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Cancer Res. Author manuscript; available in PMC 2008 February 1.

Published in final edited form as:
Cancer Res. 2007 February 1; 67(3): 1326–1334.

Peptide Vaccine Administered with a Toll-like Receptor Agonist is Effective for the Treatment and Prevention of Spontaneous Breast Tumors

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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 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 utilized 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 HER2/*neu* oncogene were vaccinated with a synthetic peptide from the rat HER2/*neu* gene product, which represents an epitope for cytotoxic T lymphocytes (CTL) in combination with a TLR agonist adjuvant. Our results show that in order to obtain tumor antigen-specific CTL responses and anti-tumor 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 HER2/*neu* product (i.e., epitope spreading). These results suggest that similar strategies should be followed for conducting clinical studies in patients.

Keywords

T regulatory cells; CpG adjuvant; peptide vaccine; CTL

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

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Conflict of interest: The authors state that there is no financial interest in this work.

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at inducing strong immune responses and therapeutic anti-tumor effects. Thus, significant work is being dedicated towards peptide vaccine optimization using animal tumor models. Early studies using some of these models indicated that in order to induce strong cytotoxic T lymphocyte (CTL) responses and anti-tumor 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 demonstrated 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 anti-tumor effects (4). Nevertheless, these experiments were performed in a somewhat artificial system, since 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 physiological 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 HER2/*neu* gene product (RNEU) in breast tissues under the MMTV promoter have been used to assess the effectiveness of tumor vaccines. The FVB-*neu*N mice (5) carry the rat HER2/*neu* proto-oncogene and develop breast tumors at 6–9 months of age. These mice have been used extensively in vaccine studies against transplantable tumors and some studies demonstrated 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 anti-tumor 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 RNEU and develops multiple spontaneous breast tumors at an earlier age (15–20 weeks, ref. 8). Using plasmid DNA vaccines, it was demonstrated 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 as compared to 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 a synthetic peptide vaccine corresponding to a CTL epitope from the RNEU antigen for its immnogenicity and anti-tumor effectiveness in BALB-neuT mice. Our results show that peptide vaccination administered in combination with a TLR ligand adjuvant was effective in inducing CTL responses with anti-tumor 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 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 weeks old BALB/c mice (*H-2*^{d}) were obtained through the NCI/Charles River program. Mice were allowed to acclimate to our animal facility for one week before beginning experiments. BALB-neuT mice (*H-2*^{d}), which were generated as described(8) were bred in our facilities. Heterozygous 6–15 week-old virgin females expressing rat HER2/*neu* as verified by PCR were used throughout this work.

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Cell lines

P815 mastocytoma cell line (*H-2*^{d}) was purchased from ATCC. 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 HER2/*neu*-transfected mouse mammary breast cancer A2L2 (*H-2*^{d}) and its parental 66.3 cell line (16) were provided by Drs. J. E. Price and L. Lachman (MD 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 analytical high-performance liquid chromatography and mass spectrometry analysis. The following peptides from the RNEU antigen were used: p66 (TYVPANASL), p304 (PYNYLSTEV), p414 (LYISAWPDSL), p557 (EYVSDLRCL), p734 (AFGTVYKGI), p784 (PYVSRLLGI), p911 (SYGVTVWEL), 989 (RFVVIQNEDL) and p1251 (EYLGLDVPV). A synthetic peptide (SYVPSAEQI) corresponding to an H-2K^d-restricted CTL epitope (17), from *Plasmodium yoelii* cicumsporozoite protein (PyCSP) was utilized as a positive control. Incomplete Freund's adjuvant (IFA) was from Sigma-Aldrich (St. Louis, MO). The immunostimulatory synthetic oligodeoxynucleotide ODN-1826 (5′-TCCATGACGTTCCTGACGTT-3′), containing two CpG motifs (referred as CpG) was prepared by the Mayo Clinic Molecular Core Facility. Monoclonal antibodies 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 5 daily subcutaneous (s.c.) injections by the nape of the neck of 100 μg of CpG (days -2, -1, 0, 1, 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 5 daily injections of CpG). For the *in vivo* cell depletion experiments, anti-CD4 mAb (0.2 mg/mouse), anti-CD8 mAb (0.5 mg/mouse) or anti-CD25 mAb (0.5 mg/mouse) were injected i.p. on days -3, -2, and -1 prior to receiving the peptide injection. Greater than 95% cell depletion for CD4 and CD8 cells and 60–80% for CD25 cells was confirmed by flowcytometry analysis with no significant depletion of other cells populations (data not shown). Immune responses were typically measured (as described below) 7–10 days after the last vaccination.

