L-BLP25 Vaccine plus Letrozole Induces a Th1 Immune Response and Has Additive Antitumor Activity in MUC1-Expressing Mammary Tumors in Mice

Neelima R. Mehta1,2, Gregory T. Wurz1, Rebekah A. Burich1, Brittany E. Greenberg1, Stephen Griffey4, Audrey Gutierrez1, Katie E. Bell3, Jamie L. McCall1, Michael Wolf5, and Michael DeGregorio1,2

Abstract

Purpose: In this study, we examine the immunomodulatory effects and antitumor activity of tamoxifen and letrozole when combined with the human epithelial mucin (hMUC1)-specific vaccine, L-BLP25, in the hMUC1-expressing mammary tumor (MMT) mouse model.

Experimental Design: Dose-finding studies were conducted for both tamoxifen and letrozole. Letrozole and L-BLP25 combination studies used 69 MMT female mice assigned to five groups: untreated, cyclophosphamide + placebo, cyclophosphamide + L-BLP25, letrozole 0.8 mg/kg, and cyclophosphamide + L-BLP25 + letrozole. Tamoxifen and L-BLP25 combination studies used 48 MMT female mice assigned to five treatment groups: untreated, cyclophosphamide + placebo, cyclophosphamide + L-BLP25, tamoxifen 50 mg/kg, and cyclophosphamide + L-BLP25 + tamoxifen 50 mg/kg group. Mice were injected subcutaneously with L-BLP25 (10 µg) weekly for 8 weeks. Serum cytokines were serially measured using a Luminex assay, whereas splenocytes at termination were analyzed by ELISpot to determine T-helper (Th)1/Th2 polarization of immune response.

Results: Daily oral doses of 50 and 0.8 mg/kg of tamoxifen and letrozole, respectively, resulted in a significant survival advantage over controls (P < 0.05). A predominant Th1-polarized immune response in vaccinated mice was seen with or without tamoxifen or letrozole treatments. In the L-BLP25 plus letrozole treatment group, statistically significant (P < 0.05) additive antitumor activity was observed, whereas tamoxifen plus L-BLP25 was not significantly different (P > 0.05).

Conclusion: The results of this study show that hormonal therapy does not interfere with L-BLP25–induced predominant Th1 response, and the combination of L-BLP25 with letrozole has additive antitumor activity in the MMT mouse model. Clin Cancer Res; 18(10); 2861–71. ©2012 AACR.

Introduction

Breast cancer is one of the leading causes of cancer death in women and accounts for one third of all cancer diagnoses worldwide (1). The majority of patients with breast cancer will develop estrogen receptor (ER)-positive tumors and will receive a hormonal agent during the course of their treatment. Unfortunately, a subset of patients will develop hormone-independent and/or triple-negative [ER-, Her2/]

Authors' Affiliations: 1Department of Internal Medicine, Division of Hematology and Oncology; 2Department of Environmental Toxicology, Pharmacology and Toxicology Graduate Group; 3Center of Comparative Medicine, University of California, Davis; 4Comparative Pathology Laboratory, University of California Davis School of Veterinary Medicine, Davis, California; and 5Merck Serono Research, Merck KGaA, Darmstadt, Germany

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Michael DeGregorio, Department of Internal Medicine, Division of Hematology and Oncology, University of California, Davis, 4501X Street Suite 3016, Sacramento, CA 95817. Phone: 916-734-2560; Fax: 916-734-2070; E-mail: mwdegregorio@ucdavis.edu
doi: 10.1158/1078-0432.CCR-12-0168
©2012 American Association for Cancer Research.
Translational Relevance

At present, tamoxifen and raloxifene are the only hormonal agents approved for reducing the risk of breast cancer. Unfortunately, these agents are only effective in women at risk of developing hormone-dependent cancer. Patients who develop hormone-independent and/or triple-negative [estrogen receptor (ER)−, Her2/neu−, progesterone receptor (PR)−] breast cancer have limited therapeutic options. A new therapeutic direction is the development of an immunotherapy that targets a tumor-associated antigen overexpressed in breast cancer, such as mucin1 (MUC1). Several vaccines such as L-BLP25 that target MUC1 are under development for a variety of epithelial cancers, such as lung, breast, and pancreatic. In this study, we examine the immunomodulatory effects and antitumor activity of L-BLP25 when combined with tamoxifen or letrozole in the hMUC1-expressing mammary tumor mouse model. The results of this study support clinical development of L-BLP25 immunotherapy combined with letrozole as a new treatment and prevention strategy for breast cancer.

