Peptide Vaccine Given with a Toll-Like Receptor Agonist Is Effective for the Treatment and Prevention of Spontaneous Breast Tumors

Pilar Nava-Parada, Guido Forni, Keith L. Knutson, Larry R. Pease, and Esteban Celis

Abstract

Our goal is to develop peptide vaccines that stimulate tumor antigen–specific T-cell responses against frequently found cancers. Previous work has shown that to generate effective T-cell responses, peptides have to be administered in combination with strong adjuvants such as Toll-like receptor agonists. However, most animal tumor model systems used to study peptide vaccines were not truly representative of malignant diseases in humans because they solely used transplantable tumor lines, and instead of true tumor antigens, they used highly immunogenic foreign proteins. Here, we describe a peptide vaccination strategy, which is highly effective in delaying or preventing the occurrence of spontaneous breast tumors. Transgenic female BALB-neuT mice that carry the activated rat HER-2/neu oncogene were vaccinated with a synthetic peptide from the rat HER-2/neu gene product, which represents an epitope for CTLs in combination with a Toll-like receptor agonist adjuvant. Our results show that to obtain tumor antigen-specific CTL responses and antitumor effects, the vaccine had to be administered repetitively, or the function of CD4/CD25 T regulatory cells had to be blocked with anti-CD25 antibody therapy. Mice that were vaccinated with this approach remained tumor-free or were able to control spontaneous tumor growth and exhibited long-lasting CTL responses, not only against the immunizing peptide but also against other peptides derived from rat HER-2/neu product (i.e., epitope spreading). These results suggest that similar strategies should be followed for conducting clinical studies in patients.

Introduction

The identification of MHC class I (MHC-I) antigen-binding peptides derived from tumor-associated antigens (TAA) has facilitated the development of T-cell epitope–based vaccines for cancer (1). Vaccines produced with synthetic peptides representing these T-cell epitopes are an attractive approach for tumor immunotherapy because they can be easily manufactured in a cost-effective manner for clinical use. However, a concern regarding the use of synthetic peptide vaccines for the treatment of cancer is their not-so-impressive track record in the clinic at inducing strong immune responses and therapeutic antitumor effects. Thus, significant work is being dedicated towards peptide vaccine optimization using animal tumor models. Early studies using some of these models indicated that to induce strong CTL responses and antitumor effects, peptide vaccines had to be administered with powerful adjuvants, such as those that stimulate Toll-like receptors (TLR; refs. 2, 3). Previous work in our laboratory showed that even in circumstances when a synthetic peptide was derived from a highly immunogenic antigen (e.g., ovalbumin), a TLR ligand was required to generate CTL responses capable of producing antitumor effects (4). Nevertheless, these experiments were done in a somewhat artificial system because the model TAA (ovalbumin) was a foreign protein (most TAA are over expressed self-proteins), and the tumors used were transplantable cell lines routinely maintained in tissue culture.

More physiologic tumor models to study the effectiveness of tumor vaccines can now be used, thanks to the existence of transgenic mouse strains such as those that target the expression of oncogenes to specific tissues, leading to the development of spontaneous tumors. Two transgenic mouse lines that preferentially express the rat HER-2/neu gene product (RNEU) in breast tissues under the mouse mammary tumor virus promoter have been used to assess the effectiveness of tumor vaccines. The FVB-neuN mice (5) carry the rat HER-2/neu proto-oncogene and develop breast tumors at 6 to 9 months of age. These mice have been used extensively in vaccine studies against transplantable tumors and some studies showed that the presence of CD4/CD25 T regulatory (Treg) cells inhibit the generation of tumor antigen–specific CTL responses (6). Removal of Treg cells with either anti-CD25 monoclonal antibodies (mAb) or low-dose cyclophosphamide increased tumor-specific CTL responses using a cytokine-expressing cell-based vaccine, resulting in significant antitumor effects against a transplantable tumor (6, 7). The other transgenic model is the BALB-neuT mouse line (BALB/c background), which express the activated form of RNU and develops multiple spontaneous breast tumors at an earlier age (15–20 weeks; ref. 8). Using plasmid DNA vaccines, it was shown that it is possible to delay or prevent spontaneous breast tumors in the BALB-neuT mice (9–13), mostly through the generation of tumor antigen–specific antibody responses. Notably, CTL responses induced by plasmid DNA vaccines in BALB-neuT mice were quite low compared with those obtained in BALB/c mice, suggesting the presence of immune tolerance and/or Treg cells in these mice (14, 15). Here, we evaluate the use of an immunogenic tumor peptide corresponding to a CTL epitope from the RNEU antigen for its immunogenicity and antitumor effectiveness in BALB-neuT mice. Our results show that peptide vaccination given in combination with a TLR ligand adjuvant was effective in inducing CTL responses with antitumor activity in both BALB/c and BALB-neuT mice. However, effective immunization of BALB-neuT mice required either removal of CD4/CD25 cells or multiple booster vaccinations. Moreover, peptide vaccination was shown to be effective in the prevention or treatment against a transplantable...
tumor as well as in showing benefit against early stages of spontaneous breast tumors arising in BALB-neuT mice. The information gathered by these studies may be of use for the implementation of peptide-based vaccines in cancer patients.

