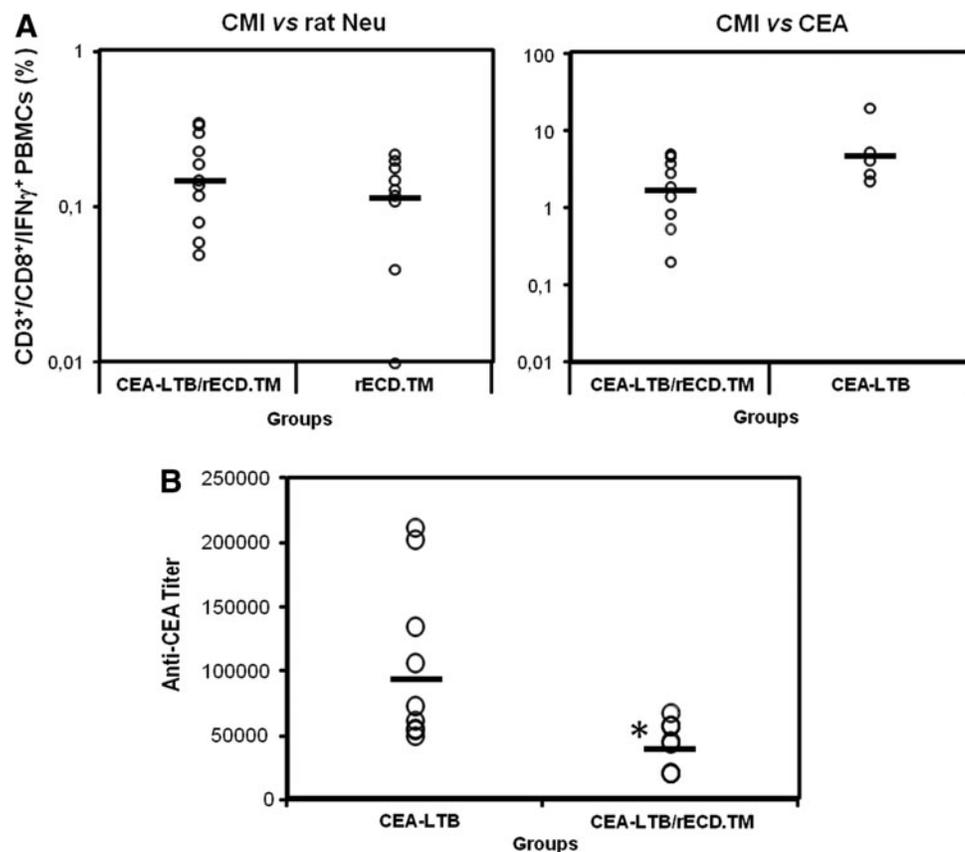
To verify whether the concurrent immunization with two plasmids leads to impaired immune responses because of antigen competition, CEA-NeuT mice were vaccinated with pV1J/ratHER2-ECD.TM, pV1J/CEA-LTB, or a mixture of the two plasmids. CMI responses against rat HER2 and CEA were not significantly affected by coimmunization ($p=0.29$ and $p=0.073$, respectively; Fig. 2A). In contrast, the anti-CEA antibody response in covaccinated mice, although still remaining high (antibody titer, approximately 1:40,000), was about 2-fold lower than on vaccination with pV1J/CEA-LTB only ($p=0.008$; Fig. 2B). These data show that the combined CEA/rat HER2 vaccine is immunogenic and capable of breaking immune tolerance.

CEA/rat HER2 vaccine protects mice from tumor development

BALB/NeuT mice spontaneously develop mammary tumor in all 10 mammary glands (Lucchini *et al.*, 1992). To study the effect of the combination of CEA/rat HER2 vaccine in an immunologically tolerant environment on spontaneous tumor development, groups of CEA-NeuT mice were treated starting at 10 weeks of age. Plasmid DNA vaccine was administered at weeks 10, 11, 12, and 13 by EGT and adenovirus was injected at weeks 15 and 17. By week 22, tumors became palpable in the control group and the development of mammary gland lesions was monitored. The vaccinated group remained tumor free until week 31 (Fig. 3A). The control group developed tumors in at least six mammary glands by week 34. The vaccine conferred a significant antitumor effect, with mice having an average of two palpable lesions at week 38 (Fig. 3B).