Measurements of immune responses

Immune responses generated by the vaccines were measured using ELISpot assays to detect CD8 T cells secreting IFN-γ (Mabtech, Inc., Mariemont, OH) using purified CD8 T cells (Miltenyi Biotec, Auburn, CA). Serial dilutions of CD8 T cells were tested against a constant number (3×10^5) 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 4hr-JAM DNA fragmentation assay (18).

Prophylactic model of TUBO challenge

Mice (5 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 2×10^6 TUBO cells. Mice were observed every other day to monitor tumor growth using a set of calipers, measuring 2 opposing diameters, including the largest diameter for each tumor. Results are presented in size as mm^2, calculated by multiplying the 2 diameters for each tumor.

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Therapeutic mode of TUBO challenge

Mice were first challenge with 2×10^6 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 week 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 is reported as tumor multiplicity (cumulative number of tumors/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, the mice were sacrificed by CO2 inhalation according to our IACUC guidelines.

Statistical analysis

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

Results

Immune responses 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 2 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-2K^d-binding motifs. We selected 9 peptides of the top 15 scoring sequences identified by both algorithms (data not shown) for synthesis and immunological evaluation. When BALB/c mice were individually vaccinated with these peptides using IFA and CpG adjuvant, 5 of the 9 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 interferon-gamma (IFN-γ) ELISpot assay (Fig. 1*A*). This response was approximately between one third to one half of the response observed using a well-known H-2K^d-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 Figure 1*B* indicate that p66 was the most effective peptide in generating CD8 T cells that reacted with 2 cell lines (TUBO and A2L2) that express RNEU. This response was antigen-specific since 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 Figure 1*C*, 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 since the effector cells did not kill the target cells not expressing RNEU.

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Anti-tumor effects of peptide vaccination in BALB/c mice

To evaluate whether the CTL responses induced by p66 vaccination were potent enough to provide an anti-tumor effect, BALB/c mice (5 per group) were vaccinated once and 7 days later they were challenged subcutaneously with live TUBO cells. Mice receiving the complete vaccine (p66 in IFA and CpG) were protected against the tumor challenge (Fig. 1*D*). 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. 1*E*). 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, 2/5 mice that received CpG alone were able to generate an anti-tumor 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 CTL responses. 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 since RNEU is expressed in the breast at a 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-*neu*N 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. 2*A*). 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 as compared to the responses observed in BALB/c mice (Fig. 2*B* versus Fig. 1*B*). Nevertheless, the low level of reactivity against TUBO and A2L2 observed in these mice was statistically significant (Fig. 2*B*). Treatment of the mice with either anti-CD4 or anti-CD25 mAb prior to vaccination increased the tumor-specific response ~5-fold (Fig. 2*B*). 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. 2*C*).

Effects of prophylactic vaccination against TUBO in BALB-neuT mice

The anti-tumor 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. 3*A*). Although p66 vaccination without CD4 or CD25 cell depletion did not appear to induce high numbers of tumor-reactive CTL responses in BALB-neuT mice (Figs. 2*B*, 2*C*), this vaccination modality had a clear and significant anti-tumor effect. Nevertheless, treatment with anti-CD25 mAb before vaccination increased the effectiveness of the vaccine. The anti-tumor effect of the vaccine seems to be provided by classical CD8-expressing CTL because mice treated with anti-CD8 mAb (prior to

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vaccination) failed to be protected against the tumor challenge (Fig. 3*A*). Notably, although CD4 cell depletion increased tumor antigen-specific CTL responses in BALB-neuT mice (Figs. 2*A*, 2*B*), this therapy did not translate to an enhancement of anti-tumor effectiveness (Fig. 3*A*), but lead to a worse outcome than the p66 vaccine alone, suggesting that some CD4 T cells, such as classical 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. While all mice in p66+CpG group had tumors (< 100 mm^2), 3/5 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, indicating that the effector T cells generated by p66 vaccination can last for at least 2.5 months (Fig. 3*B*). As before, by the termination of this experiment 3/5 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 2×10^6 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 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. 4*A*). 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 euthanatized due to the presence of large tumors. Treatment with anti-CD25 mAb, but not with anti-CD4 mAb increased the anti-tumor 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 anti-tumor effects.