MUC1 (13, 15). This vaccine generates CTLs capable of destroying MUC1-expressing tumor cells and produces T-helper (T_h)1-polarized cytokine responses (16, 17). L-BLP25 liposomal vaccine (L-BLP25, Merck KGaA) is in phase III clinical development for non–small cell lung cancer (NSCLC). L-BLP25 in NSCLC showed increased survival rates among vaccinated patients (18). In addition to NSCLCs, clinical studies evaluating the benefits of L-BLP25 immunotherapy combined with breast cancer hormonal therapies such as tamoxifen or aromatase inhibitors (e.g., letrozole, anastrozole) are being considered.

Babina and colleagues reported that tamoxifen is capable of inducing a shift from a cellular (T_h1) to a humoral (T_h2) immunity (19, 20). Tamoxifen also induces latent TGFβ in breast cancer cell lines (21) and prevents maturation of antigen-presenting cells (22). TGFβ can inhibit CTLs and induce regulatory T cells (Tregs), creating an immunosuppressive tumor microenvironment (23). The aromatase inhibitor anastrozole increased levels of proinflammatory cytokines IFN-γ and interleukin (IL)-12 (T_h1) and decreased levels of IL-4 and IL-10 (T_h2) cytokines in an experimental polyarthritis study. Anastrozole also suppressed the differentiation of naive T cells into Tregs, which are known to produce immunosuppressive cytokines in the tumor microenvironment (24, 25). These observations make it necessary to investigate the immunomodulatory properties of the commonly used hormonal therapies on the L-BLP25–induced immune response.

Materials and Methods

Chemicals

Tamoxifen citrate was a gift from the Orion Corporation, Orion-Pharma. Letrozole was purchased from Toronto Research Chemicals, Inc. Carvedilol, used as the internal standard for letrozole bioanalysis, was purchased from Tocris Bioscience. Human MUC1 peptide (BP25) STAP-PAHGVTSPDTRPAPSTAPP, scrambled human MUC1 peptide (BP L-424), L-BLP25 lyophilisate vaccine, and L-BLP25 placebo were provided by Merck KGaA. 17β-Estradiol and cyclophosphamide were obtained through Sigma Chemical Company.

Animals

The hMUC1-expressing mammary tumor (MMT) mouse model was developed by Mukherjee and colleagues (26). Breeding pairs (MTag.Tg; males and MUC1.Tg; females) were purchased from Mayo Clinic (26). Following breeding protocols, 267 female MMT mice were supplied for these studies by the University of California (UC) Davis (Sacramento, CA) Mouse Biology Program housed at the UC Davis Center for Laboratory Animal Science vivarium. All animal studies were conducted under a protocol approved by the UC Davis Institutional Animal Care and Use Administrative Advisory Committee. UC Davis is an Association for Assessment and Accreditation of Laboratory Animal Care accredited institution.

Genotype screening

MTag forward and reverse primers were 5’-TTGGATACATTTT-GCTGGTAGTG-3’ and 3’-CAGCAATCTTCG-GTTGCTT-G-5’. The MTag TaqMan probe (AACATGAATCGTCCAGCTT-G) carrying a 6-carboxyfluorescein reporter label and a 3’ MGBNFQ quencher group was used. For MUC1, forward and reverse primers were 5’-CAGCTTCTCCCCCAACCITAAGTG-3’ and 3’-GGGTGGGCTGGTGTCATG-5’. The MUC1 TaqMan probe (ACCAGTCCCC CCC-TAGC) carrying a 5’ VIC reporter label and a 3’ MGBNFQ was used. The PCR amplification program consisted of one cycle of 2 minutes at 95°C and 40 cycles of 15 seconds each at 60 and 95°C.