Because mammary tumors developing in CEA-NeuT double-transgenic mice do not express CEA (data not shown), to assess the impact of immunity against CEA groups of CEA-NeuT mice were vaccinated as described previously and then challenged with MC38-CEA (H-2^b) colon adenocarcinoma cells. As shown in Fig. 3C, all control mice developed palpable tumors by day 32 after implantation, whereas 40% of vaccinated mice remained tumor free for the entire course of the experiment. In addition, the adenocarcinoma growth rate in vaccinated tumor-bearing mice was noticeably slower than in control mice whereas there

FIG. 2. Coadministration of CEA-LTB and rat HER2/*neu* ECD.TM partially impairs the immune response. Groups of CEA-NeuT mice were vaccinated as indicated in text. After five DNA-EGT, PBMCs and serum were analyzed by intracellular staining for IFN- γ and ELISA, respectively. **(A)** CMI versus rat HER2/*neu* (left) and CEA (right). **(B)** Anti-CEA antibody titer. Each circle represents the value for a single animal; each horizontal bar represents the geometric mean of a group. The asterisk indicates a statistically significant difference ($p<0.05$).



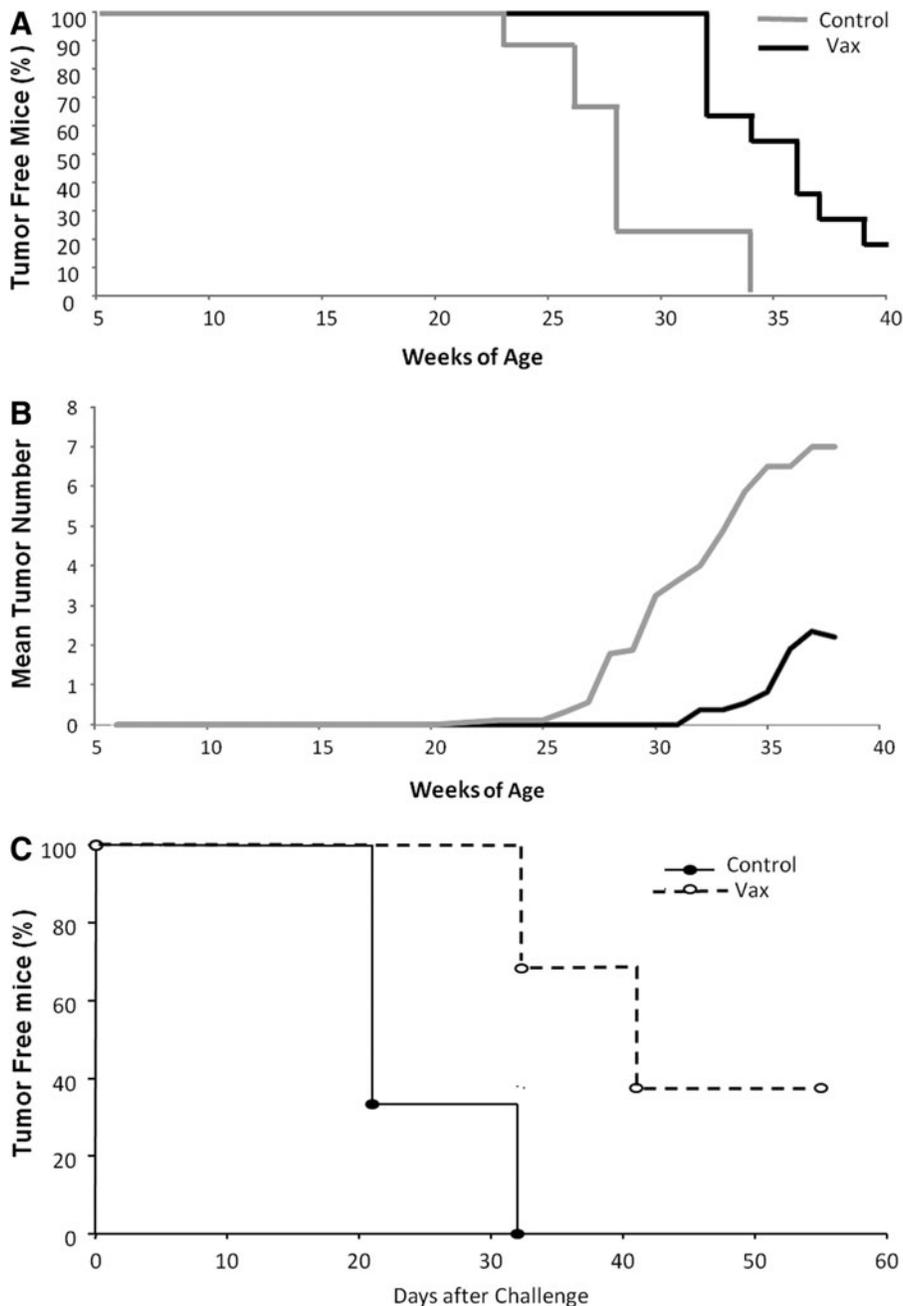


FIG. 3. Antitumor effects of dual-target/DNA-EGT and adenovirus. Groups of 10 CEA-NeuT female mice were vaccinated starting at week 10 of age as described in text and monitored once per week for tumor development. Tumor assessment was performed by palpation of the mammary glands. **(A)** Tumor-free animals. Percentage of mice with no tumors is indicated up to week 38. **(B)** Multiplicity of tumors. Average tumor number per group is shown. **(C)** Groups of CEA-NeuT male mice were vaccinated according to the same scheme described in **(A)** and challenged subcutaneously with MC38-CEA adenocarcinoma cells. Tumor challenge was performed on day 0. The percentage of tumor-free mice is indicated over time. These experiments are representative of three independent vaccinations.