Materials and Methods

**Mice.** Female, 8-week-old BALB/c mice (H-2d) 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-2d), which were generated as described (8), were bred in our facilities. Heterozygous 6- to 15-week-old virgin females expressing rat HER-2/neu were purchased at the Jackson Laboratory, and were used in all experiments. CD4, clone GK1.5; anti-CD25, clone PC61; and anti-CD8, clone 2.43) were prepared from hybridoma supernatants (obtained from ATTC) and were affinity purified on a protein G-Sepharose column.

**Cell lines.** P815 mastocytoma cell line (H-2d) was 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 (9). The rat HER-2/neu-transfected mouse mammary breast cancer A2L2 (H-2d) and its parental 66.3 cell line (16) were provided by Drs. J.E. Price and L. Lachman (M.D. Anderson Cancer Center, Houston, TX).

**Synthetic peptides, adjuvants, and antibodies.** The synthetic peptides used in these studies were purchased from A&A Labs (San Diego, CA) or prepared at the Mayo Clinic Peptide Core Facility. The purity (>95%) and identity of peptides were determined by analytic high-performance liquid chromatography and mass spectrometry analysis. The following peptides from the RNEU antigen were used: p66 (TVYVANAS), p304 (PYNYLSTEV), p414 (LYISAWPDLS), p557 (EYYSDDLRL), p734 (AFGTVYKGYK), p784 (PYYSRLGIGI), p911 (SYGVTTWELI), 989 (REFVYIQNDEI), and p1251 (EYLGLIDVPV). A synthetic peptide (SYVPSAEQI) corresponding to an H-2Kd–restricted CTL epitope (17), from Plasmadium yoelii circumsporozoite protein (PcCSP), was used as a positive control. Immune Freund's adjuvant (IFA) was from Sigma-Aldrich (St. Louis, MO). The immunostimulatory synthetic oligodeoxynucleotide ODN-1826 (5'-TCTAGACGTCATTGACGTT-3'), containing two CpG motifs (referred as CpG6), was prepared by the Mayo Clinic Molecular Core Facility. mAbs used for in vivo cell depletion (anti-CD4, clone GK1.5; anti-CD25, clone PC61; and anti-CD8, clone 2.43) were prepared from hybridoma supernatants (obtained from ATTC) and were affinity purified on a protein G-Sepharose column.

**Peptide vaccination protocol.** Mice (BALB/c or BALB-neuT) received five daily s.c. injections by the nape of the neck of 100 μg Cpgs (days 2, 1, 0, 1, and 2). On day 0, mice were immunized (s.c.) with 100 μg peptide emulsified in IFA (200 μL) at a proximal site of the Cpg injections. In some experiments, the mice received booster vaccinations, which were administered in the same manner (with five daily injections of Cpg6). For the in vivo cell depletion experiments, anti-CD4 mAb (0.2 mg per mouse), anti-CD8 mAb (0.5 mg per mouse), or anti-CD25 mAb (0.5 mg per mouse) were injected i.p. on days −3, −2, and −1 before receiving the peptide injection. More than 95% cell depletion for CD4 and CD8 cells and 60% to 80% for CD25 cells was confirmed by flow cytometry analysis with no significant depletion of other cell populations (data not shown). Immune responses were typically measured (as described below) 7 to 10 days after the last vaccination.

