

A Multiantigen Vaccine Targeting Neu, IGFBP-2, and IGF-IR Prevents Tumor Progression in Mice with Preinvasive Breast Disease

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Abstract

A multiantigen multipptide vaccine, targeting proteins expressed in preinvasive breast lesions, can stimulate type I CD4⁺ T cells which have been shown to be deficient in both patients with breast cancer and mice that develop mammary tumors. Transgenic mice (TgMMTV-neu) were immunized with a multiantigen peptide vaccine specific for neu, insulin-like growth factor-binding protein 2 and insulin-like growth factor receptor-I at a time when some of the animals already had preinvasive lesions (18 weeks of age). Although immunization with each individual antigen was partially effective in inhibiting tumor growth, immunization with the multiantigen vaccine was highly effective, blocking development of palpable lesions in 65% of mice and slowing tumor growth in the infrequent palpable tumors, which did arise. Protection was mediated by CD4⁺ T cells, and the few slow-growing tumors that did develop demonstrated a significant increase in intratumoral CD8⁺ T cells as compared with controls ($P = 0.0007$). We also combined the vaccine with agents that were, by themselves, partially effective inhibitors of tumor progression in this model; lapatinib and the RXR agonist bexarotene. Although the combination of lapatinib and vaccination performed similarly to vaccination alone ($P = 0.735$), bexarotene and vaccination significantly enhanced disease-free survival ($P < 0.0001$), and approximately 90% of the mice showed no pathologic evidence of carcinomas at one year. The vaccine also demonstrated significant clinical efficacy in an additional transgenic model of breast cancer (TgC3(1)-Tag). Chemoimmunoprevention combinations may be an effective approach to breast cancer prevention even when the vaccine is administered in the presence of subclinical disease. *Cancer Prev Res*; 6(12); 1273–82. ©2013 AACR.

Introduction

Vaccination targeting immunogenic proteins expressed by preinvasive or proliferative breast lesions offers a unique approach to breast cancer prevention. Vaccines can elicit memory T cells that remain in lymph nodes until exposed to the target antigen. After stimulation, T cells migrate to the site of antigen-expressing lesions regardless of the location and will proliferate and destroy those lesions. HER-2/neu (HER2), insulin-like growth factor-binding protein 2

(IGFBP-2) and insulin-like growth factor receptor-I (IGF-IR) are overexpressed in multiple types of breast cancer and are associated with a poor prognosis (1–4). Likewise, these proteins are immunogenic and elicit both humoral and cellular immunity in patients with breast cancer (5, 6). All three proteins are upregulated in high-risk and preinvasive breast lesions, and expression of each is associated with an increased risk of developing invasive breast cancer. We examined whether prophylactic immunization against these proteins could prevent the development of palpable breast cancer in a preclinical model where preinvasive lesions already exist in the preponderance of mice. This approach approximates the patient population of phase III breast cancer prevention trials with selective estrogen receptor modulators and aromatase inhibitors, which evaluated treatment more than 3 to 5 years to a cancer endpoint. The short period of time needed to meet that endpoint implies that a significant proportion of the women who progressed were enrolled with incipient lesions.

The FVB/N-TgN (MMTVneu)-202Mul mouse (TgMMTV-neu) develops spontaneous mammary cancer. These cancers express neu, IGF-IR, and IGFBP-2, and investigations have shown that growth of syngeneic implanted tumors can be significantly delayed by vaccinating mice with peptides,

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derived from each of these proteins, designed to elicit antigen-specific CD4⁺ T cells (6–8). The mice are tolerant to their tumors and T cells derived from tumor-draining lymph nodes do not secrete interferon (IFN- γ) when challenged with antigen (9). The lack of type I IFN- γ -secreting tumor antigen-specific T cells has also been well described in patients with breast cancer (10). IFN- γ is needed for tumor recognition through activating antigen-presenting cells (APC) to engage T cells. Several prophylactic vaccine approaches using immunization against a single antigen, neu, have been evaluated in TgMMTV-neu mice. The extent of protection in these studies was variable and vaccine efficacy decreased significantly with age at immunization (11–13).

The multiantigen multi-peptide vaccine, described here, was designed to preferentially stimulate CD4⁺ T cells. Our goal was to determine whether the vaccine could generate protective type I immunity in the TgMMTV-neu and prevent the development of palpable breast cancer destined to occur in mice with preexisting lesions. We immunized older mice to model the clinical translation of such an approach to mature adult women, with high risk of developing breast cancer who would likely be the target population for preventative vaccination. We also questioned whether combining the vaccine with agents, which are themselves partially effective in this model in inhibiting tumor progression, lapatinib and bexarotene, might enhance the antitumor efficacy of the approach.

Materials and Methods

Mice

Female TgMMTV-neu (FVB/N-TgN (MMTVneu)-202Mul; Charles River Laboratory) and TgC3(I)-Tag mice (Dr. J Green, National Cancer Institute; NCI; Bethesda, MD) were used. Procedures were in compliance with the University of Washington (Seattle, WA) Institutional Animal Care and Use Committee guidelines. Age-matched mice were used in experiments.

Expression of antigens in mammary lesions

Preinvasive lesions were identified by a veterinary pathologist (P.M. Treuting). Immunohistochemistry was performed on preinvasive and invasive tumors and cell lines. Paraffin-embedded tissues were sectioned to 5 μ m thickness. The sections were deparaffinized by washing in xylene twice for 5 minutes, dehydrated via ethanol dilutions (100%, 95%, 70%) for 3 minutes each, and washed in distilled water. Tumor cells were processed via cytospin and stained similarly. Antigen retrieval was performed by microwave heating with sodium citrate (pH 6) for 8 minutes. Tissue sections were cooled and rinsed with PBS. After blocking with 3% H₂O₂ for 5 minutes, sections were incubated with normal goat serum (10% in PBS) for one hour at room temperature. Primary antibodies against IGF-IR (C-20, Santa Cruz Biotechnology), IGFBP-2 (C-18, Santa Cruz), or neu (clone 7.16.4; ref. 14) were applied (1:500 dilution; 10% goat serum in PBS), and the slides were incubated overnight at 4°C. Isotype control immunoglob-