Dose-finding studies

Tamoxifen treatment. A total of 22 MMT female mice (4–5 weeks old) were weighed and randomly assigned to cages such that the average age and weight in each treatment arm were approximately equal. There were a total of 4 treatment groups: control (N = 7) and tamoxifen 1.0, 25, and 50 mg/kg (N = 5 at each dose).

Letrozole treatment. A total of 52 MMT female mice were weighed and randomly assigned to cages in the 4 treatment arms as described earlier. The treatment groups consisted of control and letrozole 0.08, 0.8, and 8.0 mg/kg (N = 13, all groups).

Study design

The drugs were dissolved in dimethyl sulfoxide (DMSO) and then diluted in peanut oil such that 100 μl delivered the appropriate doses. The final concentration of DMSO in all solutions was 2.0%. Control mice received a solution of peanut oil with 2% DMSO. All mice were dosed daily by oral gavage using a stainless steel, 20-gauge gavage needle (Popper & Sons, Inc.). All mice were
weighed and palpated for the presence of new mammary tumors once a week. When possible, tumors were measured in 3 dimensions using calipers, and an approximate volume was calculated using the formula \( V = \frac{1}{2} \times w \times l \times h \), where \( V \) is the volume, \( w \) the width, \( l \) the length, and \( h \) is the height of the tumor. Once the tumor(s) reached the cumulative volume of 1.5 cm\(^3\) or was abscissed or visibly impairing the mobility of the mouse, the mice were euthanized by CO\(_2\) asphyxiation. Whole blood was collected by cardiac puncture and placed in heparinized tubes. The blood was centrifuged to collect plasma for high-performance liquid chromatography (HPLC) analysis. Following blood collection, the following tissues were harvested for HPLC analysis: liver, tumor, and spleen. Mice were gavaged daily with tamoxifen, letrozole, or control. Whole blood was collected during each treatment visit, and serum was isolated for cytokine analysis. Mice achieved maximum tumor burden after the sixth dose of vaccine and were euthanized. Serum and splenocytes were collected for cytokine analysis.

**Immune phenotype in immunized female MMT mice**

This study was designed to examine the impact of immunization on T\(_{eff}\) or T\(_{reg}\) cytokine polarization in female MMT mice. A total of 16 female MMT mice were divided into 4 treatment groups: untreated, cyclophosphamide only, placebo, and vaccine (\( N = 4 \), all groups). To reduce T suppressor lymphocyte activity, all mice except those in the untreated group received a single intraperitoneal (i.p.) injection of cyclophosphamide (100 mg/kg) 3 days before beginning the L-BLP25 vaccine regimen (30–33). Mice in the vaccine group were then s.c. injected with 10\( \mu \)g of L-BLP25 (17) in 100 \( \mu \)l of empty liposomes. Following the fourth, sixth, and eighth doses of vaccine or placebo, blood was collected via submandibular bleeds. Whole blood was pooled within a treatment group and serum was isolated for cytokine analysis. Mice achieved maximum tumor burden after the sixth dose of vaccine and were euthanized. Serum and splenocytes were collected for cytokine analysis.

**Effects of hormonal therapy on L-BLP25 immune response**

A total of 60 MMT female mice (approximately 4 weeks old) were weighed and assigned to cages. On study day 119, mice were assigned to 4 treatment arms with approximately equal average weights and tumor volumes: control, tamoxifen 50 mg/kg, letrozole 0.8 mg/kg, and estradiol 0.5 mg/kg (\( N = 8 \), all groups). All mice were given a single i.p. injection of cyclophosphamide (100 mg/kg). Daily oral dosing was continued until the conclusion of the study. Three days after cyclophosphamide treatment, one mouse from each treatment group was removed and placed in a separate cage. These mice were not part of the vaccine treatment. The other 28 mice were injected s.c. with 100 \( \mu \)l of L-BLP25 (10 \( \mu \)g) weekly until the mice reached maximum tumor burden. Approximately 24 hours after the third and fourth dose of vaccine, whole blood was collected via submandibular bleeds. Whole blood was pooled within a treatment group and serum was isolated for cytokine analysis. Mice achieved maximum tumor burden after the sixth dose of vaccine and were euthanized. Serum and splenocytes were collected for cytokine analysis.