was no therapeutic impact on parental MC38 (CEA-negative) tumors (data not shown). These data indicate that the immune response elicited by CEA-rat HER2 vectors was indeed antigen specific and able to interfere with tumor progression.

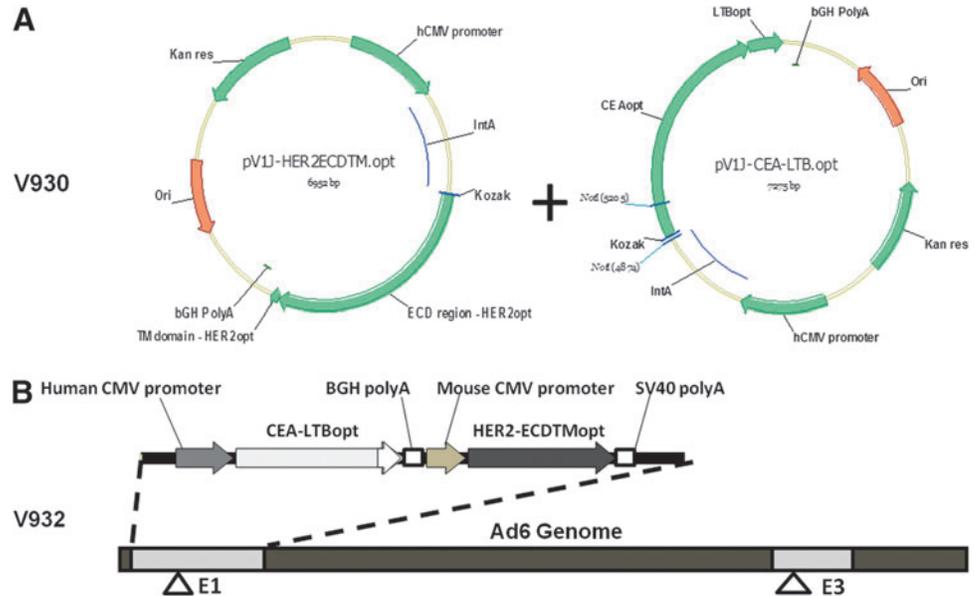
V930 and V932 immunogenicity and antitumor efficacy in wild-type mice

The mixture of plasmids described previously, expressing CEA-LTB and human HER2-ECD.TM, was designed as V930 (Fig. 4A). For clinical translation, the use of two adenoviral vectors can represent an industrial and regulatory challenge. For this reason, we sought to generate a single Ad6-based dicistronic vector (V932; Fig. 4B). This vector contains the CEA-LTB expression cassette consisting of (1) the immediate-

early gene promoter from human cytomegalovirus, (2) the coding sequence of CEA fused at its C terminus with the subunit B of *E. coli* heat-labile enterotoxin, and (3) the bovine growth hormone polyadenylation signal sequence. The CEA-LTB cassette is directly followed by the HER2-ECD.TM expression cassette consisting of (1) the immediate-early gene promoter from mouse cytomegalovirus, (2) the coding sequence of a truncated form of the human HER2/*neu* protein that encompasses the extracellular and transmembrane domain (ECD.TM), and (3) the simian virus 40 early polyadenylation signal sequence. The codon usage in the CEA-LTB and HER2-ECD.TM open reading frames was optimized for expression in human cells (Facciabene *et al.*, 2007).

