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Optimized DNA Vaccines to Specifically Induce Therapeutic CD8 T Cell Responses Against Autochthonous Breast Tumors

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Abstract

Vaccines capable of inducing CD8 T cell responses to antigens expressed by tumor cells are considered as attractive choices for the treatment and prevention of malignant diseases. Our group has previously reported that immunization with synthetic peptide corresponding to a CD8 T cell epitope derived from the rat *neu* oncogene administered together with a Toll-like receptor agonist as adjuvant, induced immune responses that translated into prophylactic and therapeutic benefit against autochthonous tumors in an animal model of breast cancer (BALB-*neuT* mice). DNA-based vaccines offer some advantages over peptide vaccines, such as the possibility of including multiple CD8 T cell epitopes in a single construct. Thus, we have evaluated the use of DNA vaccination for its ability to generate effective CD8 T cell responses against breast tumors expressing the rat *neu* oncogene. The results show that as with peptide vaccination, DNA-based vaccines were very effective in stimulating tumor-reactive CD8 T cell responses. Moreover, vaccination with modified DNA plasmids resulted in significant anti-tumor effects that were mediated by CD8 T cells without the requirement of generating antibodies to the product of rat *neu*. These results bear importance for the design of DNA vaccines for the treatment and prevention of cancer.

Keywords

DNA Vaccines; HER2/*neu*; CD8 T cells; Breast Cancer; Animal Models

Introduction

Because CD8 T lymphocytes have the capacity to recognize and eliminate tumor cells, numerous groups of investigators including ours are actively pursuing the development of vaccines capable of inducing tumor-reactive CD8 T cells [1]. The identification of MHC class I-binding peptides recognized by tumor-reactive CD8 T cells has lead to the design of epitope-based vaccines that seek to activate and expand tumor-reactive CD8 T cell responses with the goal of generating therapeutic anti-tumor effects. Initial attempts to induce effective anti-tumor immune responses in human cancer patients have been rather disappointing indicating that these vaccination strategies still require considerable optimization. Animal tumor models (mostly in mice) have been used to determine some of the critical parameters of vaccine composition (antigen type, adjuvants) and administration (route, frequency) that will play a part in generating therapeutic effects against established tumors. Unfortunately, most of these

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tumor models rely in the use of transplantable tumors that do not mimic the naturally occurring malignancies in human patients. The recent development of transgenic mouse models that target the expression of oncogenes to specific tissues, resulting in the formation of autochthonous breast tumors [2,3], has provided a more realistic alternative to evaluate vaccination strategies as compared to the use of transplantable tumors before going into the clinic.

Transgenic BALB-neuT female mice express the activated form of the rat *neu* oncogene (rNEU) at puberty in their mammary glands, leading to the formation of multi-focal adenocarcinomas [3]. By week 9 of age BALB-neuT mice already exhibit mammary hyperplasia that by week 25 has progressed to measurable, invasive tumors in all 10 mammary glands. Vaccination of young BALB-neuT mice using plasmid DNA encoding rNEU resulted in protection/delay of tumors mostly through the generation of antibody responses against the product of this oncogene [4-6]. In order to study the potential role of CD8 T cells in generating anti-tumor effects in BALB-neuT mice, our group recently evaluated the use of vaccines composed of sythetic peptides representing CD8 T cell epitopes derived from rNEU. Our results demonstrated that vaccines containing peptide p66 (TYVPANASL) administered together with a Toll-like receptor (TLR)-9 agonist (CpG) generated CD8 T cell responses capable of recognizing tumor cells expressing ratNEU [7]. More significantly, the synthetic peptide-based vaccine exhibited significant anti-tumor effects against both a transplantable tumor originated in BALB-neuT mice (TUBO) and towards spontaneously arising breast tumors in BALB-neuT mice. These results suggested that anti-tumor effects generated by therapeutic vaccination could be achieved solely through CD8 T cells in the absence of anti-rNEU antibodies. Because plasmid DNA-based vaccines represent an attractive alternative to vaccines prepared with synthetic peptide for inducing CD8 T cell responses, we have evaluated the effects of a DNA vaccine modified to stimulate tumor-reactive CD8 T cells without inducing the production of anti-rNEU antibodies. The results presented herein demonstrate that DNA vaccination was effective in eliciting strong antigen-specific CD8 T cell responses to the p66 rNEU epitope resulting in generating significant anti-tumor therapeutic responses in the absence of antibody responses. These findings could be of relevance for the design of DNA vaccination strategies that focus immune responses towards the generation of cell-based therapies.