ulin G (IgG) included; rabbit IgG (sc-2027, Santa Cruz), goat IgG (sc-2053, Santa Cruz), and rat IgG (2A3, University of California San Francisco; UCSF; Santa Cruz, CA). Sections were washed three times for 5 minutes and incubated (1:500) with a biotinylated anti-rabbit antibody for IGF-IR (BA-1000), a biotinylated anti-goat antibody for IGFBP-2 (BA-9500), or a biotinylated anti-rat antibody for neu (BA 9401) for one hour at room temperature (Vector Laboratories). After washing, slides were incubated with avidin-conjugated peroxidase (DAKO) at room temperature for 30 minutes, washed, and treated with 3,3'-diaminobenzidine before counterstaining with hematoxylin (DAKO). Images were captured using Nikon 80i Eclipse microscope with digital camera with NIS Elements Basic research imaging software.

Vaccination and tumor protection or inhibition

Eighteen-week-old female TgMMTV-neu mice, without palpable lesions, were immunized with single antigen (2–3 peptides/antigen) or a multiple antigen (total eight peptides) vaccine derived from the human HER2, IGF-IR, and IGFBP-2 proteins (Genemed Synthesis; Supplementary Table S1). Peptides ranged from 10 to 18 amino acids in length and were 70% or more homologous with the mouse homologue (median 97%, range 70%–100%). Vaccines were administered as previously described (6). Animals received vaccines consisting of 50 μ g/each peptide admixed in either Freund's (priming dose) or incomplete Freund's (boosters) adjuvant sq every 2 weeks for three immunizations. Monthly boosters were then administered until experiments were terminated at 44 to 52 weeks. The mean number of vaccinations received was six, range five to seven. Controls received adjuvant in PBS. Mice were evaluated for tumors via palpation as previously described (6). Disease-free survival (DFS) was defined as no evidence of palpable tumor. Overall survival (OS) was defined as the time period from birth to death regardless of cause; either the maximum tumor volume requiring euthanasia or termination of the experiment ($n = 15$ mice/group).

To determine vaccine efficacy in a different mammary cancer phenotype, 6- to 8-week-old TgC3(I)-Tag mice were vaccinated as described above with either the multiantigen vaccine in adjuvant or adjuvant alone every 10 to 14 days for three immunizations. Two weeks following the last immunization, 1×10^6 cells of a syngeneic tumor cell line, M6, generated from a spontaneous tumor (Dr. J. Green, NCI), was injected orthotopically into the mammary fat pads. The line was tested for antigen expression, as described above, 2 weeks before implant. Tumor growth was measured every 3 days until termination ($n = 5$ /group). Experiments were repeated with similar results.

Primary effector characterization and T-cell response

To standardize both tumor size and time from last vaccine before cell depletions, blocking studies were performed using a tumor implant model with a cell line syngeneic to TgMMTV-neu, mouse mammary cancer (MMC; ref. 14). Similar to M6, MMC was characterized for antigen expression

2 weeks before the described experiments. Mice were vaccinated three times, 14 days apart with the multiantigen vaccine. MMC cells, 1×10^6 , were implanted, 2 weeks from the last vaccine. Monoclonal antibodies were used for *in vivo* depletion [CD4⁺ and CD8⁺ T cells, natural killer (NK) and B cells] via intraperitoneal injection of the specific antibody every other day (3 \times) during the week before MMC was implanted and twice per week until the experiment was terminated. Doses used for T-cell depletion were 300 μ g of anti-CD4 clone GK1.5 and 100 μ g of anti CD8 clone 2.43 (UCSF). The depletion of B cells and NK cells was performed using 100 μ g of an anti-CD22 clone CY34.1.2 and anti-CD56 clone PK136 (UCSF). Rat IgG2b and IgG2a were used as controls. Data are shown as the mean and SEM of 5 mice/group. Experiments were repeated once with similar results. IFN- γ ELISPOT assays were performed as published (6). Antigens tested included the pools of peptides contained in the vaccine (10 μ g/mL/each peptide), an irrelevant class II peptide, HIV Gag p17 (ref. 15; 10 μ g/mL) or phytohemagglutinin (PHA; 1 μ g/mL), a nonspecific T-cell mitogen. Data are reported as mean absolute spots/well (SPW) \pm SEM for 5 mice/group.

T-cell purification and adoptive transfer

CD3⁺ T cells were purified from cells pooled from spleens of vaccinated and control mice using a Pan T-cell Isolation Kit (Miltenyi Biotec Inc). Purity of the CD3⁺ cells before infusions was an average of 92% (range 90%–94%). Naïve TgMMTV-neu mice were injected subcutaneously on the mid-dorsum with 1×10^6 of MMC 24 hours before an intravenous tail vein infusion of 10×10^6 CD3⁺ T cells derived from the experimental groups. Tumor growth was measured every other day ($n = 6$ mice/group).

Evaluation of tumor-infiltrating T cells

Tumors were frozen in Tissue-Tek OCT (16) and stored at -80°C . Frozen tumors were then sectioned (8 μ m), fixed in 75%/25% acetone/methanol for 5 minutes, and washed using PBS. Slides were incubated with normal goat serum (10% in PBS) for 1 hour at room temperature, washed, and incubated with rat anti-mouse CD8 (AbD Serotec) at 1:100 dilution in 10% goat serum/PBS overnight at 4°C . After washing, Alexa Fluor 488 goat anti-rat secondary antibody (Invitrogen) was added to the slides (1:1000) for one hour at room temperature. Prolong Gold antifade with 4', 6-diamidino-2-phenylindole (DAPI) mounting media (Invitrogen) was added after an additional wash and cover slips were attached. Positive cells and DAPI stained nuclei were counted in three random high-powered microscopic fields per slide and expressed as a mean. The number of positive cells over the total number of nuclei in the field was expressed as %CD8⁺. Data are shown as the mean and SEM for 5 mice/group.