To improve the therapeutic vaccine's efficiency we administered 2 booster immunizations. The experiment was performed in the same manner as described for Figure 4*A*, except that the mice received booster immunizations on days 24 and 38 (antibody therapy was not performed during the booster vaccinations). Significant anti-tumor effects were obtained in the mice that were vaccinated 3 times with p66+CpG (Fig. 4*B*). These effects were more pronounced than in mice that received a single vaccination (Fig. 4*A*). The therapeutic effect of the vaccine decreased if mice were treated with anti-CD8 mAb or anti-CD4 mAb prior to 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 4/5 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 were 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

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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) arose much faster in the non-vaccinated group as compared to the group that received the p66 vaccine (Fig. 5*A*). Vaccination with p66 delayed by ~5 weeks the time required for these mice develop tumors (Fig. 5*B*). 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 multi-focal *in situ* carcinomas (21). Vaccines were administered on weeks 15, 17 and 19 without cell-depleting antibodies. The data presented in Figure 5*B* demonstrates that by week 19 at least one of the mammary glands in the non-vaccinated mice had a tumor mass. In contrast, p66+CpG vaccination increased the amount of time required to develop tumors by ~15 weeks, demonstrating a significant therapeutic effect. Administration of three mock vaccinations (CpG alone) had no significant advantage over the non-vaccinated mice. By week 26 all the mice that did not received the p66 vaccine had more than 7 tumors each and because at least 1 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 2/5 were tumor-free.

Evaluation of immune responses in surviving animals

When the experiments described in Figures 5*A* and 5*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. 1*A*). The surviving mice that received a single p66+CpG vaccination (Fig. 5*A*), all of which had at least 5 tumors, had a small but significant response to p66 and to the TUBO (Fig. 6*A*). 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. 5*A*), displayed high responses to p66, TUBO and to the 4 additional RNEU peptides (Fig. 6*A*). 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. 6*A*). The surviving the BALB-neuT mice (4/5) from the experiment that evaluated the effect of 3 immunizations (Fig. 5*B*), also displayed high CD8 T responses against all RNEU peptides and TUBO cells (Fig. 6*B*).

Discussion

To our best knowledge, the present work represents the 1st 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 (TYVPANASL) was the most effective of the 9 predicted H-2K^{d}-binding candidates that we evaluated in eliciting tumor-reactive CTL in BALB/c mice (Fig. 1*B*). Previous studies reported that the homologous peptide from human HER2/*neu*, (TYLPTNASL) was effective in inducing CTL responses in normal BALB/c mice and that vaccination with peptide-pulsed APC (in combination with IL-12 administration) provided anti-tumor effect in animals subsequently challenged with a fibrosarcoma transfected with human HER2/*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 (Figs. 1*A*, 1*B*). These results suggest that these epitopes

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(p304, p734, p911 and p1251) are either not expressed in sufficient amounts as K^b/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 anti-tumor effects of vaccination, we do not know whether the CTL recognizing the p304, p734, p911 and p1251 epitopes play any role in conferring anti-tumor resistance.

Vaccination with p66 and CpG in BALB/c mice was able to elicit prophylactic and therapeutic anti-tumor responses against a challenge with the RNEU-expressing TUBO cell line (Figs. 1*D*, 1*E*). Vaccination with peptide p66 in IFA in the absence of CpG resulted in insignificant CTL responses and lack of anti-tumor 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 (5 daily injections) resulted in anti-tumor effects in 2/3 mice (Fig. 1*E*). The anti-tumor 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 shows that in order 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 as compared to BALB/c or that a large proportion of these cells are inhibited by CD4/CD25-expressing cells. When the anti-tumor 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 prior to vaccination enhanced the anti-tumor 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 (Figs. 2*B*, 2*C*). However, a plausible explanation is that a single vaccination provides effective anti-tumor 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 any vaccination strategy it would not be prudent to deplete CD4/CD25 Treg cells using anti-CD4 mAb since concomitant depletion of T helper cells would result in suboptimal anti-tumor effects. Other strategies have been considered to deplete or inhibit the function of Treg cells in order to enhance the effect of vaccination and achieve anti-tumor 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 is 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*neu*N 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 anti-tumor effect against the TUBO cells (Fig. 4*A*) or against spontaneous tumors (Fig. 5*A*).