**Measurements of immune responses.** Immune responses generated by the vaccines were measured using enzyme-linked immunosorbent spot (ELISPOT) assays to detect CD8 T cells secreting IFN-γ (Mabtech, Inc., Mariemont, OH) using purified CD8 T cells (Milltenyi Biotec, Auburn, CA). Serial dilutions of CD8 T cells were tested against a constant number of (3 × 105) stimulator cells. Spot counting was done with an AID ELISPOT Reader System (Cell Technology, Inc., Columbia, MD). Cytolytic activity of CTL derived from vaccination was measured using a 4-h JAM DNA fragmentation assay (18).

**Prophylactic model of TUBO challenge.** Mice (five animals per group) were vaccinated as described above, and 7 days after receiving the peptide, they were challenged (s.c.) a distant site of the vaccination with 2 × 106 TUBO cells. Mice were observed every other day to monitor tumor growth using a set of calipers, measuring two opposing diameters, including the largest diameter for each tumor. Results are presented in size as mm2, calculated by multiplying the two diameters for each tumor.

**Therapeutic model of TUBO challenge.** Mice were first challenged with 2 × 106 TUBO cells. When 100% of mice had an established tumor of ~3 mm diameter in the greatest dimension (5–8 days after tumor injection), peptide vaccination was initiated.

**Prevention of spontaneous tumors.** Virgin female BALB-neuT mice were selected by age to perform the immunization strategy at different time points. One group was selected at 15 weeks of age to receive the first cycle of described vaccination followed by two identical boosting on weeks 17 and 19 of age. The second group was selected at week 8 of age to receive a single immunization after treatment with anti-CD4 or anti-CD25 mAb. To monitor appearance of spontaneous tumors, the chests of the mice were shaved using an electric razor, and mammary pads were manually inspected every week. Data are reported as tumor multiplicity (cumulative number of tumors per number of mice in each group) and shown as mean ± SD as reported (9). 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.

**Statistical analysis.** Student’s t-test was applied at 95% confidence interval to determine the statistical significance of differences between groups, with P < 0.05 being considered significant. All analysis and graphics were done using GraphPad Prism, version 4 for PC (GraphPad Software, San Diego, CA).

**Results**

**Immuneresponses to RNEU peptide vaccination in BALB/c mice.** Our first task was to identify at least one CTL epitope that could be used to evaluate a peptide-based vaccine in transgenic BALB-neuT mice, which develop spontaneous breast tumors. Using two CTL epitope identification, computer-based algorithms available in the Internet (19, 20) that predict the binding of short (8–10 residues) peptide sequences to MHC-I molecules, we analyzed the RNEU protein for the presence of H-2Kd-binding motifs. We selected nine peptides of the top 15 scoring sequences identified by both algorithms (data not shown) for synthesis and immunologic evaluation. When BALB/c mice were individually vaccinated with these peptides using IFA and Cpg adjuvant, five of the nine peptides (p66, p304, p734, p911, and p1251) were found to induce antigen-specific CD8 T-cell responses against peptide-pulsed cells (P815), as measured by IFN-γ ELISPOT assay (Fig. 1A). This response was approximately between one third to one half of the response observed using a well-known H-2Kd–restricted CTL epitope from P. yoelii (PyCSP). Peptides p66, p304, p734, p911, p1251, and p989 (as a negative control) were then evaluated in BALB/c mice for their capacity to induce CD8 T-cell responses capable of recognizing tumor cells expressing the RNEU protein. The results presented in Fig. 1B indicate that p66 was the most effective peptide in generating CD8 T cells that reacted with two cell lines (TUBO and A2L2) that express RNEU. This response was antigen specific because these T cells failed to respond to tumor cells not expressing RNEU: P815 and 66.3 (the parental line of A2L2). In addition, mice immunized with PyCSP did not produce CD8 T cells that reacted with either the TUBO or the A2L2 cells. As shown in Fig. 1C, the CD8 T cells derived from mice vaccinated with p66 displayed high cytolytic activity against target cells that were either pulsEd with synthetic peptide (P815+p66) or target cells naturally expressing RNEU (TUBO and A2L2). This response was antigen specific because the effector cells did not kill the target cells not expressing RNEU.
Antitumor effects of peptide vaccination in BALB/c mice. To evaluate whether the CTL responses induced by p66 vaccination were potent enough to provide an antitumor effect, BALB/c mice (five per group) were vaccinated once, and 7 days later, they were challenged s.c. with live TUBO cells. Mice receiving the complete vaccine (p66 in IFA and CpG) were protected against the tumor challenge (Fig. 1D). On the other hand, mice that were not vaccinated developed tumors that grew at a fast rate. In addition, mice treated with CpG alone (no peptide) also failed to reject the tumor challenge. Being aware that a prophylactic vaccination protocol does not come close to reflecting the circumstances of most human malignancies, we tested the effectiveness of peptide vaccination in a therapeutic mode. Mice were first challenged with live TUBO, and when tumors reached a ~3 mm diameter size, the animals received the vaccine (p66+CpG), were treated with CpG alone, or were left untreated. Peptide vaccination with CpG was also effective in the therapeutic mode (Fig. 1E). Although the tumors continued to grow for ~1 week after, the vaccine caused total tumor regression, and the animals remained free of disease for 120 days. In mice that were left untreated, the tumors grew fast and had to be euthanatized when the tumors exceeded 2 cm in diameter. Interestingly, two of five mice that received CpG alone were able to generate an antitumor response but were unable to completely eradicate the tumor.