**Effects of combining L-BLP25 with hormonal therapy on overall survival**

Letrozole and L-BLP25 combination studies used 69 MMT female mice assigned to 5 groups (\( N = 13–14 \)): untreated, cyclophosphamide + placebo, cyclophosphamide + L-BLP25, letrozole 0.8 mg/kg, and cyclophosphamide + L-BLP25 + letrozole. Tamoxifen and L-BLP25 combination studies used 48 MMT female mice assigned to 5 treatment groups (\( N = 9–10 \)): untreated, cyclophosphamide + placebo, cyclophosphamide + L-BLP25, tamoxifen 50 mg/kg, and cyclophosphamide + L-BLP25 + tamoxifen 50 mg/kg group. All mice were dosed by oral gavage on a daily basis with tamoxifen 50 mg/kg, letrozole 0.8 mg/kg, or control according to treatment group assignment starting from the age of 4 weeks. All mice except for those in untreated groups were given a single i.p. injection of cyclophosphamide (100 mg/kg). Three days after cyclophosphamide treatment, mice were injected s.c. with 100 \( \mu \)l (delivering 10 \( \mu \)g) of L-BLP25 or placebo according to treatment group once each week for 8 weeks. Daily oral dosing was continued until the conclusion of the study. The tamoxifen combination study was terminated on day 135 after approximately 70% of untreated mice had reached maximum tumor burden. The letrozole combination study was first analyzed on study day 116, when 85% of the untreated mice had reached maximum tumor burden. The letrozole combination study was first analyzed on study day 137, when 85% of mice in the cyclophosphamide + vaccine and cyclophosphamide + placebo groups had reached maximum tumor burden. Serum and splenocytes were collected for cytokine analysis.

**Immunohistochemistry**

The breast tumors, left kidney, and spleen were harvested from each mouse at the time of sacrifice. Tissues were then paraffin-embedded and step-sectioned at 4 \( \mu \)m for immunohistochemical analysis. Immunohistochemistry was carried out using a MUC1 antibody (CD227, 550486; 1:400; BD Pharmingen). ER-\( \alpha \) staining was conducted using a 1:1,200 diluted rabbit polyclonal primary antibody (MC-20; Santa Cruz).
Western blotting analysis
The following antibodies were used to assess protein expression: ER-α (E115; Novus Biologicals NB110-56961 lot#YF112101C, 1:1,500), ER-β (Abcam, ab3576, lot#GR14900-1, 1:1,500), and β-actin (AC-15; Sigma A5441, 1:50,000). All images were obtained on a FluorChem E System with AlphaView (SA) software, version 3.2.2 (Cell Biosciences).

Luminex
We used the mouse cytokine 10-plex panel (Invitrogen) to evaluate the cytokine levels in collected mouse serum. The cytokines assessed consisted of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IFN-γ, granulocyte macrophage colony—stimulating factor (GM-CSF), and TNF-α. The assay was conducted according to manufacturer’s protocol. Cytokine concentrations were acquired on a BioPlex system using BioPlex software version 5.0 (BioRad).

ELISpot assay
Splenicocytes from vaccinated and unvaccinated mice were examined for Th1/Th2 polarization by analyzing 2 key cytokines: IFN-γ for Th1 and IL-4 for Th2 polarization. BD ELISPOT mouse IFN-γ (BD) and dual color ELISpot mouse IFN-γ/IL-4 (R&D systems) kits were used for this assay. The BP25 peptide and scrambled peptide (BP 1-424) were prepared at a final concentration of 500 ng/mL in culture medium. The lymphocytes were incubated with no peptide (medium only), BP25, or scrambled peptide at 37°C overnight.