To verify the ability of V932 to elicit immune responses to both target antigens two studies were carried out. In the first

FIG. 4. (A) V930 DNA plasmids and (B) V932 adenoviral vector encoding human HER2/*neu* and CEA. V930 is a bivalent DNA plasmid vaccine consisting of a plasmid expressing the extracellular domain (ECD) and transmembrane (TM) domains of HER2 and a plasmid expressing CEA fused to the B subunit of *E. coli* LTB. V932 adenovirus encodes human CEA fused to LTB and the truncated version of human HER2 tumor antigen (HER2-ECD.TM). CEA-LTB expression is driven by the human CMV immediate-early (IE) promoter, whereas the mouse CMV IE promoter drives the expression of HER2-ECD.TM. Color images available online at www.liebertpub.com/hum



one, groups of BALB/c and C57BL/6 mice were immunized with different doses of V932 on days 0 and 14. Two weeks later, the immune response to human CEA and human HER2/*neu* was analyzed by IFN- γ enzyme-linked immunospot (ELISPOT) assay. As shown in Fig. 5A, significant immune responses against CEA, LTB, and HER2/*neu* were detected in both mouse strains. In BALB/c mice the immune response was directed mainly against the N-terminal region of CEA and HER2/*neu* as shown by the higher degree of immunoreactivity of the Neu-1 and CEA-1 peptide pools. In contrast, the immune response in C57BL/6 mice was targeted mainly to the C-terminal region of CEA (CEA-2). In C57BL/6 mice both HER2/*neu* peptide pools showed some reactivity, albeit to a lower extent than was observed in BALB/c mice with the Neu-1 peptide pool. LTB reactivity was observed in both mouse strains.

To further characterize the immunogenic potency of V932 and assess whether the two expression cassettes in the same backbone may interfere with each other, groups of C57BL/6 mice were immunized with V932, individual monocistronic vectors (Ad6-hCMV-CEA-LTB or Ad6-mCMV-hHER2-ECD.TM), or their combination. The immune response was determined by intracellular staining for IFN- γ . As shown in Fig. 5B, the mixture of the two adenoviral vectors was equally effective in the induction of a CD8⁺ response. Notably, the amplitude of the response obtained with V932 was even higher, especially against LTB. Similar results were observed for a CD4⁺ response, albeit the responses were not significantly different among the groups (Fig. 5C). Therefore, these data demonstrate that V932 is immunogenic and can elicit a CMI response to both target antigens in different mouse strains.

To verify the antitumor efficacy of the combined DNA-EGT/Ad immunization regimen, 5 μ g of V930 plasmid was injected into C57BL/6 mice. Two weeks later, mice were treated with 10⁸ VP of V932. These doses were selected as previously shown to be efficacious, using the CEA monocistronic vectors in this nontolerant mouse model (data not shown). Two weeks later, mice were challenged with a subcutaneous injection of 10⁶ MC38-hCEA/hHER2 tumor

cells. This syngeneic colon carcinoma cell line was engineered to express both human target antigens. As shown in Fig. 5D, all vaccinated mice were completely protected from tumor progression. Thus, these data demonstrate that the V930/V932 prophylactic vaccination protocol is able to protect mice from tumor growth.

Immunogenicity and antitumor efficacy of V930/V932 in double-transgenic human CEA/HER2 model

To further characterize the immunogenic potency of V930/V932 in a tolerant setting, human HER2 and human CEA double-transgenic (hCEA-HER2) mice were generated (see Materials and Methods) and used as the animal model for an immunization study. A group of hCEA-HER2.Tg mice received four weekly injections of V930 (50 μ g) and 15 days later received two biweekly intramuscular injections of 1 \times 10¹⁰ VP of the V932 vector. Fifteen days later, a subgroup of mice was killed and splenocytes were analyzed by intracellular staining for HER2/*neu* and CEA-specific IFN- γ production. As shown in Fig. 6A, significant immune response was measured against CEA and HER2/*neu* peptide pools, particularly against the C-terminal region of CEA (CEA-2) and HER2-ECD.TM (Neu-2). Response against LTB was similarly high. Two weeks later, mice were challenged with a subcutaneous injection of 10⁶ MC38-hCEA/hHER2 tumor cells as described previously. As shown in Fig. 6B, the control group developed palpable tumors by day 80 post-challenge whereas 50% of V930/V932-vaccinated mice were completely protected ($p < 0.0001$).

These data demonstrate that the V930/V932 regimen is indeed able to break tolerance to both TAAs and provides therapeutic effects against a tumor cell line expressing both antigens.