Immune responses to peptide vaccination in BALB-neuT mice. We proceeded to study whether peptide vaccination in BALB-neuT mice would induce antigen-specific immunity. We considered the possibility that CTL responses to the RNEU peptides in these mice could be absent or much lower than in BALB/c mice because RNEU is expressed in the breast at a...
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relatively young age (~6 weeks), and some degree of immune tolerance at the CTL level has been observed (14, 15, 21). In addition, as reported in FVB-neuN mice, the presence CD4/CD25 Treg cells leads to suppression of CTL responses to the RNEU antigen (6, 7, 22). Vaccination of BALB-neuT mice with p66 or with PyCSP resulted in a significant CD8 T-cell response against peptide-pulsed antigen-presenting cells (APC), but the response to p911 was much lower and failed to reach statistical significance (Fig. 2A). Being cognizant that tumor-specific CTL responses would be difficult to achieve in BALB-neuT mice due to the potential inhibitory effects of CD4/CD25 Treg cells, we assessed the immune responses to p66 vaccination in mice that were treated with either anti-CD4 or anti-CD25 mAb, which in our hands consistently eliminated ~90% and 60%, respectively, of lymphocytes expressing these molecules (data not shown). The results show that the tumor-specific CTL response to p66 vaccination in BALB-neuT mice (in the absence of depleting antibodies) was not as effective compared with the responses observed in BALB/c mice (Fig. 2B versus Fig. 1B). Nevertheless, the low level of reactivity against TUBO and A2L2 observed in these mice was statistically significant (Fig. 2B). Treatment of the mice with either anti-CD4 or anti-CD25 mAb before vaccination increased the tumor-specific response ~5-fold (Fig. 2B). Lastly, the CD8 T-cell responses to p66 vaccination in CD4 or CD25-depleted BALB-neuT mice displayed significant cytolytic activity against peptide-pulsed target cells or RNEU-expressing tumor cells (Fig. 2C).

Effects of prophylactic vaccination against TUBO in BALB-neuT mice. The antitumor effect of peptide vaccination was first evaluated in BALB-neuT mice in the prophylactic setting. Animals receiving p66+CpG were significantly protected against a subsequent challenge with live TUBO tumor cells (Fig. 3A). Although p66 vaccination without CD4 or CD25 cell depletion did not seem to induce high numbers of tumor-reactive CTL responses in BALB-neuT mice (Fig. 2B and C), this vaccination modality had a clear and significant antitumor effect. Nevertheless, treatment with anti-CD25 mAb before vaccination increased the effectiveness of the vaccine. The antitumor effect of the vaccine seems to be provided by classic CD8-expressing CTL because mice treated with anti-CD8 mAb (before vaccination) failed to be protected against the tumor challenge (Fig. 3A). Notably, although CD4 cell depletion increased tumor antigen-specific CTL responses in BALB-neuT mice (Fig. 2A and B), this therapy did not translate to an enhancement of anti-tumor effectiveness (Fig. 3A) but lead to a worse outcome than the p66 vaccine alone, suggesting that some CD4 T cells, such as classic T helper cells, may play an important role in conferring protection. Animals that were treated with anti-CD25 mAb or anti-CD4 mAb and received CpG alone (no peptide) were not protected to any extent against the tumor challenge (data not shown). By day 70 after tumor challenge, all of the mice in the p66+CpG– and p66+CpG/anti-CD25 mAb–treated groups remained alive. Although all mice in p66+CpG group had tumors (~100 mm²), three of five mice in the p66+CpG/anti-CD25 mAb–treated group remained tumor-free.

