Addition of anti-estrogen therapy to anti-HER2 dendritic cell vaccination improves regional nodal immune response and pathologic complete response rate in patients with ER\textsuperscript{pos}/HER2\textsuperscript{pos} early breast cancer

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ABSTRACT
HER2-directed therapies are less effective in patients with ER\textsuperscript{pos} compared to ER\textsuperscript{neg} breast cancer, possibly reflecting bidirectional activation between HER2 and estrogen signaling pathways. We investigated dual blockade using anti-HER2 vaccination and anti-estrogen therapy in HER2\textsuperscript{pos}/ER\textsuperscript{pos} early breast cancer patients. In pre-clinical studies of HER2\textsuperscript{pos} breast cancer cell lines, ER\textsuperscript{pos} cells were partially resistant to CD4\textsuperscript{+} Th1 cytokine-induced metabolic suppression compared with ER\textsuperscript{neg} cells. The addition of anti-estrogen treatment significantly enhanced cytokine sensitivity in ER\textsuperscript{pos}, but not ER\textsuperscript{neg}, cell lines. In two pooled phase-I clinical trials, patients with HER2\textsuperscript{pos} early breast cancer were treated with neoadjuvant anti-HER2 dendritic cell vaccination; HER2\textsuperscript{pos}/ER\textsuperscript{pos} patients were treated with or without concurrent anti-estrogen therapy. The anti-HER2 Th1 immune response measured in the peripheral blood significantly increased following vaccination, but was similar across the three treatment groups (ER\textsuperscript{neg} vaccination alone, ER\textsuperscript{pos} vaccination alone, ER\textsuperscript{pos} vaccination + anti-estrogen therapy). In the sentinel lymph nodes, however, the anti-HER2 Th1 immune response was significantly higher in ER\textsuperscript{pos} patients treated with combination anti-HER2 vaccination plus anti-estrogen therapy compared to those treated with anti-HER2 vaccination alone. Similar rates of pathologic complete response (pCR) were observed in vaccinated ER\textsuperscript{pos} patients and vaccinated ER\textsuperscript{pos} patients treated with concurrent anti-estrogen therapy (31.4% vs. 28.6%); both were significantly higher than the pCR rate in vaccinated ER\textsuperscript{pos} patients who did not receive anti-estrogen therapy (4.0%, \(p = 0.03\)). Since pCR portends long-term favorable outcomes, these results support additional clinical investigations using HER2-directed vaccines in combination with anti-estrogen treatments for ER\textsuperscript{pos}/HER2\textsuperscript{pos} DCIS and invasive breast cancer.

Introduction
Human epidermal growth factor receptor 2 (HER2) protein is overexpressed in approximately 20–30% of invasive breast cancers, and 80–90% of high-grade DCIS lesions.\textsuperscript{1–4} HER2 overexpression is associated with an increased rate of progression from \textit{in situ} to invasive breast cancer, increased rates of recurrence and metastasis, and worse survival.\textsuperscript{1,2,5–7} Nearly 50% of HER2\textsuperscript{pos} breast cancers also overexpress hormone receptors.\textsuperscript{8,9} Bidirectional crosstalk between the HER2 and estrogen receptor (ER) signaling pathways leads to mutual activation and enhanced cell proliferation and survival.\textsuperscript{10,11} Preclinical studies have shown that breast cancer cell lines transfected with HER2 develop resistance to anti-estrogen (AE) treatment,\textsuperscript{8,11} and clinical studies have shown that patients with HER2 overexpressing tumors who are treated with AE have worse outcomes compared with similarly treated patients with HER2\textsuperscript{neg} tumors.\textsuperscript{12–14} These observations have provided the rationale for therapeutic approaches targeting both HER2 and ER.

The antigen-presenting capacity of dendritic cells (DC) led to investigation of potential use in antitumor vaccination. However, the observed clinical responses to DC vaccines have been disappointing.\textsuperscript{15–20} Early efforts employed DC vaccination as salvage therapy after conventional therapeutics had been exhausted. We have previously argued that such vaccines may not be well suited to tackle advanced or bulky disease, particularly when the patients are in declining health and subject to the immunosuppressive effects of advanced malignancy and its treatments.\textsuperscript{21} In contrast, patients with early stage disease and preserved anti-HER2 Th1 immunity,\textsuperscript{22} may represent a more suitable cohort for immune-based interventions.\textsuperscript{21} We designed a HER2 peptide-pulsed autologous DC vaccine engineered to promote anti-HER2 Th1 sensitization.\textsuperscript{23} We previously
reported on the feasibility, safety, and preliminary clinical results following neoadjuvant vaccination in patients with HER2pos DCIS. Complete tumor regression was induced in 19% of patients; however, these responders predominantly had ERneg tumors. Patients with ERpos DCIS were seemingly less responsive to the anti-HER2 DC vaccine.