CTL assay
From an ongoing study, we took female MMT mice belonging to 3 treatment groups: untreated, vaccine, and placebo. All mice in the vaccine and placebo groups had received a single i.p. injection of cyclophosphamide (100 mg/kg) 3 days before beginning the L-BLP25 vaccine regimen. One day after the fifth weekly dose of vaccine/placebo was administered, the mice were euthanized and the spleens were collected for CTL assay. For effector cells, spleens were harvested aseptically from mice given L-BLP25 or placebo and ground through 100-mesh sterile sieves into 5 mL of 1× PBS. For separation of CD8-positive T cells, Isolation Kit II (Miltenyi Biotec, cat. no. 130-095-236) was used. Cell concentration was adjusted to 1×10⁷ cells/mL. For target cells: hMUC1-expressing murine breast cancer cells were derived from MMT tumors. Cells were resuspended in PanToxiLux wash buffer (OncoImmunin Inc., cat. no. PTL 802-8), at a final concentration of 2×10⁶ cells/mL. Flow cytometry was conducted on an LSRFortessa (BD Biosciences) and data were collected using BD FACSDIVA software. Data were analyzed using FCS Express 4 (De Novo Software) software.

Statistical analysis
Overall survival was compared graphically for different treatment groups using Kaplan–Meier estimates. Kaplan–Meier survival curves and log-rank tests for significance were generated using GraphPad Prism 5 software. Bonferroni’s adjustment for multiple tests was used to lessen the likelihood of a false-positive result. For the ELISpot assay, spot-
forming cells (SFC) are presented as the mean value of triplicate wells. The differences in SFC levels were analyzed by 2-way ANOVA.

Results

MMT tumors respond to SERM/AI treatment

To determine the most effective doses of tamoxifen and letrozole in the MMT mouse model, we conducted a series of dose-finding studies. In the tamoxifen dose-finding study, animals in the highest dose group (50 mg/kg) showed a significant survival advantage over controls ($P = 0.0210$; Fig. 1A). For the letrozole dose-finding study, both the 0.8 and 8.0 mg/kg treatment groups were associated with significantly improved survival compared with the control group ($P = 0.0085$ and $P = 0.0052$), respectively (Fig. 1B). No survival advantage was seen with 8 versus 0.8 mg/kg. The bioavailability of tamoxifen and letrozole was verified after oral dosing using HPLC bioanalytic methods (data not shown).

ER status

To examine the ER status and potential antiestrogen sensitivity of the MMT tumors, mice were allowed to develop tumors, which were then isolated for Western blotting. ER-$\alpha$ and hMUC1 expression in an MMT tumor was confirmed by immunohistochemistry (Supplementary Fig. S1). Using selected samples from various studies, we confirmed that ER-$\alpha$ and hMUC1 expression were retained. A cell line established from an MMT tumor (MMT-494) shows preservation of the same protein expression patterns of ER-$\alpha$ and ER-$\beta$ in vitro (Fig. 1C).

L-BLP25 induces predominant $T_{H1}$ cytokine response

Noticeable differences were observed in cytokine levels of vaccinated versus untreated, cyclophosphamide, and placebo-treated mice. The levels of IFN-$\gamma$ were greater in vaccinated mice than in placebo-treated mice at 24 hours after the fourth, sixth, and eighth doses of vaccine (Fig. 2). Levels of IL-4 in vaccinated mice were not different at any time point. The levels of IL-6 were elevated in vaccinated mice in comparison with placebo-treated mice. Although levels of TNF-$\alpha$ were not elevated in all vaccinated mice, a trend was evident. The observed elevated levels of IL-6 and TNF-$\alpha$ in vaccinated mice may be related to the adjuvant (monophosphoryl lipid A) used in the vaccine and support immunogenicity of the vaccine. In summary, the results indicate a predominant $T_{H1}$ polarization of cytokines following vaccination.