V932 is immunogenic in NOD/scid-DR1 mice engrafted with the human immune system. Next, we decided to assess whether V932 was able to induce an immune response in mice with a humanized immune system. Groups of NOD/scid-DR1 mice that received intrahepatic injection of

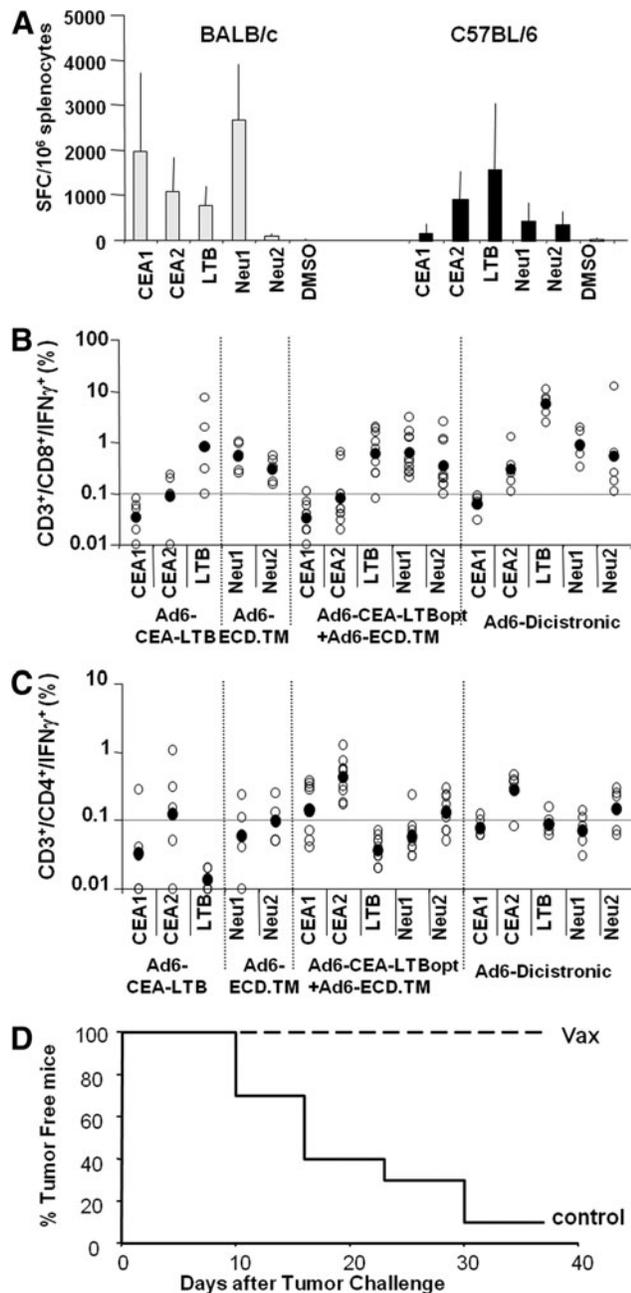


FIG. 5. Immunogenic potency and prophylactic efficacy of Ad6/CEA-LTB-HER2-ECD.TM. **(A)** Groups of BALB/c and C57BL/6 mice were immunized with two injections of Ad6 vector encoding human CEA-LTB and HER2-ECD.TM (10^8 VP). Immune response was measured by IFN- γ ELISPOT assay 2 weeks after the last injection. Each column and error bar indicate the group average and relative standard deviation. **(B)** C57BL/6 mice were vaccinated as described previously. Two weeks later, splenocytes were stimulated with peptide pools and analyzed by intracellular staining for IFN- γ . The percentage of CD8⁺/IFN- γ ⁺ cells is indicated. **(C)** Percentage of CD4⁺/IFN- γ ⁺ splenocytes. The line was arbitrarily set at 0.1% and indicates the threshold for immune responders' identification. **(D)** Groups of 10 C57BL/6 mice were vaccinated with 5 μ g of V930 plasmid and 2 weeks later with 10^8 VP of V932. Two weeks later, mice were challenged with a subcutaneous injection of 10^6 MC38-hCEA/hHER2. Tumor growth was monitored weekly up to day 38. The graph shows the percentage of tumor-free mice.