Cancer incidence and pathologic evaluation

Age at the time of first palpable tumor was determined in 50 female TgMMTV-neu mice. Fifteen mice, 18 weeks old, with no palpable disease, were subject to microscopic

examination of mammary fat pads. Tissues were processed routinely, stained with hematoxylin and eosin, and examined for the presence of hyperplasia and neoplasia using published methods (17). Similar methods were used to evaluate tissues from treated mice (Fig. 4C).

Combination chemoprevention

Fifteen mice, ages 18 weeks, per group were assigned to: (i) adjuvant only, (ii) 30 mg/kg bexarotene (ONYX Scientific) administered for the duration of the experiment, (iii) vaccine, administered as described above, with adjuvant, and (iv) vaccine with adjuvant administered with 30 mg/kg bexarotene for the duration of the experiment. Bexarotene, in 200 μ L sesame oil, was given by gavage 5 days/week. The adjuvant control group was gavaged with 200 μ L sesame oil alone. Tumor growth was measured. For experiments using lapatinib (Dr. C. Grubbs, University of Alabama; Tuscaloosa, AL), the same groups were repeated but in place of bexarotene, lapatinib was administered at 75 mg/kg. One group received the vaccine with concurrent lapatinib and another received lapatinib alone. For administration of lapatinib, mice were gavaged 5 days/week with the drug in 200 μ L of sesame oil. The adjuvant control group was gavaged with 200 μ L of sesame oil alone. Lapatinib or oil control treatment was given for the duration of the experiment. The doses of agents used were those that have been shown to induce shown antiproliferative activity in mice. Each experimental group was composed of 15 mice.

Statistical analysis

Kaplan–Meier curves were generated to show the probability of DFS and OS. Curve comparisons were performed by the Gehan–Breslow–Wilcoxon test. The Student *t* test or Mann–Whitney test was used to evaluate significance. Kruskal–Wallis test was used to assess equivalence across multiple groups. In all cases, a $P < 0.05$ was considered significant. All statistical analyses were performed using GraphPad Prism version 3.02 (GraphPad Software).

Results

A prophylactic multiantigen vaccine, targeting proteins expressed in preinvasive mammary lesions, can inhibit cancer development and increase survival in TgMMTV-neu mice

The mouse homologues of all three proteins, IGFBP-2, IGF-IR, and neu, demonstrated expression in hyperplastic mammary lesions in TgMMTV-neu mice (Supplementary Fig. S1). Fifty percent of multiantigen immunized mice did not develop breast cancer as compared with 0% of controls ($P < 0.0001$). The median DFS of vaccinated animals was significantly longer than those mice receiving adjuvant alone, 46 versus 30 weeks [HR 3.959; confidence interval (CI), 2.018–7.768; Fig. 1A). At experiment termination, OS was also significantly different between the two groups ($P < 0.0002$). The median OS of the vaccinated animals had not been reached, whereas the median OS of the adjuvant control group was 40 weeks (HR 4.335; CI, 1.886–9.962; Fig. 1B).

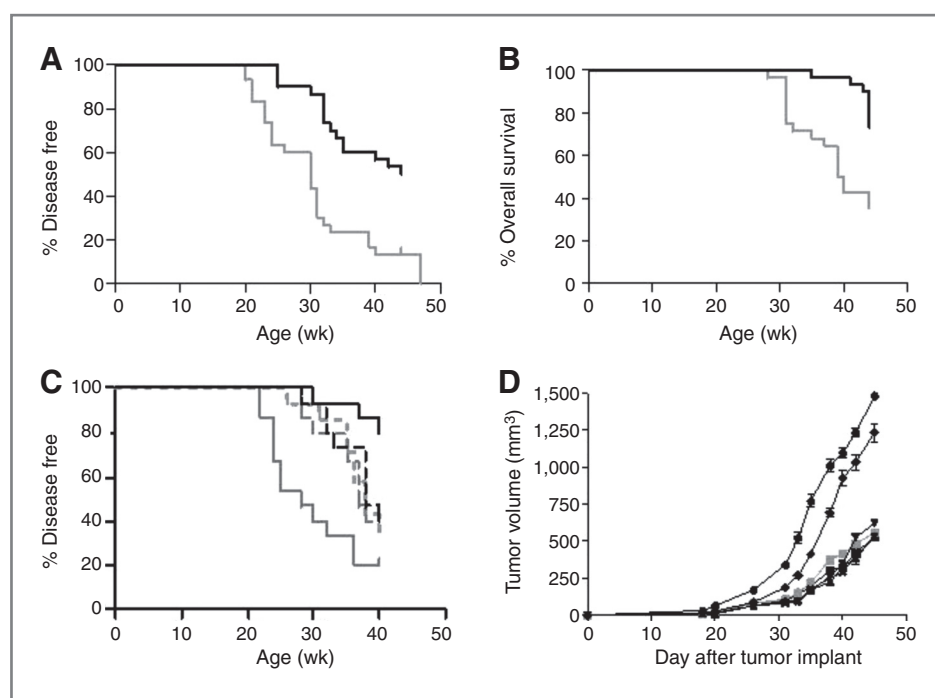


Figure 1. A prophylactic multiantigen vaccine, targeting proteins expressed in preinvasive mammary lesions, can inhibit cancer development and increase survival in TgMMTV-neu mice. **A**, percentage of mice with DFS (*y*-axis) and **B** OS (*y*-axis) after vaccination by weeks (*x*-axis) from birth. Solid gray line, adjuvant controls; solid black line, multiantigen vaccinated mice, $n = 15/\text{group}$. Data are representative of three separate experiments. **C**, percentage of mice with DFS after vaccination (*y*-axis) by age (*x*-axis). Solid gray line, controls; solid black line, multiantigen vaccine; dashed black line, neu-specific vaccine; dotted gray line, IGF-IR-specific vaccine; and dashed gray line, IGFBP-2-specific vaccine. **D**, tumor volume (*y*-axis) by day after MMC implant (*x*-axis) is shown for mice depleted of CD4⁺ T cells (◆) and CD8⁺ T cells (▼), B cells (○), NK cells (△), and isotype controls IgG2a (●) and IgG2b (■). Animals receiving vaccine alone (—◆—) and adjuvant controls (—●—) are depicted. Data are representative of two separate experiments.