Materials and methods

Mice

Female, 6 to 8-week-old BALB/c mice (*H-2^d*) were obtained through the National Cancer Institute/Charles River program. Mice were allowed to acclimate to our animal facility for 1 week before beginning experiments. BALB-neuT mice (*H-2^d*), which were generated as described [3], were bred in our facility. Heterozygous 6- to 15-week-old virgin females expressing rNEU as verified by PCR were used throughout this work.

Cell lines

P815 mastocytoma and A20 B cell lymphoma cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA). The TUBO (Turin-Bologna) tumor is a cloned cell line established *in vitro* from a lobular carcinoma that arose spontaneously in a BALB-neuT mouse [4]. The rNEU-transfected mouse mammary breast cancer A2L2 [8] and its parental 66.3 cell line [9] were provided by Drs. J.E. Price and L. Lachman (M.D. Anderson Cancer Center, Houston, TX). The rNEU-expressing mouse fibroblast 3T3-NKB [10] and its parental 3T3 cell line were provided by Dr. W.Z. Wei (Karmanos Cancer Institute, Detroit, MI).

Peptide, antibodies, and tetramer

The synthetic peptides, p66 (TYVPANASL) defined as one of the dominant epitopes of rNEU in previous study [7], and T helper epitope derived from ovalbumin (Ova₃₂₃₋₃₃₉: ISQAVHAAHAEINEAGR, ref. [11]) were purchased from A&A Labs (San Diego, CA). The purity (>95%) and identity of peptides were determined by analytic high-performance liquid chromatography and mass spectrometry analysis. mAbs used for *in vivo* cell depletion (anti-CD4, clone GK1.5; and anti-CD8, clone 2.43) were prepared from hybridoma supernatants (cells obtained from ATTC). Phycoerythrin (PE)-conjugated-H-2K^d/rNEUp66 tetramer was produced and kindly provided by the National Institute of Allergy and Infectious Disease (NIAID) Tetramer Facility at the Emory University Vaccine Center (Atlanta, GA).

Plasmid DNA and vaccination protocol

pcDNA3 vector, pEC-TM^{neu} encoding extracellular domain and transmembrane domains of rNEU was provided by Dr G. Forni (University of Torino, Torino, Italy). A plasmid encoding the amino terminal end of rNEU, residues 1-170 (pEC₁₋₁₇₀^{neu}) and a similar construct containing the Ova₃₂₃₋₃₃₉ T helper epitope proximal to p66 (pEC₁₋₁₇₀^{neu/Ova}), were constructed by PCR-based cloning. A plasmid encoding for the complete chicken ovalbumin antigen designated as pOva was used as negative control in some experiments. All constructs, which were in a pcDNA3 backbone were confirmed by DNA sequencing and purified in an endotoxin-free formulation for vaccination using an EndoFree Plasmid Mega Kit (Qiagen, Valencia, CA). Mice (BALB/c or BALB-neuT) were immunized with a total of 100 µg of plasmid DNA resuspended in normal saline and injected into two sites in the quadriceps femoris muscles. Immunization was followed immediately by electroporation of the injected area (95 V, 4 pulses of 65 ms with re-poling) using an Electro Square Porator device (BTX, model TX830; San Diego, CA). After 3 weeks, mice were given in identical booster immunization together with electroporation. Immune responses were typically measured (as described below), either 12 days after primary immunizations or 8 days after the booster vaccination.