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Recently it was reported that prolonged administration of anti-CD25 mAb to BALB-neuT mice (weekly from week 6 to week 24 of age) significantly delayed, but not prevented, the occurrence of mammary tumors (30). The present results show that a single vaccination of peptide+CpG administered after 3 daily injections of anti-CD25 mAb completely prevented the occurrences of spontaneous tumors in these mice to up to 35 weeks of age (Fig. 5*A*). To achieve this "persistent" anti-tumor effect it was not necessary to continue the administration of the anti-CD25 mAb, which could result in the generation of autoimmune pathology. Thus, it appears that once tolerance has been broken by an effective vaccination strategy, in the absence (or in the presence of low numbers) of Treg cells, effector tumor-specific CD8 T cells will persist in sufficient numbers to maintain a tumor-free status.

Perhaps the most significant finding of the present work was the observation that peptide vaccination helps in the prevention and treatment of spontaneous breast tumors. Our results indicate that p66 vaccination was extremely effective in preventing the occurrence of spontaneous tumors (Fig. 5), even when the vaccine was administered at an age where the mice are known to already have *in situ* carcinomas (21). The most effective prophylactic vaccination regimen was when mice were vaccinated once at week 8 (at this age most mice have atypical breast tissue hyperplasia, (21) in combination with anti-CD25 mAb therapy, where by 35 weeks of age none of the mice had evidence of disease (Fig. 5*A*). In the case where the p66 vaccine was applied once or 3 times without anti-CD25 mAb, a significant delay in the occurrence of tumors was observed and when tumors arose, these grew at a slower rate (Figs. 5*A*, *5B*). It remains to be determined whether additional booster vaccinations would further delay, or ultimately prevent the emergence of tumors in the absence of anti-CD25 mAb therapy.

Preliminary studies indicate that peptide vaccination at more advanced stages of disease, was less effective at obtaining anti-tumor effects. Only 50% of mice that were vaccinated when tumor masses were large (~0.5 cm diameter) mounted significant tumor-specific CTL responses and were able to control to some extent the progression of the tumors (P. Nava, unpublished). It is likely that the suboptimal effect of vaccination under these circumstances is due to immune suppressive activities generated by the presence of large tumors (31–35). It has been shown that the number of CD11b+/Gr1+ immature myeloid cells with immune suppressive activity increases significantly in BALB-neuT mice starting at weeks 16–20 of age and that this increase correlates with tumor multiplicity (36). For the above-mentioned reasons a strong case has been made to preferentially use cancer vaccines in the prophylactic setting (37,38), or in a relatively disease-free condition that could be achieved by early detection, or post-surgery, radiation or chemotherapy.

Acknowledgements

The authors would like to thank Adam Herron and Virginia Van Keulen for technical assistance. Supported in part by NIH grants R01CA80782 and R01CA103921.