The DFS of mice immunized with the multiantigen vaccine was statistically higher than that mediated by any single antigen vaccine. Mice immunized with a neu-specific vaccine demonstrated a median DFS of 38 weeks as compared with 28 weeks in adjuvant controls ($P = 0.251$ between groups; Fig. 1C). The DFS was 38 weeks for IGF-IR-immunized mice ($P = 0.367$) and 37 weeks for IGFBP-2-vaccinated animals ($P = 0.251$) compared with adjuvant controls. In the multiantigen vaccinated cohort, 80% of the mice remained tumor free and the median DFS for the multiantigen vaccinated mice was not reached at the time of experiment termination ($P = 0.0013$ compared with adjuvant control). In addition, although the DFS of the single antigen vaccinated animals was not different from each other ($P = 0.965$), the multiantigen vaccine significantly prolonged DFS compared with any of the single antigen approaches ($P = 0.018$).

Tumor protection was mediated by CD4⁺ T cells (Fig. 1D). Splenocytes from immunized mice were tested for their ability to facilitate rejection of the graft tumor cells derived from TgMMTV-neu mice. Elimination of CD8⁺ T cells, NK cells, or B cells had no impact on the antitumor response elicited by the multiantigen vaccine without T-cell depletion ($P = 0.99$ all groups compared with vaccination alone). Similarly, isotype controls administered after vaccination were not significantly different from vaccine alone ($P > 0.1$). Depletion of CD4⁺ T cells within 2 weeks of

completing immunizations, however, resulted in a significant loss of the tumor inhibitory effect of the multiantigen vaccine ($P = 0.013$ compared with vaccine without depletion). Indeed, vaccination followed by depletion of CD4⁺ T cells resulted in an antitumor effect similar to administration of adjuvant alone ($P = 0.450$).

To evaluate whether antigen coverage provided by the vaccine would effectively inhibit the growth of additional mammary cancer phenotypes, TgC3(I)-Tag mice, a model with mammary tumors that are neu⁻ and estrogen receptor negative (ER⁻), were immunized and then challenged with syngeneic tumor. Tumor growth was significantly inhibited ($P < 0.00001$) in the multiantigen vaccinated mice as compared with the adjuvant controls (Supplementary Fig. S2A). Indeed, tumor inhibition occurred despite the fact spontaneous tumors in these mice show expression of only IGF-IR and IGFBP-2 but not neu (Supplementary Fig. S2B).

Multiantigen vaccine induced T cells, from both protected and unprotected mice, demonstrate significant antitumor activity

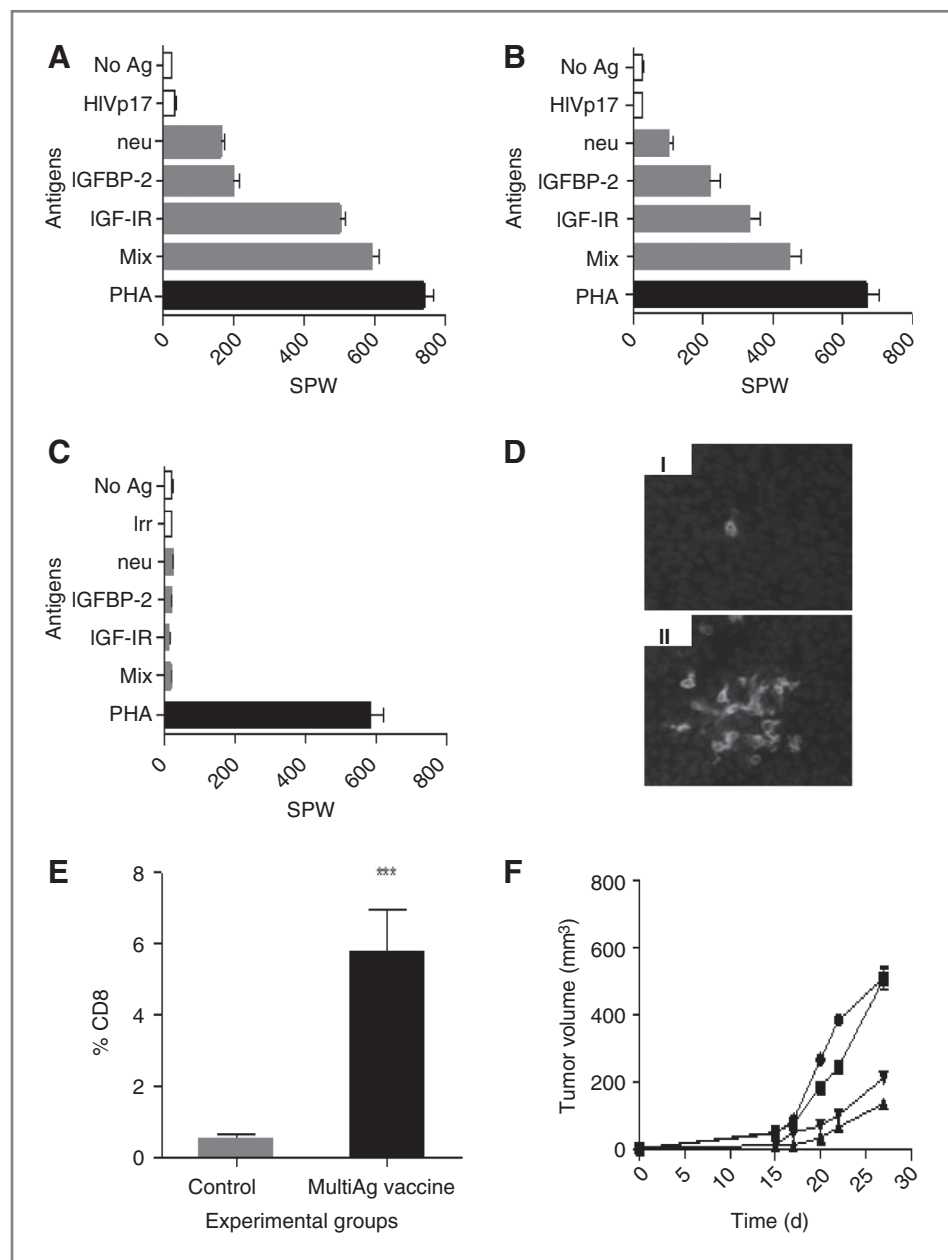
As vaccine efficacy was mediated by CD4⁺ T cells, to determine a mechanism for vaccine failure, we first evaluated potential differences in the cellular immune response between groups. For those mice protected by multiantigen vaccination (protected), the mean IFN- γ -secreting cell