Figure 2. Serum cytokine levels in vaccinated MMT females versus placebo/untreated and cyclophosphamide (CTX)-treated mice. Whole blood was collected via submandibular bleeds 24 hours after the fourth (A), sixth (B), and eighth (C) doses of vaccine or placebo. Whole blood was pooled within each treatment group (at least 3 mice per group) and serum was analyzed using Luminex technology. The levels of IFN-$\gamma$ were noticeably higher in vaccinated mice than in placebo-treated mice at all time points. IL-4 levels in vaccinated mice compared with placebo-treated mice were not noticeably different at any time point. Serum levels of IL-5 appeared to be higher in all vaccinated mice than in placebo-treated mice. Concentration values are means of duplicate samples and error bars represent the range. Missing bars represent values below detection limits.
Hormonal therapy does not interfere with L-BLP25–induced immune response

This study was conducted to examine the impact of pretreatment with hormonal therapy on the overall immune response induced by L-BLP25 vaccine. Serial cytokine analysis of serum specimens after the third and fourth doses of vaccine showed a clear Th1 response with noticeably higher levels of IFN-γ in vaccinated mice from all treatment groups (Fig. 3). The levels of IL-4 in vaccinated mice were unchanged compared with unvaccinated mice.

Terminal serum cytokines levels in each treatment group were analyzed (Fig. 4). These results confirmed the serial cytokine response observed showing that the vaccinated mice have elevated IFN-γ levels compared with unvaccinated mice. Serum IL-5 levels were noticeably elevated in all vaccinated mice. There was a trend of elevated IL-6 and TNF-α levels in vaccinated mice, which is associated with the immunogenicity of the vaccine. The IL-10 levels were unchanged in vaccinated mice compared with nonvaccinated mice, whereas IL-12 levels were higher in only vaccinated mice. Although the IL-4 and IL-5 levels were elevated in some vaccinated mice, on balance, the predominant immune response was Th1-polarized as evidenced by the IFN-γ and IL-12 cytokine response.

These data were supported by the ELISpot analysis of splenocytes from the aseptically collected spleens. Control vaccinated mice showed a highly significant increase in IFN-γ SFC levels (P < 0.001) in comparison with nonvaccinated control mice (Fig. 5A). The IL-4 SFC levels were not statistically different from those of media/scrambled peptide-stimulated splenocytes (P > 0.05; Fig. 5C–D). These results confirm that the Th1 polarization is retained 120 hours after vaccination. The difference between Luminex and ELISpot is probably because of the delayed nature of splenocytes compared with transient plasma cytokine changes.

In a subset of 32 tumor-bearing mice, tamoxifen 50 mg/kg, letrozole 0.8 mg/kg, and estradiol 0.5 mg/kg (N = 8, all groups) were examined for antitumor activity. Survival was compared between vaccine treatment alone with antiestrogen aromatase inhibitor-treated and vaccinated treatment groups. The tumor progression rates for vaccinated mice treated with tamoxifen or letrozole were not statistically different from the vaccine alone (log-rank test: P = 0.5117 and 0.886; Supplementary Fig. S2A and S2B). Mice which were vaccinated and treated with estradiol had significantly shorter survival compared with vaccine treatment alone (log-rank test: P = 0.0429; Supplementary Fig. S2C).

Letrozole and tamoxifen in combination with L-BLP25

The main aim of these L-BLP25 combination studies was to determine whether combining L-BLP25 with hormonal therapy provides any overall survival advantage over L-BLP25 or hormonal therapy alone. These studies were designed to use untreated and cyclophosphamide + placebo groups as controls. As in the dose-finding studies, survival data were compiled for both combination studies to generate Kaplan–Meier survival curves.

Letrozole. Interim analysis on study day 113, as measured by mean tumor volumes for all surviving mice, showed that treatment with L-BLP25 in combination with letrozole reduced mean tumor burden (Fig. 6A). In the letrozole and L-BLP25 combination study on day 116, when 85% of the untreated mice had reached maximum
tumor burden, mice in the cyclophosphamide + L-BLP25 and letrozole + cyclophosphamide + L-BLP25 treatment groups showed a clear and significant survival advantage over untreated mice (log-rank test: \( P < 0.001 \)), whereas the cyclophosphamide + placebo group was not significantly different from the untreated group (log-rank test: \( P = 0.3457 \)). Letrozole in combination with L-BLP25 showed significantly improved survival compared with letrozole alone (log-rank test: \( P = 0.0405 \); data not shown).