Measurement of cellular immune responses

For assessing antigen-specific T cell responses, peripheral blood was taken from the submandibular vein and the RBCs were lysed by brief treatment with ammonium chloride buffer followed by staining with peptide/MHC tetramer. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated MHC class II, PerCP Cy5.5-conjugated CD8a (eBioscience; San Diego, CA), and PE-conjugated H-2K^d/rNEUp66 tetramer for 30 min. After washing with three times with 1% BSA/PBS solution, the fluorescence intensity was evaluated using FACSCalibur (BD Biosciences) flowcytometer and analyzed using FlowJo software. For detection of CD8 T cells secreting IFN-γ, enzyme-linked immunosorbent spot (EliSpot) assays were performed as described previously [7] using CD8 T cells from spleens that were purified by positive selection using antibody-coated magnetic beads following the directions provided by the vendor (Miltenyi Biotec; Auburn, CA). Responder (CD8 purified) cells were incubated at 3×10^5 , 1×10^5 and 3×10^4 per well together with 5×10^4 stimulator cells (P815, plus/minus peptide, A2L2, 66.3 or A20). Cultures were incubated at 37 °C for 20 h and spots (IFN-γ producing cells) were developed as described by the EliSpot kit manufacturer (Mabtech, Inc., Mariemont, OH). Spot counting was done with an AID EliSpot Reader System (Autoimmun Diagnostika GmbH, Strassberg, Germany).

Assessment of anti-rNEU antibody

Eight days after the booster immunization, the production of anti-rNEU antibody was measured as previous described [12,13]. Briefly, sera collected from vaccinated BALB/c and BALB-neuT mice was diluted 1:10 in 1%BSA/PBS and used to stain 5×10^5 3T3-rNEU⁺ (3T3-NKB) or 3T3-rNEU⁻ cells for 20 min. After extensive washing, the cells were incubated for 20 min

with FITC-conjugated goat anti-mouse Ig (IgG+IgM) antibodies (KPL, Gaithersburg, MD) and resuspended in 1% BSA/PBS containing 1 mg/ml propidium iodide. The fluorescence intensity was evaluated in live cells using FACSCalibur flowcytometer and analyzed using FlowJo software. Normal mouse serum was used as the negative control and anti-c-ErbB2/*neu* (Ab4) mouse monoclonal antibody (Calbiochem; San Diego, CA) as positive control.

Assessment of anti-tumor effects of vaccination (therapeutic mode using TUBO)

Mice were injected s.c. with 3×10^5 TUBO cells. When all of mice had an established measurable tumor (3-5 mm diameter @ ~ 10 days after tumor injection), the animals were randomized, divided two groups and plasmid DNA vaccination was initiated. To assess the tumor-specific memory responses, the vaccinated mice that rejected the established tumors were rechallenged with 1×10^6 TUBO cells on the opposite flank ~3 weeks after the initial tumor regression.

Assessment of anti-tumor effects of vaccination (prevention of autochthonous tumors)

Virgin female BALB-*neuT* mice were selected by age to perform a plasmid DNA vaccination strategy at different time points. One group was selected at 10 weeks of age to receive DNA vaccination followed by identical boosting on weeks 13 of age. The second group was selected at week 7 of age to start DNA immunization after treatment with anti-CD4 or anti-CD8 monoclonal antibodies (mAb) followed by identical boosting on weeks 10, 13, 19 and 23 of age. Measurable/palpable masses >2 mm in diameter were regarded as tumors. In all cases, when mice had tumors >20 mm in the greatest dimension, or when skin ulceration occurred, mice were sacrificed by CO₂ inhalation according to our Institutional Animal Care and Use Committee guidelines. For the *in vivo* cell depletion experiments, anti-CD4 mAb (0.2 mg per mouse) or anti-CD8 mAb (0.5 mg per mouse) were injected intraperitoneally on days -2 and -1 before receiving plasmid DNA injection, and followed by injections twice per week throughout the duration of the experiments. More than 95% cell depletion for CD4 and CD8 cells was confirmed by flowcytometry analysis with no significant depletion of other cell populations (data not shown).