response to the mix of epitopes was 591 SPW compared with 33 SPW for the HIV peptide ($P < 0.0001$; Fig. 2A). Animals who were immunized, but developed tumors (unprotected) demonstrated a mean IFN- γ -secreting cell response to immunizing peptides of 450 SPW, significantly higher than the response to the HIV peptide (24 SPW; $P < 0.001$), but not as robust as the protected group ($P = 0.006$; Fig. 2B). In both groups, single antigen-specific responses were more than the adjuvant controls ($P < 0.0001$). Animals immunized with the adjuvant alone did not develop antigen-specific immunity to either the epitope mix or single antigen pools (Fig. 2C) with the mean IFN- γ response similar to the HIV peptide ($P = 0.621$). There was

no significant difference in PHA response in animals receiving vaccines ($P = 0.319$).

Vaccine-induced CD4⁺ T cells have the capability of indirectly eliciting antigen-specific CD8⁺ T cells via the activation of tumor localized APC (18). The activated APC stimulate CD8⁺ T cells, through a process called cross-priming, resulting in CD8⁺ T cells of multiple antigenic specificities responding to the tumor. An evaluation of the tumors of mice that developed mammary cancer after multiantigen vaccination demonstrated that adjuvant vaccinated controls contained significantly less intratumoral CD8⁺ T cells (mean $0.520 \pm 0.12\%$; Fig. 2D, I and E) compared with vaccinated mice (mean $5.8 \pm 1.1\%$),

Figure 2. Multiantigen vaccine-induced T cells, from both protected and unprotected mice, demonstrate significant antitumor activity. IFN- γ -secreting cells quantified as SPW \pm SEM (x-axis) for (A) vaccinated mice protected from mammary cancer development, (B) vaccinated mice not protected from mammary cancer development, and (C) adjuvant control mice. For all groups (y-axis), white bars are the negative controls (no antigen; No Ag) and irrelevant HIV peptide (HIVp17); gray bars, responses to single antigen peptide pools (neu, IGFBP-2, IGF-IR), or to the pool of all antigen vaccinating peptides (Mix); and black bars, PHA response; $n = 5$ mice/group, assayed in quadruplicates. D, representative immunofluorescence for intratumoral CD8⁺ T cells. I; adjuvant control tumor, II; vaccinated/unprotected tumor. E, percentage of CD8⁺ cells in relation to the total number of nuclei/400X HPF (y-axis). Gray bar, controls; black bar, multiantigen vaccinated tumors (x-axis); mean and SEM of 5 mice/group; ***, $P < 0.001$. F, mean and SEM for tumor growth (mm²; y-axis), days (x-axis) after adoptive transfer of CD3⁺ cells from experimental groups. -●-, naïve; -■-, adjuvant control vaccinated; -▲-, vaccinated/protected; and -▼-, vaccinated/unprotected derived T cells (6 mice/group). The data are representative of two separately executed experiments.



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$P = 0.0007$ (Fig. 2D, II and E). This observation infers that vaccine-induced T cells could traffic to tumor. Moreover, the kinetics of tumor growth in vaccine failures was significantly slower than adjuvant controls at both 20 days ($P = 0.021$) and 40 days ($P = 0.007$) after tumor detection. The mean rate of tumor growth in control animals was $16 \text{ mm}^3/\text{day}$ as compared with $8 \text{ mm}^3/\text{day}$ in vaccinated but unprotected animals.

To assess the functionality of the entire T-cell compartment, CD3^+ T cells derived from either protected or unprotected animals were adoptively transferred to tumor-bearing mice. The tumors in mice treated with naïve cells were no different in size ($509.5 \pm 37.9 \text{ mm}^2$) than those animals treated with cells derived from adjuvant only group ($506.67 \pm 32.7 \text{ mm}^2$), $P = 0.749$ (Fig. 2F). The tumors from mice treated with T cells derived from the vaccinated animals were significantly smaller than either control, whether cells were derived from protected ($134.4 \pm 6.1 \text{ mm}^2$), $P = 0.004$, or unprotected ($212.1 \pm 13.6 \text{ mm}^2$), $P = 0.027$, mice. T cells obtained from the protected animals behaved similarly to the T cells from unprotected mice in inhibiting tumor growth ($P = 0.425$).

Vaccine failure may be due to the presence of subclinical disease at the time immunizations are initiated

As significant levels of T cells were generated by immunization and seemed to be functional, we questioned whether mice administered vaccines at 18 weeks of age had existing preinvasive lesions. We evaluated the natural history of tumor development in 50 consecutive TgMMTV-neu mice born in our colony, contemporaneous to the experiments described here, and determined that 16% of animals were found to have palpable mammary cancers between 16 to 20 weeks; similar to the time we initiated immunizations (Fig. 3A). We next examined selected serial sections of the mammary tissue harvested from 18-week-old mice that did not seem to have evidence of palpable mammary masses and determined that only half of the mice (53%) were histologically free of any mammary disease (Fig. 3B). Forty-seven percent of 18-week-old animals had histologic evidence of subclinical mammary lesions (Fig. 3B); 13% demonstrated focal hyperplasia, 27% multifocal hyperplasia, and 7% bore adenomas. Thus, many of the mice had lesions or presumably developed lesions early during the vaccination process.