To evaluate the capacity of peptide vaccination to induce a protective tumor-specific memory immune response, BALB-neuT mice were immunized with p66 peptide and challenged with live TUBO tumor cells 10 weeks after a single peptide vaccination. The results of this experiment were similar to those obtained when mice had been challenged with tumors 7 days after vaccination,

Figure 2. Vaccination of BALB-neuT mice with peptide p66 and CpG induces CD8 T-cell responses but requires anti-CD4 or anti-CD25 mAb treatment for tumor-specific reactivity. A, groups of 3 BALB-neuT female mice were vaccinated with 100 μg of either p66, p911, or PyCSP peptide emulsified in IFA and CpG as described in Materials and Methods, and 7 d later, the immune responses of CD8 T cells from pooled splenies to each corresponding peptide were measured using an IFN-γ ELISPOT assay. B, peptide p66 (or PyCSP tested as negative control) was evaluated for its capacity to induce CD8 T-cell responses to RNEU-expressing tumors (TUBO and A2L2) and RNEU-negative controls (P815 and 66.3). As indicated, some mice were treated with either anti-CD4 or anti-CD25 mAb before vaccination (see Materials and Methods for details). Numbers above the columns represent Ps of TUBO versus P815 or A2L2 versus 66.3. Column, average of two to three wells containing 1×10⁶ cells per well; bars, SD. C, cell-mediated cytotoxic responses of CD8 T cells derived from p66-immunized BALB-neuT mice with and without the indicated mAb therapy measured by the JAM assay (see Materials and Methods) against various [3H]thymidine-labeled target cells. Points, mean of triplicate determinations; bars, SD. Representative results of data obtained from two to three different experiments.
indicating that the effector T cells generated by p66 vaccination can last for at least 2.5 months (Fig. 3B). As before, by the termination of this experiment, three of five mice in the p66+CpG/anti-CD25 mAb–treated group remained tumor-free.

**Effects of therapeutic vaccination against TUBO in BALB-neuT mice.** Next, we evaluated the effect of p66 peptide vaccination in BALB-neuT mice in the therapeutic setting. Mice were injected with 2 x 10⁶ live TUBO cells, and when the tumors were visible (≥3 mm diameter, ~10 days later), the mice were vaccinated. We also evaluated the effect of treating the mice prior to vaccination with either anti-CD25 or anti-CD4 mAb. The results show that by day 20, all of the control, unvaccinated mice had developed large tumors and did not survive (Fig. 4A). However, a single therapeutic vaccination with p66+CpG significantly delayed the tumor growth, but ultimately by day ~70, all of the mice had to be euthanized due to the presence of large tumors. Treatment with anti-CD25 mAb, but not with anti-CD4 mAb, increased the antitumor effect of the vaccine. Although the tumors were not eradicated, they ceased to grow in the anti-CD25 mAb–treated mice. In contrast, anti-CD4 mAb therapy reduced the effectiveness of the vaccine. Antibody therapy (anti-CD25 or anti-CD4) in combination with CpG had no therapeutic effect. These results suggest that removal or inhibition of cells expressing CD25, most likely Treg lymphocytes, increases the effectiveness of peptide vaccination, and that conventional CD4 T helper cells may play an important role in the generation of antitumor effects.

To improve the therapeutic efficiency of the vaccine, we administered two booster immunizations. The experiment was done in the same manner as described for Fig. 4A, except that the mice received booster immunizations on days 24 and 38 (antibody therapy was not done during the booster vaccinations). Significant antitumor effects were obtained in the mice that were vaccinated thrice with p66+CpG (Fig. 4B). These effects were more pronounced than in mice that received a single vaccination (Fig. 4A). The therapeutic effect of the vaccine decreased if mice were treated with anti-CD8 mAb or anti-CD4 mAb before vaccination. By day 125, when the experiment was terminated, all of the mice in groups p66+CpG and p66+CpG/anti-CD25 mAb were alive, and four of five mice in the latter group remained tumor-free (all the mice in the p66+CpG group had small tumors). Again, no therapeutic advantage was observed in mice that received CpG alone, even when anti-CD4 or anti-CD25 was administered (data not shown).