Results

Design of a cDNA construct to specifically induce CD8 T cell responses to rNEU

The goal of the present studies was to evaluate the effectiveness of an rNEU-based DNA vaccine in inducing CD8 T cell responses without generating antibodies to rNEU that could have an anti-tumor effect. This would allow us to determine specifically the therapeutic effectiveness of CD8 T cells generated by DNA vaccination against tumors expressing rNEU. Vaccination with cDNA of rNEU containing the complete extracellular and transmembrane domains (EC-TM^{neu}) has been reported to elicit both CD8 T cell responses to p66 epitope as well as antibodies to rNEU [5]. In our hands, we observed that vaccination in BALB/c mice followed by a booster immunization (administered 3 weeks after the prime) with EC-TM^{neu} (100 µg plasmid) generated a strong CD8 T cell response as measured by the % CD8 T cells reactive with a H-2K^d/rNEUp66 tetramer (Fig. 1A). However, as expected these mice also produced antibodies that reacted against cell surface rNEU (Fig. 1B). In view of these results we cloned a fragment of EC-TM^{neu} containing residues 1 to 170 (EC₁₋₁₇₀^{neu}), with the expectation the corresponding plasmid would remain immunogenic for CD8 T cells but would not generate anti-rNEU antibodies. In addition, we also generated a plasmid containing residues 1-170 or rNEU, but with the addition a strong T helper epitope derived from ovalbumin (Ova₃₂₃₋₃₃₉), which was inserted proximal to the p66 epitope (EC₁₋₁₇₀^{neu/Ova}). We hypothesized that the inclusion of a strong T helper epitope in the vaccine would increase the magnitude of the CD8 response to rNEU. The results presented in Figure 2A show that BALB/c mice immunized (prime-boost, 3 weeks apart) with EC₁₋₁₇₀^{neu} or EC₁₋₁₇₀^{neu/Ova} induced

strong p66-specific CD8 T cell responses (of similar magnitude as those obtained with EC-TM^{neu}, shown in Fig. 1A). Most significantly, as shown in Figure 2B (upper panel) the resulting CD8 T cells were quite effective in recognizing rNEUp66 pulsed target cells and breast tumor cells expressing rNEU (A2L2) but not rNEU-negative control breast tumor cells (66.3). Nevertheless, no significant increase of the responses was observed by the inclusion of the Ova T helper epitope in the cDNA construct, even though the EC₁₋₁₇₀^{neu/Ova} plasmid generated a strong CD4 T cell response to Ova₃₂₃₋₃₃₉ (Fig. 2B, lower panel). Lastly as anticipated, no significant antibody responses to rNEU were detected in the sera of mice vaccinated with either EC₁₋₁₇₀^{neu} or EC₁₋₁₇₀^{neu/Ova} (examples shown in Fig. 2C).

Next, we proceeded to assess the capacity of plasmids EC₁₋₁₇₀^{neu} or EC₁₋₁₇₀^{neu/Ova} to induce CD8 T cell responses in BALB-neuT mice, which exhibit a high degree of immune tolerance to rNEU [7]. The data presented in Figure 3A shows that antigen specific (tetramer positive) CD8 T cell responses were elicited by the DNA vaccines in these mice, although the intensity of the responses was significantly lower (~80 %) as compared to the wild type BALB/c mice. Nevertheless, the CD8 T cells induced by DNA vaccination were quite effective in recognizing peptide-pulsed targets and rNEU-expressing breast tumor cells as determined by EliSpot assays (Fig. 3B). As with BALB/c mice, the BALB-neuT mice vaccinated with the EC₁₋₁₇₀^{neu} or EC₁₋₁₇₀^{neu/Ova} plasmids did not produce antibodies capable of binding to cell surface expressed rNEU (data not presented). The results so far indicate that plasmids EC₁₋₁₇₀^{neu} and EC₁₋₁₇₀^{neu/Ova} are appropriate candidates of DNA vaccines to specifically evaluate anti-tumor effects of CD8 T cells against tumors expressing rNEU in the absence of antibodies.