Bexarotene, but not lapatinib, in combination with a multiantigen polyepitope vaccine significantly enhances vaccine efficacy

We hypothesized combining vaccination with known chemoprevention agents, lapatinib and bexarotene, that have been shown to inhibit progression of preinvasive to invasive disease in the TgMMTV-neu mouse would increase vaccine efficacy (19, 20). Vaccination in combination with lapatinib was no more effective than vaccination alone (Fig. 4A). Detectable disease was present in 53% of the group that received lapatinib/vaccine as compared with 60% with

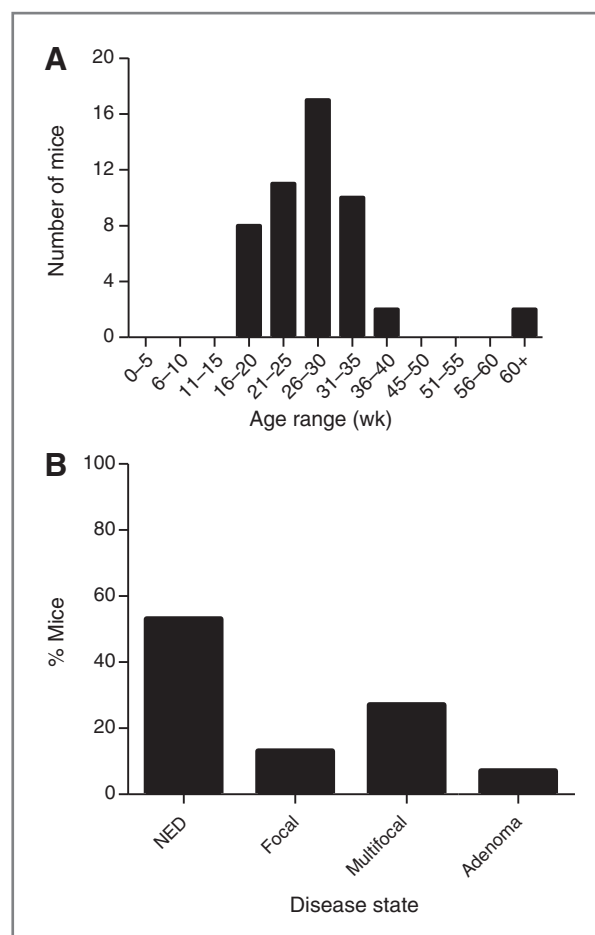


Figure 3. Vaccine failure may be due to the presence of subclinical disease at the time immunizations are initiated. A, the number (y-axis) of TgMMTV-neu mice at specific age ranges (x-axis) at the time of first palpable mammary tumor ($n = 50$). B, histologic evaluation of resected mammary tissue at the age of 18 weeks ($n = 15$) showing the percentage of mice (y-axis) with NED, focal hyperplasia (focal), multifocal hyperplasia (multifocal), or adenoma (x-axis).

vaccination alone ($P = 0.735$). Both groups had a significantly improved DFS (not reached) versus adjuvant controls ($P = 0.022$). Twenty-seven percent of mice treated with lapatinib alone were disease free (median DFS 40 weeks) as compared with 13% of adjuvant control mice ($P = 0.386$).

In contrast, vaccine combined with bexarotene was highly effective (Fig. 4B). At study termination, the group that received combination prophylaxis had not yet reached a median DFS as compared with a median DFS of 25 weeks in the adjuvant control ($P < 0.0001$; HR 20.14; CI, 6.420–63.19). Vaccination alone was significantly better than adjuvant control with a median DFS of 49 weeks ($P = 0.016$). The addition of bexarotene to vaccine significantly enhanced DFS versus vaccine alone ($P = 0.024$; Fig. 4B). The DFS outcome of the bexarotene alone treated group was similar to the adjuvant controls ($P = 0.307$).

One hundred percent of adjuvant control animals, 87% of bexarotene-treated mice, and 66% of vaccine-treated animals, but only 13% of mice who received a combination