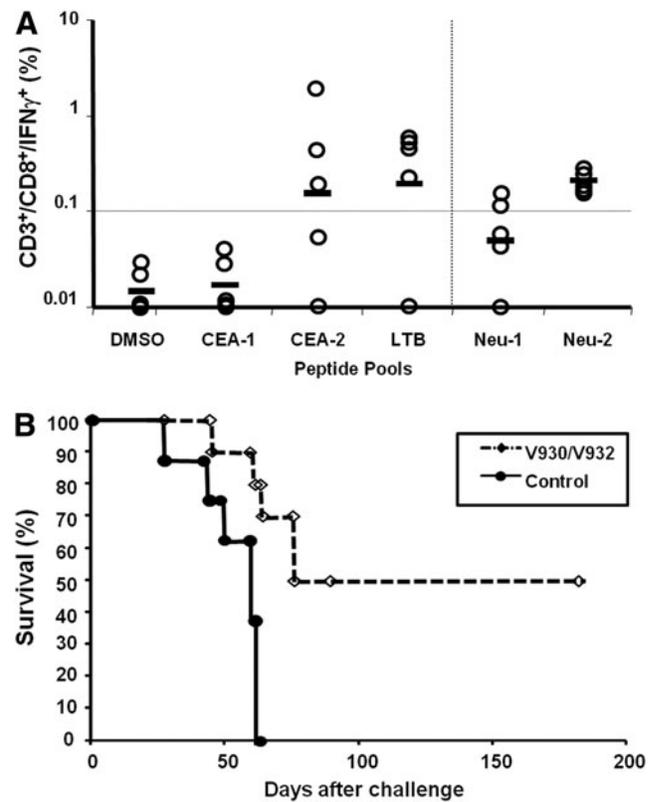


FIG. 6. V930/V932 regimen breaks tolerance to both target antigens and confers significant antitumor effects. **(A)** Groups of six hCEA/HER2 double-transgenic mice were immunized with four weekly injections of V930 followed by two injections of 10^{10} VP of V932. Immune response was measured by IFN- γ intracellular staining on mouse splenocytes 14 days after the last injection. Each circle indicates a single mouse value. Geometric mean group values are also shown (bar). **(B)** Groups of 10 hCEA/HER2.Tg mice were treated as previously described or left untreated and then challenged with a subcutaneous injection of 10^6 MC38-hCEA/hHER2. Tumor growth was monitored weekly up to day 180 postchallenge.

HLA-A*0201⁺ cord blood mononuclear cells (CBMNCs) were immunized intramuscularly with two injections of V932 (10^{10} VP), 2 weeks apart. Fourteen to 21 days postimmunization, mice were killed and splenocytes were prepared for intracellular staining for human IFN- γ and incubated with CEA peptides for a cytotoxicity assay. Figure 7 shows a representative experiment on three mice engrafted with HLA-A*0201⁺ CBMNCs from donors. As previously observed in this chimeric model (Camacho *et al.*, 2007), the engraftment of human T cells was variable (10.3–30.5% CD3⁺) and generally higher for CD4⁺ ($70.2 \pm 9.5\%$) than for CD8⁺ ($20.8 \pm 5.9\%$). As shown in Fig. 7A, vaccinated mice developed a strong and specific T cell response against the TAAs. Both mouse 1 and mouse 2 (same CBMNC donor) developed both a CD8⁺ and a CD4⁺ response against CEA (CEA-1 plus CEA-2 pools) and HER2/neu (Neu-1 plus Neu-2 pools). On the other hand, mouse 3 (different CBMNC donor) mounted mainly a strong CD8⁺-specific response against both antigens.

To determine whether the elicited T cells were endowed with cytotoxic properties, a CTL assay was performed using