Therapeutic DNA vaccination against a transplantable breast tumor (TUBO)

We first examined whether the CD8 T cell responses induced by plasmid DNA vaccination without the production of antibodies would offer a therapeutic benefit against an established rNEU-expressing tumor. In the first experiment BALB/c mice received a single DNA vaccination when subcutaneous TUBO tumors measured 3-5 mm in diameter. The results (Fig. 4A) show that although the tumors continued to grow for ~10 days after the vaccine was administered, 80% (4/5) of the vaccinated mice were able to reject the TUBO tumors. In addition, tumor growth rate of the mouse that failed to reject the tumor was substantially reduced as compared to the control (non-immunized) mice. When the experiment was repeated in mice bearing slightly larger tumors (5-7 mm diameter), we observed that 57% (4/7) mice were able to reject the tumors and that the tumor growth in the non-responder mice was delayed as compared to the controls (Fig. 4B). In both experiments, the vaccinated mice that rejected the established TUBO tumors were re-challenged at the termination of the experiment (day 60) with a second dose of live TUBO tumor cells and in all cases (with the exception of naïve control mice) the tumors failed to grow (data not shown), indicating the presence of an effective CD8 T cell memory response. Thus, these results demonstrate that plasmid DNA vaccination that induces strong CD8 T cell responses but no antibody response to rNEU is effective in curtailing established (transplantable) breast tumors.

Evaluation of DNA Vaccination Against Autochthonous Tumors in BALB-neuT Mice

We proceeded to determine whether DNA vaccination using the plasmid constructs that specifically induce CD8 T cell responses would have a therapeutic effect against autochthonous tumors arising in BALB-neuT mice. The results shown in Figure 5A demonstrate that mice immunized with either plasmids EC₁₋₁₇₀^{neu} or EC₁₋₁₇₀^{neu/Ova} received significant therapeutic benefit against developing mammary tumors. Not only the number of tumors per mouse (tumor multiplicity) in the treatment groups decreased as compared to the controls that were vaccinated with an irrelevant plasmid (pOva), but several of mice became protected indefinitely. Specifically, 30% (4/13) of mice that received 2 immunization of the EC₁₋₁₇₀^{neu} plasmid and 27% (4/15) vaccinated with EC₁₋₁₇₀^{neu/Ova} did not develop any tumors up to 58 weeks of age

(data not presented). In addition, the overall survival (time to euthanasia) of mice vaccinated with EC₁₋₁₇₀^{neu} or EC₁₋₁₇₀^{neu/Ova} that developed tumors was approximately 10 weeks longer as compared to the controls that received the irrelevant vaccine (Fig. 5B) indicating that tumors in the vaccinated mice grew at a slower rate than in the controls. Some of the mice in this experiment were monitored for the production of antigen-specific (tetramer positive) CD8 T cells in blood after each vaccination. The results shown in Figure 5C indicate that the mice immunized with either EC₁₋₁₇₀^{neu} or EC₁₋₁₇₀^{neu/Ova} had approximately 3% H-2K^d/rNEUp66 tetramer positive cells in peripheral blood, while mice immunized the control plasmid (pOva) did not have any measurable tetramer positive cells. However, there was no correlation between the levels of tetramer positive cells with survival and time to tumor development.

In order to validate the role of CD8 T cells in the effectiveness of DNA vaccination using the modified plasmids, we carried a similar experiment as the one described in Figure 5 in BALB-neuT mice that were depleted of CD8 T cells or CD4 T cells before vaccination and throughout the entire experiment by repeated administration with monoclonal antibodies. In addition, in this experiment mice received a total of 5 vaccinations in order to determine if the effectiveness of the vaccine against autochthonous tumors could be increased. The results presented in Figure 6 show that 75% (3/4) of the BALB-neuT non-depleted mice remained tumor-free up to 35 weeks of age. In addition, the only mouse in this group that was not tumor free developed one single slow-growing tumor (Fig. 6A, left panel). In contrast, all mice in the CD8-depleted group developed multiple (4-7) fast growing tumors (Fig. 6A, central panel) and by the age of 35 weeks had to be euthanatized because of exceeding the maximal tumor size (>2 cm diameter). Depletion of CD4 T cells resulted in 50% (2/4 mice) overall tumor protection (Fig. 6A, right panel). However, as with the non-depleted group, mice with tumors in the CD4 T cell-depleted group had slow growing masses indicating a therapeutic benefit of the vaccine even in the absence of CD4 T cells. These results indicate that CD8 T cells are mostly responsible for the anti-tumor effectiveness of DNA vaccination in a system that utilizes reengineered plasmids designed not to induce antibody immunity.