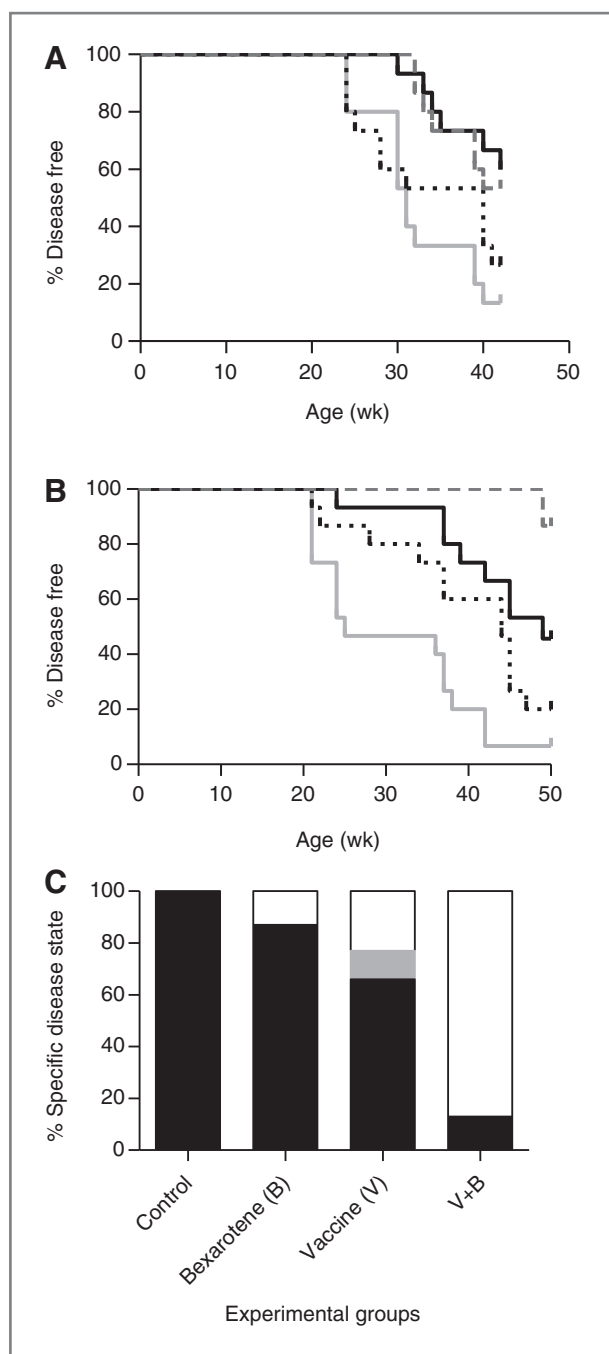


Figure 4. Bexarotene, but not lapatinib, in combination with a multiantigen polyepitope vaccine significantly enhances vaccine efficacy. A, percentage of mice with DFS after vaccination (y-axis) by weeks from birth (x-axis). Solid gray line, adjuvant controls; solid black line, multiantigen vaccinated mice; dotted black line, lapatinib alone; dashed gray line, vaccine plus lapatinib; $n = 15$ mice/group. Data are representative of two separate experiments. B, percentage of mice with DFS after vaccination (y-axis) by weeks from birth (x-axis). Solid gray line, adjuvant controls; solid black line, multiantigen vaccinated mice; dotted black line, bexarotene alone; dashed gray line, vaccine plus bexarotene, $n = 15$ mice/group. Data are representative of two separate experiments. C, percentage specific disease states; adjuvant controls, bexarotene alone (B), vaccine (V), vaccine and bexarotene (V+B). Black bars, detectable cancer; gray bars, preinvasive disease; white bars, histologic NED.

of bexarotene and multiantigen vaccination had palpable disease at study termination (Fig. 4C). We analyzed the mammary tissue from representative surviving mice with no evidence of palpable tumors. The highest proportion of mice without histologic evidence of mammary cancer or preinvasive disease, that is no evidence of disease (NED) was in the group that received combination prophylaxis, all mice evaluated (5/5) were NED (Fig. 4C).

Oral bexarotene increases the number of CD8⁺ tumor-infiltrating lymphocytes in treated animals

We evaluated the number of CD8⁺ TIL from representative mice in each group that developed mammary cancer (Fig. 5). Both the vaccine alone ($P = 0.003$) and the bexarotene alone groups ($P = 0.015$) significantly increased the number of intratumoral T cells over adjuvant controls, whereas there was no difference between the controls and animals treated with lapatinib ($P = 0.100$). Indeed, the addition of lapatinib to vaccination had no effect on the number of tumor-infiltrating CD8⁺ T cells and was equivalent to vaccination alone ($P = 0.604$). The combination of the multiantigen vaccine and bexarotene, however, resulted in a significant increase in the level of intratumoral CD8⁺ T cells over vaccine alone ($P = 0.0014$, $P = 0.0006$ compared with adjuvant control) nearly doubling the relative number of tumor-infiltrating CD8⁺ cells (Fig. 5).

Discussion

Data presented here demonstrate that vaccines stimulating antigen-specific Th cells, and composed of a limited number of immunogenic proteins expressed in high-risk lesions, can inhibit the development/progression of

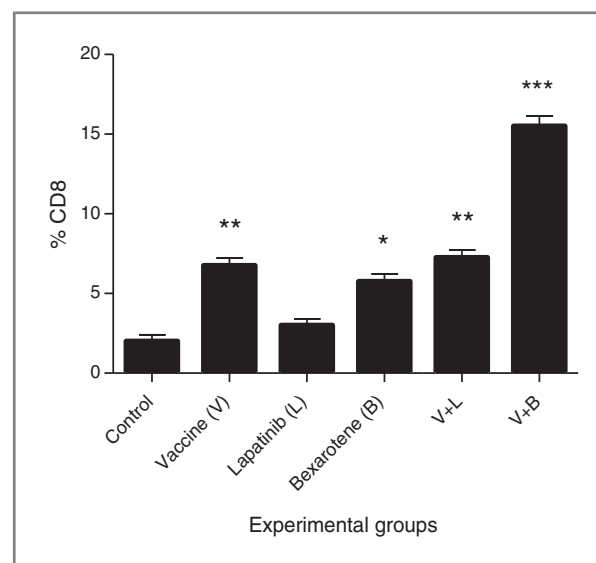


Figure 5. Oral bexarotene increases the number of tumor-infiltrating CD8⁺ T cells in treated animals. Percentage of CD8⁺ cells in relation to the total number of nuclei (y-axis). Black bars (x-axis) represent the mean and SEM of the mice/experimental group; adjuvant control, vaccine (V), lapatinib alone (L), bexarotene alone (B), vaccine +lapatinib (V+L), vaccine+bexarotene (V+B). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

mammary cancer. Furthermore, vaccination may be an effective strategy for breast cancer prophylaxis, even in high risk and older women who may be bearing occult preinvasive disease. Finally, our data suggests that some chemoprevention agents may act synergistically with vaccines. The synergistic effect of bexarotene with immunization may be due to the inherent immune stimulatory properties of RXR modulation.

HER2, one of the most commonly studied breast cancer antigens, is expressed in ductal carcinoma *in situ* (DCIS), and expression of the protein has been associated with a higher risk of the development of subsequent invasive disease (21, 22). In addition, HER2⁺ DCIS is more likely to be ER⁻ (23). Investigators have immunized patients with DCIS against HER2, before definitive resection, and demonstrated some resolution of lesions at the time of surgery (24). Remaining lesions, however, were antigens negative either due to the generated immune response eliminating antigen-expressing cells or immunity having no effect on cells which were HER2 negative. To mitigate the limited efficacy of single antigen vaccination, we focused on both extending antigenic coverage and formulating a vaccine to stimulate CD4⁺ T cells (25). The IGF family of proteins has been shown to play a role in the progression of normal mammary tissue to preinvasive lesions (26). We have previously published that IGFBP-2 is immunogenic in patients with breast cancer and more than 40% of patients have an increased level of expression of IGFBP-2 in their tumors (3, 6). IGFBP-2 has also been shown to have increased expression in atypical hyperplasia and DCIS as compared with normal glandular cells (27). Increased expression of IGF-IR in normal breast tissue has been linked with a greater risk of developing breast cancer (28, 29). Moreover, increased IGF-IR expression is correlated with non-invasive high-risk breast lesions that are hormonally driven such as atypical ductal hyperplasia (30). We have recently demonstrated that patients with breast cancer have preexistent immunity to IGF-IR (8). We reasoned a vaccine targeting both HER2 and IGF family members may give broad phenotypic coverage of ER⁺ and ER⁻ preinvasive and high-risk lesions. The observation that transgenic mouse models express similar proteins in preinvasive mammary lesions provided a stringent model in which to evaluate immune prophylactic approaches for breast cancer prevention.