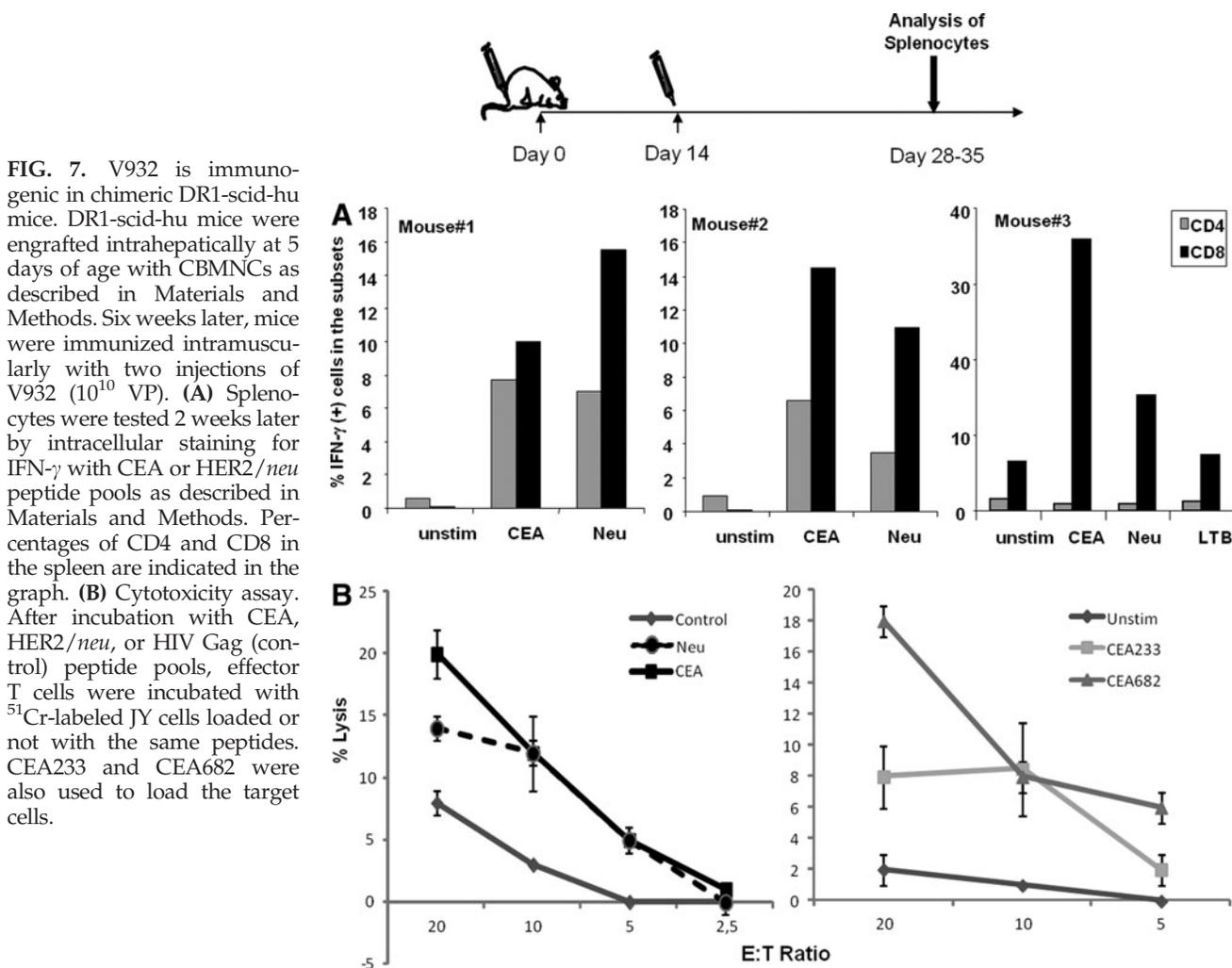


FIG. 7. V932 is immunogenic in chimeric DR1-scid-hu mice. DR1-scid-hu mice were engrafted intrahepatically at 5 days of age with CBMNCs as described in Materials and Methods. Six weeks later, mice were immunized intramuscularly with two injections of V932 (10^{10} VP). **(A)** Splenocytes were tested 2 weeks later by intracellular staining for IFN- γ with CEA or HER2/*neu* peptide pools as described in Materials and Methods. Percentages of CD4 and CD8 in the spleen are indicated in the graph. **(B)** Cytotoxicity assay. After incubation with CEA, HER2/*neu*, or HIV Gag (control) peptide pools, effector T cells were incubated with ^{51}Cr -labeled JY cells loaded or not with the same peptides. CEA233 and CEA682 were also used to load the target cells.

JY cells, an HLA-A*0201⁺ EBV-transformed cell line, as target. Splenocytes were incubated with CEA or HER2/*neu* peptide pools. When loaded with CEA and HER2/*neu* peptides, JY cells were efficiently lysed by effectors. Similarly, HLA-A*0201-selected CEA peptides (Conforti *et al.*, 2009; Fridman *et al.*, 2012) were recognized and significant lytic activity was observed (Fig. 7B).

These data indicate that V932 was able to induce an *in vivo* TAA-specific immune response in the presence of the human immune system.

Discussion

Cancer immune therapy and its translation to the clinic need the development of efficient vaccine technologies and delivery systems. In addition, these novel strategies must be evaluated in appropriate preclinical models. In this study, we have developed a novel heterologous prime-boost genetic vaccination strategy cotargeting two well-known tumor antigens, CEA and HER2/*neu*, and tested its immunogenicity and therapeutic efficacy in immune-tolerant mice.