Discussion

We have utilized the BALB-neuT mouse breast tumor model to assess the effectiveness of 2 types of CD8-inducing vaccines against autochthonous breast tumors. We previously reported that synthetic peptide-based vaccines administered with repeated injections of a TLR-L (CpG) was effective in generating CD8 T cell responses associated with anti-tumor effects against transplantable tumors (TUBO) and autochthonous tumors that develop in BALB-neuT mice [7]. Those studies revealed that in order generate anti-tumor CD8 T cell responses in BALB-neuT mice, immune suppression mediated by the presence of CD4/CD25 double positive T regulatory (Treg) cells had to be overcome by either anti-CD25 antibody treatment or repeated immunizations. The studies presented here were carried out with the purpose of evaluating DNA plasmid vaccines for their capacity to induce tumor-reactive CD8 T cell responses that translate into anti-tumor therapeutic benefit. Although DNA vaccination was previously studied in this tumor model, the plasmid utilized (EC-TM^{neu}) induced both T cell and antibody responses [5]. However, those studies indicated that the therapeutic effect of DNA vaccination with EC-TM^{neu} in BALB-neuT mice depended in great part in the production of antibody responses to rNEU. Studies using a recombinant adenovirus expressing EC-TM^{neu} also showed therapeutic benefits against rNEU-expressing tumors that were mediated mostly by anti rNEU antibodies [14]. Our results demonstrate that in circumstances where the plasmid DNA is modified to avoid the induction of antibodies (at least to the native protein), CD8 T cell responses that lead to a significant therapeutic benefit in BALB-neuT mice can be generated. Although vaccination with the modified plasmids EC₁₋₁₇₀^{neu} and EC₁₋₁₇₀^{neu/Ova} did not induce measurable antibody responses to rNEU in both BALB/c and BALB-neuT mice immediately after immunization (Fig. 2A and data not shown) the possibility existed that these antibody

responses could arise after a longer time period, specially in those BALB-neuT mice that became protected and did not develop tumors. Nevertheless, when we tested the sera of those mice that received multiple immunizations and had not developed tumors (experiment described in Fig. 6) long after they had been vaccinated, we could not detect the presence of anti-rNEU antibodies (data not shown). Thus, our results and those utilizing EC-TM^{neu} appear to differ with regards to the necessity of generating anti-rNEU antibodies to attain therapeutic benefit in BALB-neuT mice. The most likely explanation for this disparity may be due to the use of different plasmid constructs. On one hand, our modified plasmids EC₁₋₁₇₀^{neu} and EC₁₋₁₇₀^{neu/Ova} represent only a fragment of the EC-TM^{neu} sequence, so in addition to not inducing antibodies to the native rNEU protein, these plasmids encoding a portion of rNEU could be focusing the immune response towards the generation of more effective CD8 T cell responses as compared to the EC-TM^{neu} plasmid. At the present time we do not know the exact mechanism by which plasmids EC₁₋₁₇₀^{neu} or EC₁₋₁₇₀^{neu/Ova} do not induce antibody responses to the native rNEU protein. The most likely explanation is that these fragments do not fold correctly and may not be secreted by the transfected muscle cells after vaccination. However, these possibilities remain to be shown. It is clear that immunization strategies that are intended to generate CD8 T cell responses have the option of removing large portions of the cDNA not coding for the peptide epitope, which would not only help focus the immune response to desired epitopes, but could also remove potentially immune suppressive sequences of the tumor antigen, for example MHC class II binding peptide sequences that could stimulate inhibitory CD4 T cells such as Th2 and Treg lymphocytes.