As per our design, we continued the study until 85% of mice in the cyclophosphamide + placebo group were euthanized because of excessive tumor burden, at which time (day 137) survival data were calculated again for this study (Fig. 6B). Confirming our previous findings, animals in the cyclophosphamide + L-BLP25 and letrozole + cyclophosphamide + L-BLP25 treatment groups showed a clear and significant survival advantage over the untreated group (log-rank test: \( P = 0.0086 \) and \( P < 0.0001 \), respectively). Letrozole in combination with L-BLP25 showed significantly improved survival compared with the cyclophosphamide + L-BLP25 alone treatment group (log-rank test: \( P = 0.0024 \)). Letrozole in combination with L-BLP25 was, however, not significantly different when compared with the letrozole-alone treatment group (log-rank test: \( P = 0.0895 \)).

Tamoxifen. Interim analysis on study day 113, as measured by mean tumor volumes for all surviving mice, showed that treatment with L-BLP25 in combination with tamoxifen reduced mean tumor burden, although not to the same extent as tamoxifen monotherapy (Fig. 6C). On study day 135, only the mice in the tamoxifen 50 mg/kg group showed a significant survival advantage over the untreated group (log-rank test: \( P < 0.0083 \); Fig. 6D). It was also noted that neither cyclophosphamide + L-BLP25 nor tamoxifen alone were significantly different from the cyclophosphamide + L-BLP25 plus tamoxifen treatment group, \( P = 0.1229 \) and 0.1451, respectively (Fig. 6D). Although not significantly different, both interim tumor analysis and overall survival data showed that combining L-BLP25 with tamoxifen does not provide any added advantage over tamoxifen monotherapy.

L-BLP25 elicited CTLs specific for hMUC1

To determine whether CTL activity was induced, we exposed splenocytes (effector cells) from vaccinated mice to hMUC1-expressing breast cancer cells established from an MMT tumor (target cells). With a single-cell cytotoxicity assay kit that detects both granzyme B and upstream caspase activation, a higher cytotoxicity was observed with effector cells (CD8\(^+\) T cells) from vaccinated mice in comparison with placebo and untreated mice (Supplementary Fig. S3). We also saw 35% cytotoxicity in cyclophosphamide plus placebo treatment group. One explanation for such a high cytotoxicity in this treatment group may be that the cyclophosphamide treatment eliminates T suppressor cells, therefore allowing tumor-sensitized T cells to attack the tumor cells to a greater extent (34). These mice already had existing tumor burden, and the presence of MUC1-specific CTLs was confirmed by the 18% cytotoxicity we saw in the untreated group. Cyclophosphamide followed by the vaccine
regimen augmented this existing cytotoxicity to approximately 45%.

Discussion

In this study, we investigated the immunomodulatory effect of tamoxifen and letrozole on the L-BLP25-induced immune response in a MUC1-expressing breast cancer mouse model. Our results showed that both tamoxifen and letrozole do not interfere with the T11-polarized cytokine response induced by L-BLP25, although only letrozole in combination with L-BLP25 showed a significant survival advantage. To our knowledge, this is the first demonstration of a hormonal therapy combined with a vaccine that achieved additive antitumor activity and survival benefit in a preclinical model.

Mukherjee and colleagues first used the MMT model to test a vaccine formulation composed of L-BLP25 and human recombinant IL-2 (26). In this study, MMT mice were vaccinated with liposomal MUC1 formulation every 2 weeks starting at 7 weeks of age for a total of 6 doses. The immunized mice were positive for T cells that express intercellular INF-γ, were reactive with MHC class I H-2Dβ/MUC1 tetramer, and were cytotoxic to MUC1-expressing tumor cells in vitro. Immunized MMT mice had significantly lower tumor burden at 18 weeks of age than controls. However, by 20 to 24 weeks of age, no significant difference in tumor burden was observed between immunized and control mice (26). In a recent study by this group, MUC1.Tg mice were immunized with a glycosylated MUC1-derived glycopeptide covalently linked to a Toll-like receptor (TLR) agonist (6). After 35 days, mice were transplanted with $1 \times 10^6$ MMT mammary tumor cells, followed by additional vaccination 7 days after implantation. On day 14, postimplantation mice were euthanized and the efficacy of the vaccine was determined by tumor wet weight. The results of this study suggest that the vaccine elicited potent antitumor response (6).