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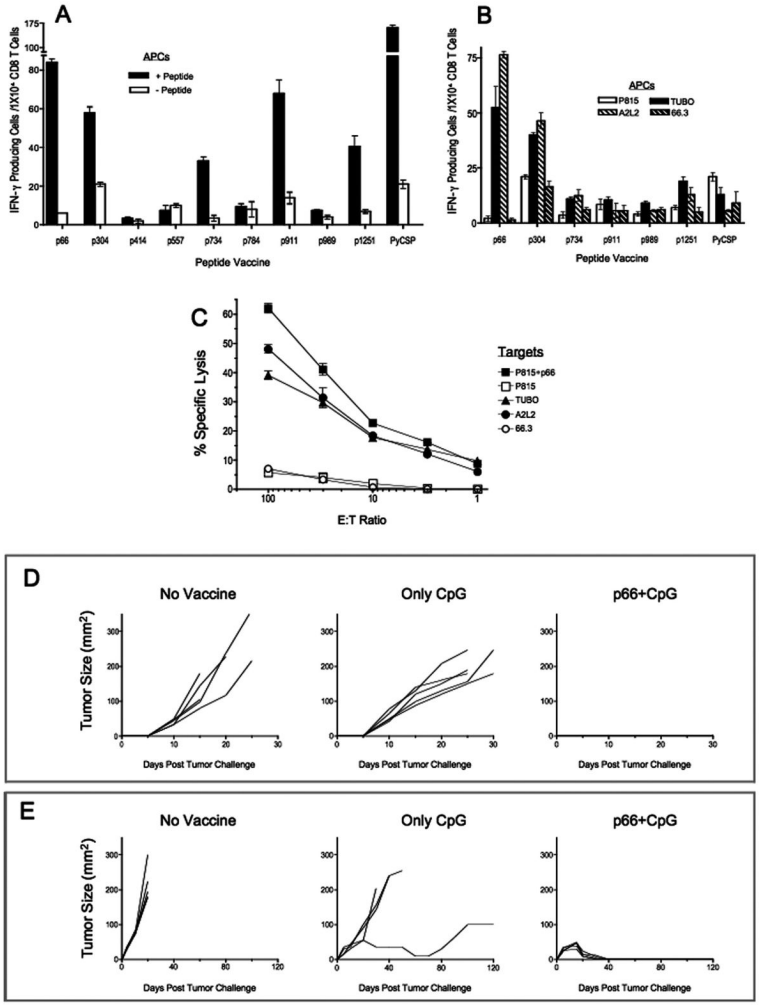

Figure 1. Vaccination of BALB/c mice with RNEU peptides induces CD8 T cell responses and tumor antigen-specific immunity

A, Groups of 3 mice were vaccinated with 100 μg of the indicated synthetic peptide emulsified in IFA and CpG as described in "Materials and Methods" and 7 days later the immune responses of CD8 T cells from pooled spleens to each corresponding peptide was evaluated using an IFN-γ ELISpot assay. *B*, Peptides that induced significant CD8 T cell responses (Fig. 1*A*) were studied for their capacity to induce CD8 T cell responses to RNEU-expressing tumors (TUBO and A2L2) and RNEU negative controls (P815 and 66.3). Data for both panels *A* and *B* represent the average ± SD of 2–3 wells containing 1×10^4 cells/well. *C*, Cell-mediated cytotoxic responses of CD8 T cells derived from p66-immunized BALB/c mice measured by the JAM assay (see Materials and Methods) against various ^{3}[H]-thymidine labeled target cells. Each point represents the mean ± SD of triplicate determinations. *D*, Prophylactic peptide p66+CpG vaccination against TUBO cells. BALB/c mice (*n=5*) received p66 peptide (100 μg) in IFA and CpG, CpG alone or no vaccine and 7 days later they were challenged s.c. with 2×10^6 live TUBO cells. *E*, Therapeutic peptide p66+CpG vaccination against TUBO cells. BALB/c mice (*n=5*) were injected s.c. with 2×10^6 live TUBO cells and 8 days later they received p66 peptide (100 μg) in IFA and CpG therapy, CpG alone or no vaccine. For both panels *D* and *E* tumor growth was measured (2 opposing diameters) and recorded every other day as explained in detail in "Materials and Methods". Each line represents the tumor size in area (mm^2) of an

individual mouse. All of these results are representative of data obtained 2–3 different experiments.

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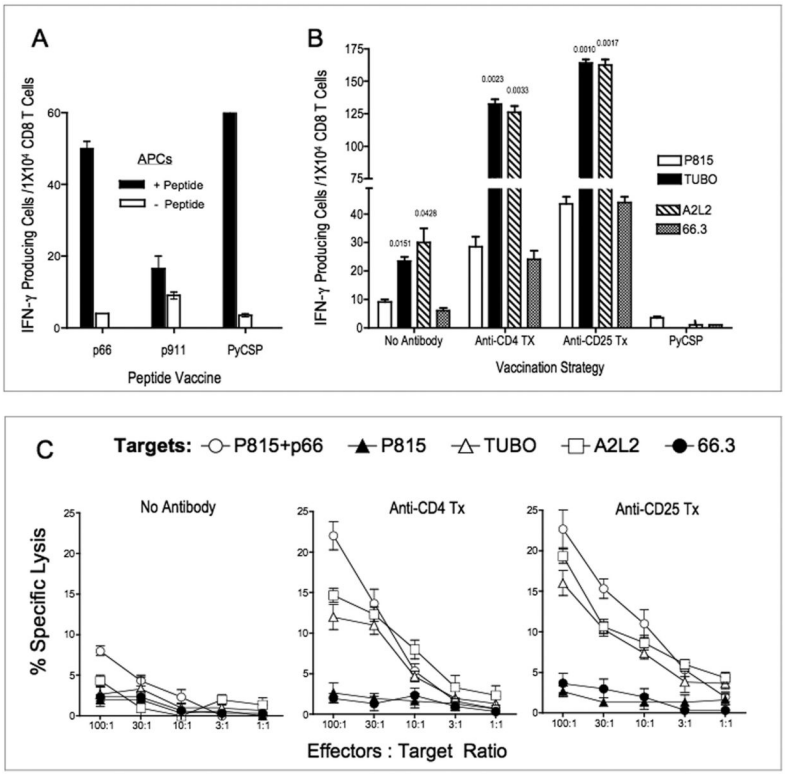