It was important to note that CD8 T cell responses induced by DNA vaccination when measured by tetramer analysis were significantly lower in BALB-neuT mice as compared to those obtained in BALB/c mice (3% versus 15%). On the other hand, the differences were not as dramatic when the CD8 T cell responses were measured by EliSpot assays (compare Figs. 2B and 3B). Thus, it appears that the vaccine may be inducing different subsets of CD8 T cells in both strains of mice and that a large proportion of CD8 T cells in BALB-neuT mice that are capable of recognizing antigen and producing cytokine may not bind tetramer well. This possibility is reinforced by the recent report of the remarkable differences existing in the T cell repertoires between BALB/c and BALB-neuT mice [15]. Our results show that CD8 T cells are responsible for the therapeutic benefit observed by fragment vaccination (Fig. 6). It is clear that the effector mechanisms of CD8 T cells against tumors are not limited to cell-mediated cytotoxicity, but other activities such as secretion of high levels of cytokines may be involved in limiting tumor cell growth [16].

Our work in the BALB-neuT autochthonous breast cancer model demonstrates that both the synthetic peptide and DNA epitope based vaccines are effective in generating CD8 T cell responses that translate into therapeutic benefit. Being cognizant that any kind of epitope based vaccination strategy will ultimately require the inclusion of multiple epitopes, restricted to most common MHC-I alleles and derived from several tumor antigens in order to offer broad population coverage, we believe that developing DNA vaccines could be more advantageous than using peptide cocktails since a single plasmid could be designed to contain large numbers of epitopes by fusing minigenes to generate “a string of beads” type of vaccine [17].

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Abbreviations

rNEU

rat *neu* oncogene

TLR

Toll-like receptor

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Fig. 1.

Immunization of BALB/c mice with plasmid, pEC-TM^{neu} encoding the extracellular and transmembrane domains of rNEU elicits both CD8⁺ T cell and antibody responses to rNEU. **A**, Mice (n=3 per group) were vaccinated on days 0 and 21 as described in “Materials and methods” with pEC-TM^{neu} followed by electroporation. On days 12 and 29 peripheral blood lymphocyte samples were evaluated for the presence of antigen-specific CD8 T cells. Each data point represents the % of p66-specific cells of CD8 T cells for each individual mouse and horizontal line represents the average value of the group. **B**, The production of anti-rNEU antibodies by the vaccinated mice was evaluated by flowcytometry as described in “Materials and methods”. Eight days after second immunization, serum samples (diluted 1:10) were incubated with rNEU-expressing (3T3-RNEU⁺, clear histogram) or rNEU-negative (3T3-RNEU⁻; shaded histogram) cell lines. Result in left panel is a representative example of a mouse vaccinated with pEC-TM^{neu}. Middle panel is the result using sera from a non-vaccinated (No Vax) mouse. Right panel represents the positive control obtained by staining the cells with 0.5 µg/ml of mouse monoclonal antibody Ab-4. These are representative results of data obtained from two different experiments.

Fig. 2.

Immunization of BALB/c mice with plasmids encoding a fragment of rNEU (residues 1-170) induces epitope-specific CD8⁺ and CD4⁺ T cell responses, without the production of antibodies to rNEU. Mice (n=3 per group) were vaccinated with plasmids pEC₁₋₁₇₀^{neu} and pEC₁₋₁₇₀^{neu/Ova} and immune responses were evaluated in the same manner as described in Figure 1. **A**, Percentage of tetramer positive CD8 T cells in individual mice (data points) and average per groups (horizontal lines) shown after prime and boost. **B**, Eight days after the boost, CD8 and CD4 T cells were purified from pooled splenocytes and antigen-induced IFN- γ secretion was evaluated in ELiSpot assays. As stimulator, p66-loaded P815 (P815/p66) and rNEU-expressing tumor (A2L2) were used to evaluate CD8 T cell responses. Ova₃₂₃₋₃₃₉-loaded A20 (A20/Ova₃₂₃) were used for the CD4 T cell responses. rNEU-negative tumor (66.3) and mock-treated P815 and A20 cells were used as negative controls. Results represent the average number of spots from triplicate wells with SD (error bars) of the means. Representative results of data obtained from 2 different experiments. **C**, Absence of anti-rNEU antibodies in mice vaccinated with pEC₁₋₁₇₀^{neu} (left panel) and pEC₁₋₁₇₀^{neu/Ova} (right panel) determined as described in the legend of Figure 1. Positive and negative controls gave identical results as those shown in Figure 1B.