Vaccines designed to stimulate CD4⁺ T cells may also function to enhance antigenic coverage. Type I cytokine secretion by Th, particularly IFN- γ , upregulates MHC class I expression on tumor cells, as well as APC, facilitating tumor recognition by CD8⁺ T cells (31). Tumor localized Th, secreting IFN- γ (Th1), will enable cross-presentation of tumor-associated antigens by APC or dendritic cells to both CD4⁺ and CD8⁺ T cells (18). Our data showing depletion of CD4⁺ T cells immediately after vaccine priming, resulting in the abrogation of an antitumor effect, suggest that the long-term tumor inhibition observed in the TgMMTV-neu was due to an initial vaccine-induced CD4⁺ T-cell response. The significant increase in intratumoral CD8⁺ T cells, docu-

mented in vaccinated unprotected animals, suggests that endogenous priming of tumor-specific CD8 T cells occurred. The ability of vaccine-induced Th1 to modulate the tumor environment may have important consequences for breast cancer prophylaxis. A type II T-cell (Th2) signature has been reported in DCIS (32). Studies indicate that Th2 may dominate the immune infiltration in breast cancer, secreting cytokines, such as interleukin (IL)-13 and IL-4, which are conducive to breast cancer progression (33). Thus, induction of a Th1 response may partially overcome the immunoinhibitory signature observed in progressing preinvasive lesions.

The target population for a prophylactic breast cancer vaccine includes high risk and postmenopausal women. Furthermore, the standard phase III breast cancer prevention trial has used a cancer endpoint within 3 to 5 years implying many individuals may have preexisting lesions. For this reason, we immunized older, mature mice many of which have preinvasive disease. Routinely, therapeutic vaccination has been marginally effective in eradicating established tumors. Analysis of prophylactic mastectomies, derived from women with a high risk of developing breast cancer, suggests a significant incidence of subclinical breast disease in many populations. A prospective evaluation of 67 women, almost 70% with known genetic mutations, demonstrated that 57% harbored a high-risk lesion or cancer with some women having multiple lesions (34). A similar study found the incidence of occult carcinoma *in situ* or invasive disease to be almost 20% (35). In more than 200 postmortem examinations in women with no known breast cancer risk, the incidence of both breast hyperplasia and carcinoma *in situ* was nearly 15% (36). Furthermore, we hoped that the multiantigen approach would be effective against a variety of subtypes of breast cancer. We show activity in neu overexpressing and neu⁻/ER⁻ mammary tumors. We are presently testing the vaccine in an ER⁺ model of breast cancer in rats.

We determined that coadministration of agents, which were themselves partially effective in this model, could improve vaccine efficacy. The agents we combined with vaccination were lapatinib and bexarotene. Lapatinib is a dual tyrosine kinase inhibitor, which blocks the activation of both HER2 and EGF receptor. Using the same dose, we used for this study, investigators have demonstrated that lapatinib could significantly delay progression of hyperplastic lesions to invasive disease in TgMMTV-neu mice (20). Similarly, bexarotene, an RXR-selective ligand has been shown to delay tumor progression when used as a chemoprevention agent in the model (19). Treatment with either lapatinib or bexarotene alone delayed the development of tumors in mice; however, DFS was not statistically different between the agents and controls when initiated in 18-week-old mice. Concurrent vaccination and oral bexarotene increased the number of disease-free mice at 1 year by almost 50% compared with vaccination alone, suggesting that bexarotene acts synergistically to enhance immune function. Lapatinib plus vaccination was not significantly different than vaccination alone.

There is evidence to indicate that treatment with bexarotene can both activate T cells as well as prevent activation-induced apoptosis. *In vitro* studies, incubating T-cell lymphoma lines with bexarotene, demonstrated a significant increase in IL-2 receptor (R) α and β on the surface of treated cells (37). Th1, secreting IL-2, may activate other T cells via IL-2R. This activation could be augmented by bexarotene treatment. In addition, RXR agonists have been shown to enhance the survival of T-cells *in vivo* potentially preventing the "activation induced cell death" T cells may undergo when chronically exposed to antigen (38, 39). Examination of tumors from mice who failed prophylaxis with concurrent vaccination and bexarotene show a marked 2-fold increase of tumor infiltrating CD8⁺ T cells versus vaccination alone. Indeed, oral bexarotene, on its own, increased CD8⁺ TIL to the level of what was present in animals receiving vaccination alone implying that immune stimulation may be a significant contributor to the efficacy of bexarotene in multiple models of breast cancer. Lapatinib seemed to have little impact on the influx of CD8⁺ TIL.

A vaccine for breast cancer prevention could offer a significant advantage over other types of chemoprevention as, if effective, will generate lifelong protective immunity potentially requiring only occasional booster vaccines. The identification of antigens expressed in preinvasive breast disease has allowed the development of a multiantigen vaccine immunizing against early tumor-promoting proteins in a manner analogous to vaccinating against the most important strains of a virus to prevent infection. Combination immune-chemoprophylaxis may significant-

ly improve the protective efficacy of vaccines designed to prevent breast cancer in high-risk populations and postmenopausal women.

Disclosure of Potential Conflicts of Interest

M.L. Disis has ownership interest (including patents) in University of Washington. No potential conflicts of interest were disclosed by the other authors.

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