Figure 2. Vaccination of BALB-neuT mice with peptide p66 and CpG induces CD8 T cell responses but requires anti-CD4 or anti-CD25 mAb treatment for tumor-specific reactivity

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 days later the immune responses of CD8 T cells from pooled spleens to each corresponding peptide was evaluated 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 prior to vaccination (See Materials and Methods for details). Numbers above the columns represent p values of TUBO vs. P815 or A2L2 vs. 66.3. Data for both panels *A* and *B* represent the average ± SD of 2–3 wells containing 1×10^4 cells/well. *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 ^{3}[H]-thymidine labeled target cells. Each point represents the mean ± SD of triplicate determinations. All of these results are representative of data obtained 2–3 different experiments.

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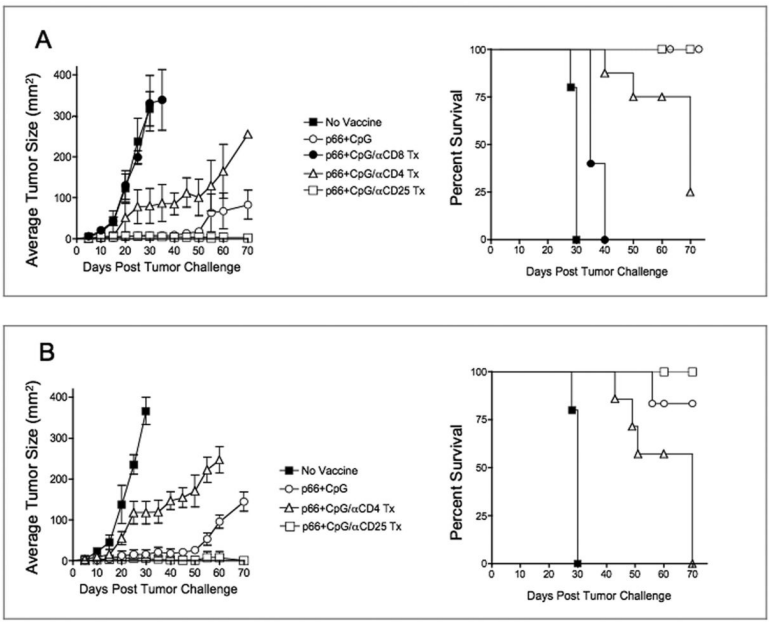

Figure 3. Prophylactic immunization with peptide p66 and CpG confers protection in BALB-neuT mice against a tumor challenge

A, Tumor growth and Kaplan-Mayer survival curves for BALB-neuT mice *(n=5/group)* vaccinated with peptide p66 and CpG, with or without the indicated mAb treatment, was performed 7 prior to challenge with live TUBO cells. Each point in the tumor growth curves represents the average tumor size (in mm^2) with SD. *B*, Similar experiment as described in panel *A*, except that mice were challenged 10 weeks after vaccination. These experiments were repeated twice with similar results.