**Therapeutic vaccination against spontaneous mammary tumors.** The effect of p66 vaccination in the prevention of spontaneous mammary tumors that naturally arise in BALB-neuT mice was evaluated. First, we studied the effect of a single peptide vaccination, which was administered to the mice at week 8 from birth when diffuse atypical hyperplasia is already evident in the mammary glands but before in situ carcinomas are evident (21). The average number of tumors (tumor multiplicity) increased much faster in the nonvaccinated group compared with the group that received the p66 vaccine (Fig. 5A). Vaccination with p66 delayed by ~5 weeks the time required for these mice develop tumors (Fig. 5A). Treatment with anti-CD25 mAb increased significantly the effect of the p66+CpG vaccine. By week 35, all of the mice that were vaccinated with p66+CpG remained alive, and those mice that were treated with anti-CD25 mAb were tumor-free. In contrast, by week 26, all of the mice in the control groups had large tumors and required euthanization.

Next, we assessed the p66 vaccine in older (15-week-old) BALB-neuT female mice, which presumably already have multifocal in situ carcinomas (21). Vaccines were administered on weeks 15, 17, and 19 without cell-depleting antibodies. The data presented in Fig. 5B show that by week 19, at least one of the mammary glands in the nonvaccinated mice had a tumor mass. In contrast, p66+CpG vaccination increased the amount of time required to develop tumors by ~15 weeks, showing a significant therapeutic effect. Administration of three mock vaccinations (CpG alone) had no significant advantage over the nonvaccinated mice. By week 26, all the mice that did not received the p66 vaccine had more than
seven tumors each, and because at least one tumor had reached the 2-cm diameter size limit, they all had to be euthanatized. In contrast, on week 45, all mice that received p66 vaccine remained alive, and two of five mice were tumor-free.

**Evaluation of immune responses in surviving animals.** When the experiments described in Fig. 5A and B were terminated, the CD8 T-cell responses of the surviving mice were evaluated against p66 and TUBO. In addition, to assess the possibility of epitope spreading, we evaluated the CD8 T-cell responses to the RNEU peptides that were able to generate peptide-reactive T-cell responses in BALB/c mice (Fig. 1A). The surviving mice that received a single p66+CpG vaccination (Fig. 5A), all of which had at least five tumors, had a small but significant response to p66 and to TUBO (Fig. 6A). In addition, these mice also exhibited a small response to p1251. In contrast, mice that received anti-CD25 mAb therapy and a single p66+CpG vaccination, which remained tumor-free throughout the experiment (Fig. 5A), displayed high responses to p66, to TUBO, and to the four additional RNEU peptides (Fig. 6A). The animals that were treated with anti-CD4 mAb and received one p66+CpG vaccine exhibited a significant CD8 T-cell response to p66 and TUBO but not to any of the additional RNEU peptides (Fig. 6A). The surviving the BALB-neuT mice (four of five)
from the experiment that evaluated the effect of three immunizations (Fig. 5E) also displayed high CD8 T responses against all RNEU peptides and TUBO cells (Fig. 6B).

**Discussion**

To our best knowledge, the present work represents the first attempt to evaluate a peptide vaccine representing a CTL epitope derived from a true TAA in mouse model of spontaneous cancer. The peptide epitope selected for these studies, p66 (TVYVPANASL), was the most effective of the nine predicted H-2Kd–binding candidates that we evaluated in eliciting tumor-reactive CTL in BALB/c mice (Fig. 1B). Previous studies reported that the homologous peptide from human HER-2/neu, (TYLPTNASL) was effective in inducing CTL responses in normal BALB/c mice, and that vaccination with peptide-pulsed APC [in combination with interleukin-12 (IL-12) administration] provided antitumor effect in animals subsequently challenged with a fibrosarcoma transfected with human HER-2/neu (23). In our studies, peptides p304, p734, p911, and p1251, which also scored high in the computer-based algorithms, were able to induce T-cell responses against peptide-pulsed APC, but the responses against the RNEU-expressing tumor cells were not as effective as those obtained with p66 (Fig. 1A and B). These results suggest that these epitopes (p304, p734, p911, and p1251) are either not expressed in sufficient amounts as Kb/peptide complexes on these APC (TUBO and A2L2 cells) to allow for T-cell recognition, or that immune tolerance mechanisms have deleted the high avidity CTL that would recognize naturally processed antigen. However, T-cell responses were observed to p304, p734, p911, and p1251 in BALB-neuT mice that successfully responded to p66-CpG vaccination. Although this epitope spreading correlated with antitumor effects of vaccination, we do not know whether the CTL recognizing the p304, p734, p911, and p1251 epitopes play any role in conferring antitumor resistance.