Fig. 3.

Immunization of BALB-neuT mice with plasmids encoding a fragment of rNEU (residues 1-170) elicits CD8⁺ T cell responses to the rNEUp66 epitope. 8-week old mice (4 per group) were vaccinated with plasmids pEC₁₋₁₇₀^{neu} or pEC₁₋₁₇₀^{neu/Ova} and immune responses were evaluated in the same manner as described for the previous experiments. **A**, Percentage of tetramer positive CD8 T cells in individual mice (data points) and average per groups (horizontal lines) shown after prime, after boost and in non-vaccinated mice (No Vax, 3 animals). **B**, Eight days after the boost immunization, CD8 T cells were purified from pooled splenocytes, and antigen-induced IFN- γ secretion was evaluated in ELISpot assays in the same manner as described in Figure 2. Representative results of data obtained from two different experiments.

Fig. 4.

Therapeutic effects induced by plasmid DNA immunization against established tumors.

BALB/c mice (A: 5 per group, B: 7 per group) were s.c. inoculated with 3×10^5 live TUBO cells, and vaccinated with plasmid pEC₁₋₁₇₀^{neu/Ova} (shown as the vertical arrow) either 1 time (A) or 2 times (B). Non-vaccinated mice (No Vax) were used as negative controls for each experiment. Tumor growth was measured (two opposing diameters) and recorded every other day. Lines, tumor size in area (mm²) of each individual mouse.

Fig. 5.

Immunization of BALB-neuT mice with plasmids encoding a fragment of rNEU results in delay of autochthonous mammary neoplasms. Mice (n=13 per group) were vaccinated at 10 and 13 weeks of age, with pEC₁₋₁₇₀^{neu}, pEC₁₋₁₇₀^{neu/Ova} or pOva (negative control). All animals were monitored for tumor appearance by manual examination of the mammary glands every 5 d. Measurable masses >2 mm diameter were regarded as tumors. **A**, mean number of tumors per mouse in each group (tumor multiplicity) bars, SD. Arrows represent the day when the vaccine was administered. **B**, Kaplan-Mayer survival curves for all 3 groups of BALB-neuT mice. Log-rank test analyses (Mantel-Cox) between the negative control (pOva) and pEC₁₋₁₇₀^{neu} or pEC₁₋₁₇₀^{neu/Ova} revealed a statistical significance of $p < 0.0001$. **C**, Some of mice that were repeatedly vaccinated were monitored for antigen-specific CD8 T cell responses in peripheral blood lymphocytes by tetramer analysis.

Fig. 6.

Role of T cell subsets in the anti-tumor effects of DNA immunization in BALB-neuT mice. Mice (n=4 per group), were depleted of CD8 T cells (middle panel), CD4 T cells (right panel) or not depleted of T cells (left panel) using monoclonal antibodies before the vaccination as described in "Materials and methods". All mice were vaccinated with plasmid pEC₁₋₁₇₀^{neu/Ova} administered at 7, 10, 13, 19, and 23 weeks of age. All animals were monitored for tumor appearance by manual examination of the mammary glands every 5 d. **A**, Tumor growth assessed by the average tumor size in each mouse. Numbers at the end of each line represent the number of tumors per mouse. Arrows, day when the vaccine was administered. **B**, Kaplan-Meier survival curves for this experiment. Log-rank test analyses (Mantel-Cox) between the non-depleted control and CD8 T cell depleted group revealed a statistical significance of $p < 0.0067$, and between the non-depleted control and CD4 T cell depleted group revealed a statistical significance of $p < 0.0558$.