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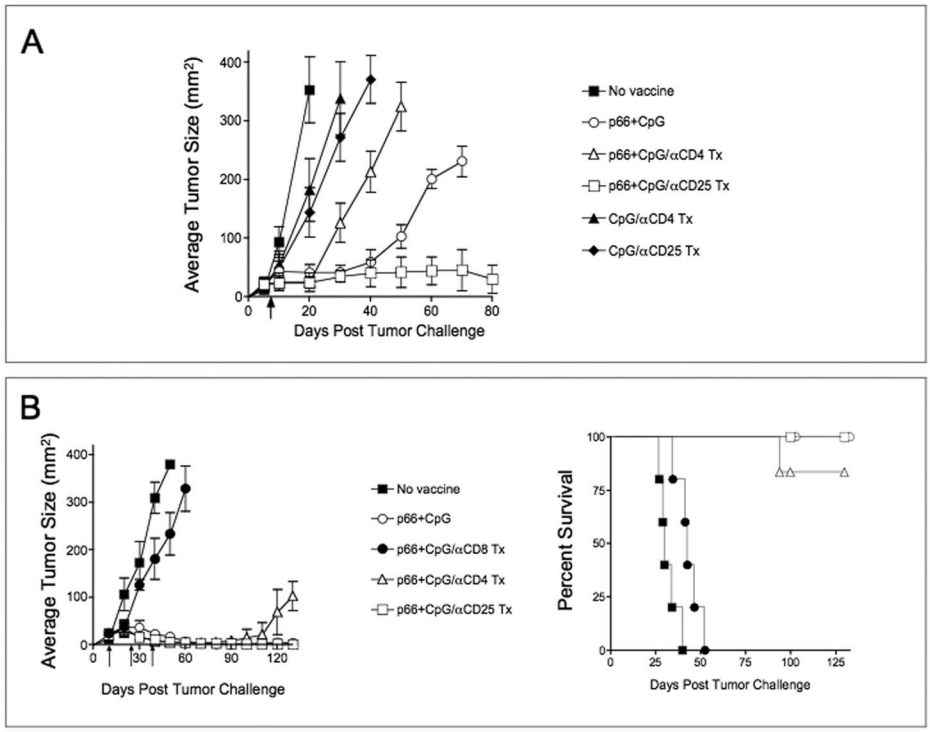

Figure 4. Therapeutic immunization with peptide p66 and CpG confers anti-tumor effect in BALB-neuT mice against TUBO

A, Tumor growth curves for BALB-neuT mice *(n=5/group)* vaccinated with peptide p66 and CpG, with or without the indicated mAb treatment, were performed 10 days after challenge with live TUBO cells. Arrows represent the day when the vaccine was administered. Each point represents the average tumor size with SD. *B*, Similar experiment as described in panel *A*, except that mice received 2 additional booster vaccinations (without mAb therapy) on days 24 and 38 post-tumor challenge. Kaplan-Mayer curves are presented to indicate the % survival for this experiment. These experiments were repeated twice with similar results.

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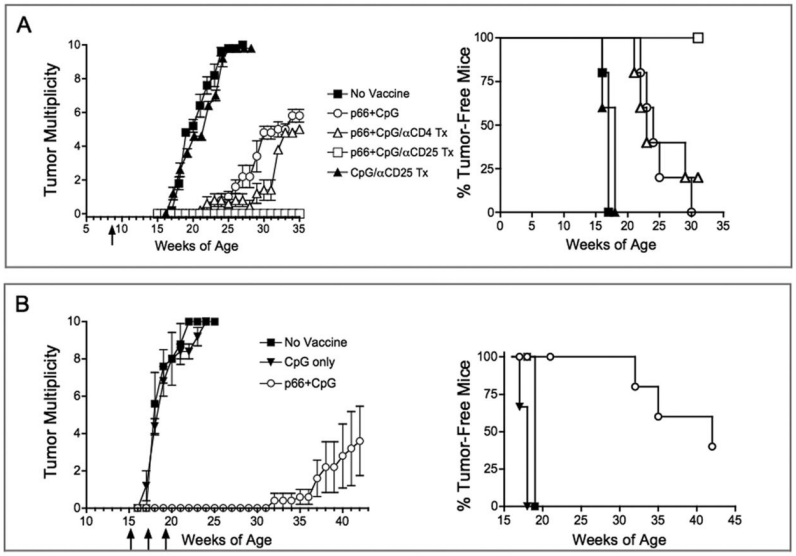

Figure 5. Vaccination with p66 and CpG results in prevention and delay of spontaneous mammary neoplasms in BALB-neuT mice

A, A single peptide immunization of BALB-neuT female mice (*n*=*5*) given at 8 weeks of age applied with and without mAb therapy. All animals were monitored for tumor appearance by manual examination of the mammary glands every 5 days. Measurable masses >2 mm diameter were regarded as tumors. Results are presented as Tumor Multiplicity, which is the mean number of tumors in each group (left panel) with SD (error bars) and as % tumor-free mice (right panel). Arrows represent the day when the vaccine was administered. *B*, Repeated peptide immunization of BALB-neuT female mice (*n*=*5*) given at week 15 of age followed by two boosters (at 17 and 19 weeks of age) in the absence of mAb therapy. Results were evaluated as described in panel *A*. These experiments were repeated twice with similar results.