In agreement with previous findings of Mukherjee and colleagues (26), our study showed that vaccination with L-BLP25 does not produce a durable antitumor response when administered to mice with large tumor burdens. An increase in tumor burden is associated with increasing Treg populations and an overall immunosuppressive tumor microenvironment which can affect a vaccine-induced immune response. It is also well known that the elicitation of cancer-specific immunity is best when tumor burden is at its lowest (35–37), and perhaps the timing of our vaccinations influenced the results of this study. Furthermore, it is becoming more common to integrate strategies that enhance T-cell responsiveness and downregulate existing immune suppression for the purpose of augmenting cancer immunotherapies (38). In our studies, mice were administered cyclophosphamide before beginning the L-BLP25 vaccine regimen to reduce T suppressor lymphocyte activity.

Of interest is the relationship between pregnancy and reduced incidence of breast cancer (39, 40). Many multiparous women have circulating T cells which function in a
It is thought that hormone levels, such as estrogens, during pregnancy contribute to the reduced risk, but other factors such as age at first completed pregnancy also contribute (42). In fact, it is estimated that a woman must have a full-term pregnancy before the age of 25 to reduce the lifetime risk of developing breast cancer (43). Recent work has shown that high estrogen levels are associated with an increased risk of breast cancers diagnosed before age 40 and a decreased risk after the same age (44). The authors conclude that this increased risk is associated with hormone-negative tumors and therefore their findings mainly apply to premenopausal women. Their data also support the theory that pregnancy protects against hormone receptor–positive cancers (44). Other work has shown that multiparous women have an even lower risk of developing breast cancer than uniparous women (43). The reduction of breast cancer risk after full-term pregnancy may be related to a MUC1 response. Vaccines that elicit an MUC1 immune response may be useful in mimicking a multiparous woman as a preventive strategy.

In conclusion, we showed that (i) TH1 polarization of L-BLP25–induced immune response is unaffected by concurrent administration of tamoxifen or letrozole and (ii) L-BLP25 vaccine when combined with letrozole has additive antitumor activity. Both observations support the clinical testing of a combination therapy of L-BLP25 with letrozole for the treatment and prevention of breast cancer.

Disclosure of Potential Conflicts of Interest
M. DeGregorio has received a commercial research grant from Merck-KGaA. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: N.R. Mehta, G.T. Wurz, R.A. Burich, A. Gutierrez, M. Wolf, M. DeGregorio
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N.R. Mehta, G.T. Wurz, R.A. Burich, B.E. Greenberg, A. Gutierrez, J.L. McCall, M. DeGregorio
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N.R. Mehta, G.T. Wurz, R.A. Burich, S. Griffey, A. Gutierrez, J.L. McCall, M. DeGregorio
Writing, review, and/or revision of the manuscript: N.R. Mehta, G.T. Wurz, R.A. Burich, B.E. Greenberg, S. Griffey, A. Gutierrez, K.E. Bell, J.L. McCall, M. Wolf, M. DeGregorio
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N.R. Mehta, G.T. Wurz, B.E. Greenberg, K.E. Bell, M. DeGregorio
Study supervision: M. DeGregorio

Acknowledgments
The authors thank Kate Wasson (UC Davis, Mouse Biology Program) and Beverly Packard (OncoImmunin, Inc.) for their valuable technical assistance. The authors also thank Dr. Oscar Kashala (EMD Serono, Inc.) for his expert opinions and feedback.
Grant Support

This work was supported by a grant from Merck KGaA.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 20, 2012; revised March 9, 2012; accepted March 13, 2012; published OnlineFirst March 20, 2012.

References

L-BLP25 Vaccine plus Letrozole Induces a Th1 Immune Response and Has Additive Antitumor Activity in MUC1-Expressing Mammary Tumors in Mice

Neelima R. Mehta, Gregory T. Wurz, Rebekah A. Burich, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-12-0168

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2012/05/10/1078-0432.CCR-12-0168.DC1

Cited articles
This article cites 43 articles, 16 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/18/10/2861.full.html#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
/content/18/10/2861.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.