Vaccination with p66 and CpG in BALB/c mice was able to elicit prophylactic and therapeutic antitumor responses against a challenge with the RNEU-expressing TUBO cell line (Fig. 1D and E). Vaccination with peptide p66 in IFA in the absence of CpG resulted in insignificant CTL responses and lack of antitumor effects in both BALB/c and BALB-neuT mice (data not presented), indicating that CpG serves a critical role in generating effective tumor-specific CTL responses. It has been shown that CpG not only activates APC to serve as better stimulators of T-cell responses (2), but it also prevents activation-induced cell death in T cells facilitating their expansion (4, 24). It should be noted that in the therapeutic vaccination mode, administration of CpG alone without peptide (five daily injections) resulted in antitumor effects in two of three mice (Fig. 1E). The antitumor effects of CpG monotherapy have been reported in several tumor model systems (25, 26) and could be the result of enhancing immune responses to antigens derived from the tumor challenge.

Our data show that to obtain tumor antigen-specific T-cell responses in BALB-neuT mice, either booster immunizations or depletion of CD4- or CD25-expressing cells was necessary. These findings indicate that tumor-reactive CTL specific for the p66 epitope are either present in much lower numbers in BALB-neuT mice compared with BALB/c, or that a large proportion of these cells are inhibited by CD4/CD25–expressing cells. When the antitumor effects of peptide vaccination were evaluated in the prophylactic setting, we were surprised to find that BALB-neuT mice vaccinated a single time with p66-CpG were protected to a great extent against a subsequent challenge with TUBO cells (Fig. 3). As expected, depletion of CD25-expressing cells before vaccination enhanced the antitumor effect of the vaccine, but in contrast, depletion of CD4-expressing cells had an opposite effect. These results, to some extent, contradict the results that measured the effect of vaccination in eliciting CD8 T-cell responses (Fig. 2B and C). However, a plausible explanation is that a single vaccination provides effective antitumor effect could be that a low number of CTL derived from the vaccine are able to generate additional antigen as they kill some of the tumor cells, which helps to expand these cells or to stimulate new CTL precursors. In addition, the tumor challenge itself may provide antigen (in the form of dead tumor cells), which could function as a booster vaccination. The negative effect of depleting CD4-expressing cells could be explained by the loss of CD4 T helper lymphocytes, which are known to enhance CTL responses to vaccination. Thus, these findings indicate that with any vaccination strategy, it would not be prudent to deplete CD4/CD25 Treg cells using anti-CD4 mAb because...
concomitant depletion of T helper cells would result in suboptimal antitumor effects. Other strategies have been considered to deplete or inhibit the function of Treg cells to enhance the effect of vaccination and achieve antitumor responses. Low-dose chemotherapy, mainly using cyclophosphamide, has been shown to reduce the numbers and function of suppressor CD4/CD25 Treg cells (7, 27). Another approach to block the inhibitory effects of Treg cells for cancer immunotherapy is with the use of an IL-2 immunotoxin (28, 29). In recent studies, it was shown administration of IL-2 immunotoxin to FVB/neuN mice resulted in immune-mediated rejection of transplantable tumors, even in the absence of vaccination, and helped to overcome CTL tolerance to the RNEU antigen (22). The results presented here in the BALB-neuT system indicate that reduction of CD25 cell numbers without vaccination, even when CpG was administered, did not have any antitumor effect (Fig. 4).

When CpG was administered, did not have any antitumor effect that reduction of CD25 cell numbers without vaccination, even (22). The results presented here in the BALB-neuT system indicate rejection of transplantable tumors, even in the absence of vaccination, and helped to overcome CTL tolerance to the RNEU antigen (22). The results presented here in the BALB-neuT system indicate that reduction of CD25 cell numbers without vaccination, even when CpG was administered, did not have any antitumor effect (Fig. 4).

4 P. Nava, unpublished.

**References**


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