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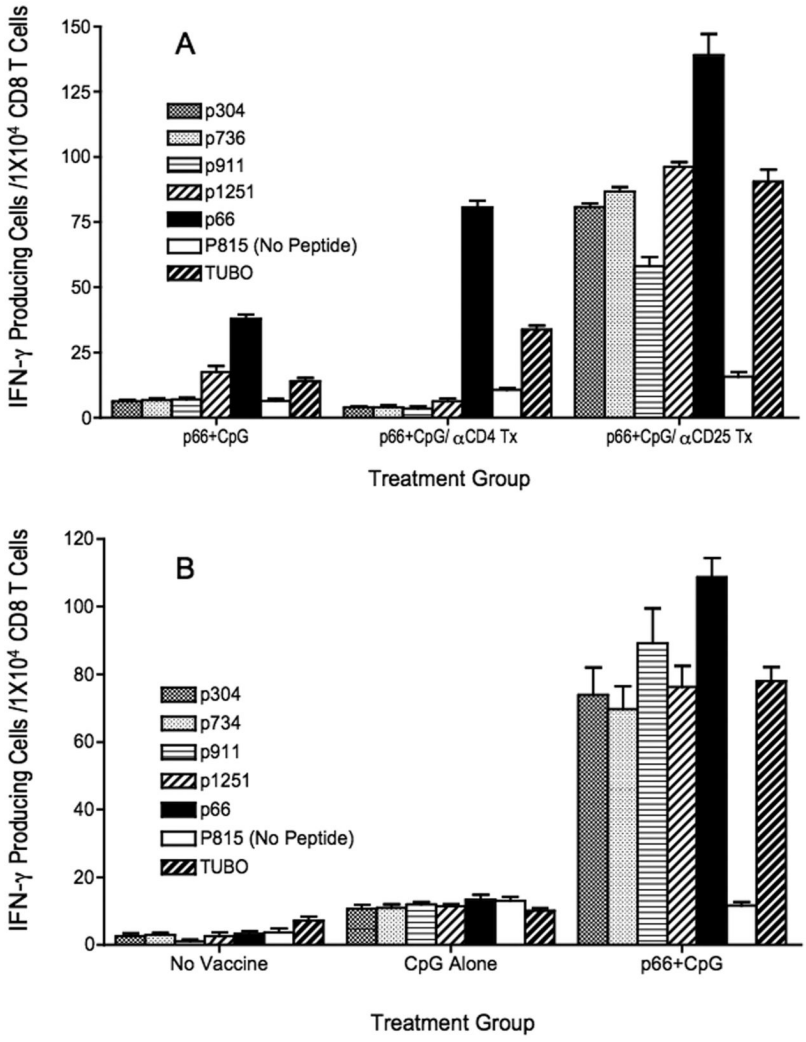

Figure 6. Immune responses and evidence of epitope spreading in vaccinated BALB-neuT mice that displayed significant effects against spontaneous tumors

A, Surviving mice from the experiment presented in Figure 5*A* were euthanatized and their splenocytes were pooled, CD8 T cells purified and evaluated for immune responses against RNEU peptides and TUBO cells using an IFN-γ release ELISpot assay. Responses against individual peptides were measured using peptide-pulsed P815 cells. *B*, Surviving mice from the experiment presented in Figure 5*B* were euthanatized and their splenocytes were pooled, CD8 T cells purified and evaluated for immune responses as described in panel *A*. The "No Vaccine" and "CpG Alone" groups for this experiment were recreated as controls, since the mice in the experiment in Figure 5*B* had all succumbed when the experiment was terminated. Mice of ~15 weeks of age similar age were mock vaccinated with CpG alone (for 3 times) or left unvaccinated and were used in these experiments as controls to compare the responses of the p66+CpG mice.