CEA.Tg mice express CEA with a tissue distribution similar to that of humans (Eades-Perner *et al.*, 1994) and provide a particularly relevant model to critically verify the potential efficacy of a human CEA-based vaccine (Hance

et al., 2005). Similarly, BALB/NeuT mice allow assessment of active cancer immunotherapy against HER2/*neu*. In this model, overexpression of the activated (V664E) form of the rat HER2/*neu* oncogene, under the control of the mouse mammary tumor virus promoter, leads to progressive development of invasive mammary adenocarcinoma in all 10 mammary glands (Lucchini *et al.*, 1992). Both models have been extremely useful for evaluating vaccine therapies targeting individually HER2/*neu* or CEA, respectively. Induction of immune responses to self-antigens can be monitored in these mice, along with overt toxicities connected with them. Furthermore, vaccine efficacy on spontaneous tumor development can be examined in BALB/NeuT mice.

To determine the efficacy of plasmid DNA and/or adenovirus vaccination against both CEA and HER2, we generated double-transgenic mice through crossing of BALB/NeuT and CEA.Tg mice. These mice are immunologically tolerant to both target antigens and suitable for antitumor studies. A heterologous prime-boost DNA-EGT/Ad regimen with a mixture of vectors encoding CEA-LTB and rat HER2 was able to break immune tolerance (Figs. 1 and 2). Both a cell-mediated immune response and high antibody titers were detected. Most importantly, the vaccine was able to confer a therapeutic effect in spontaneous rat HER2⁺ addicted mammary tumors (Fig. 3A and B) and CEA⁺ colon

tumors implanted subcutaneously (Fig. 3C). These data prompted us to generate vectors able to elicit an immune response against the human antigens. In particular, DNA and adenoviral vectors encoding human HER2 ECD.TM were generated and a dicistronic Ad6 vector was designed to minimize the amount of injected viral particles and simplify the scale-up procedure (Fig. 4). The Ad6 serotype was chosen because it belongs to the subgroup C human adenoviruses; like the more common serotype Ad5, Ad6 has the same elevated degree of immunogenicity, but its seroprevalence in the human population is significantly lower (Colloca *et al.*, 2012). V932 was able to induce a strong T cell immune response against both TAAs in wild-type mice, and its immunogenicity was comparable to that elicited by the two monocistronic vectors (Fig. 5A–C). In this setting, V932 was able to confer complete tumor protection (Fig. 5D). To assess the immunogenic potency of V930/V932 in a tolerant environment, human HER2 and human CEA double-transgenic mice were generated and used as the animal model for an immunization study. These mice are derived from CEA.Tg mice crossed with a human HER2 transgenic line. The latter mice carry the full-length wild-type cDNA of human HER2 under the control of the whey acidic protein (WAP) promoter. HER2/*neu* protein expression is detected in the secretory mammary epithelia during pregnancy and lactation and expressed constitutively in the Bergman glial cells within the molecular layer of the cerebellum. No neoplastic transformation is detected in any tissue (Piechocki *et al.*, 2003), and thus this model needs tumor implantation for therapeutic assessment. Again, a significant immune response and antitumoral effects were observed in this model (Fig. 6). Finally, the immunogenicity of V932 was also evaluated in an NOD/scid-DR1 model engrafted with the human immune system. This model, albeit complex, provides an excellent tool with which to evaluate vaccine potency and characterize immunogenic epitopes. The strong T cell immune response and the cytotoxic activity indicated that V932 was strongly immunogenic in this surrogate model (Fig. 7). Unfortunately, we were not able to assess the potency of V930. It would be of interest to test the heterologous prime-boost strategy as well as the antitumoral effects by using HLA-A*0201⁺, CEA⁺, and/or HER2/*neu*⁺ tumor xenografts in this mouse model.

The findings of our study provide the basis for evaluation of the DNA-EGT/Ad combination in human clinical trials. We have reported the results of two phase 1 trials aimed at evaluating the safety/tolerability and immunogenicity of V930 with EGT alone and followed by V932 (Diaz *et al.*, 2013). Importantly, the immunization regimens were safe and well tolerated. However, only the immune response to the bacterial portion of the vector (LTB) was detected and none of the vaccinated patients had detectable cell-mediated responses to either CEA or HER2/*neu*. It must be pointed out that a rather heterogeneous population of patients at different clinical stages and with different cancer diagnoses was enrolled in this trial. Such heterogeneity likely prevented any meaningful conclusions. Therefore, this points to the consideration that encouraging results from preclinical testing can be dampened by the overwhelming difficulty of clinical testing. Because V930/V932 was indeed safe and immunogenic (at least against LTB), it would be of great interest to evaluate the vaccine in patients with cancer at the same

clinical stage and tumor type who have completed curative intent therapy and have a high risk of recurrent disease.

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Author Disclosure Statement

No competing financial interests exist.

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