We further investigated the immune mechanism by which the vaccine induced destruction of the tumors and found that there was extensive lymphocytic infiltration in the tumor bed of vaccinated patients; however, in contrast to the widely held hypothesis that CD8+ cytotoxic T lymphocytes (CTLs) serve as the most important effector lymphocytes for tumor control, the majority of lymphocytes were CD4+ Th1 cells. We also identified a key role for Th1 immunity in HER2pos breast cancer development and response to treatment. Along the continuum of HER2pos breast cancer, patients without disease had a robust anti-HER2 Th1 immune response, patients with HER2pos DCIS had a diminished anti-HER2 Th1 immune response, and the anti-HER2 Th1 immune response was nearly absent in patients with HER2pos invasive breast cancer. Moreover, patients with invasive HER2pos breast cancer who achieved complete tumor regression (pathologic complete response, pCR) following treatment with neoadjuvant chemotherapy plus trastuzumab had higher levels of anti-HER2 Th1 immunity compared with patients who failed to achieve pCR following neoadjuvant treatment. Additionally, the higher level of anti-HER2 Th1 immunity correlated with longer disease-free survival. These observations suggest that CD4+ Th1 cells are critically important in HER2pos breast cancer development.

Although the exact mechanism by which CD4+ Th1 cells impact tumor survival is not fully understood, several studies have suggested that Th1 cytokines, IFNγ and TNF-α, have direct tumoricidal effects. Braumuller et al. demonstrated in vitro treatment of murine and human cancer cells with IFNγ and TNF-α induced cancer cell senescence; such growth arrest was maintained when transferred into murine models. Recently, our group showed that Th1 cytokines inhibit metabolic activity, decrease proliferation, and induce apoptosis in HER2pos breast cancer cell lines, mimicking the biologic effects seen following DC1 vaccination and providing an in vitro model for our vaccine.

We hypothesized that the addition of AE therapy to anti-HER2 vaccination would improve pathologic response rates in patients with HER2pos/ERpos early breast cancer. This hypothesis was studied in vitro using breast cancer cell lines treated with Th1 cytokines, IFNy and TNF-α, and AE metabolites. We then amended our ongoing clinical protocol to include AE treatment in ERpos/HER2pos patients undergoing HER2-pulsed DC1 vaccination. We compared clinical and immune responses in patients with ERneg tumors who received vaccination alone (ERneg), patients with ERpos tumors who received vaccination alone (ERpos without AE), and patients with ERpos tumors who received both vaccination and concurrent AE therapy (ERpos with AE).

**Results**

*In vitro treatment of HER2pos/ERpos breast cancer cells with combination Th1 cytokines and anti-estrogen treatment suppresses metabolic activity*

The SKBr-3 human breast cancer cells (HER2pos 3+/ERneg) demonstrated strongly diminished metabolic activity in response to Th1 cytokines (p < 0.001) compared with no treatment (Fig. 1, upper left panel); the AE drug, fulvestrant, however, had no effect (p = 1.00), nor did it significantly enhance metabolic expression when combined with Th1 cytokines (p = 0.17). In contrast, treatment of BT-474 cells (HER2pos 3+/ERpos) with Th1 cytokines (Fig. 1, upper right panel) generated a statistically significant, yet more modest, suppression of metabolic activity (p < 0.001). Fulvestrant alone had a similar modest effect (p < 0.001). However, when both treatments were combined, cellular metabolic activity was suppressed dramatically (p < 0.001), to a level nearly identical to that seen in SKBr-3 cells treated with Th1 cytokines alone.

![Figure 1](image-url) Anti-estrogen compounds strongly enhance Th1 cytokine effects for estrogen receptor-expressing breast cancer cells. SKBr-3 (HER2pos 3+/ERneg) and BT-474 (HER2pos 3+/ERpos) human breast cancer cell lines were cultured with no additives (No Tx), treated with recombinant Th1 cytokines (‘Cyto’ IFNγ and TNF-α, 20 ng/mL each), anti-estrogen drugs including Fulvestrant (‘Fulv’ 0.5 μM; upper panels) and the active tamoxifen metabolite 4-Hydroxy-Tamoxifen (‘4HT’ 5 μM; lower panels), or the combination of Th1 cytokines and the respective anti-estrogen drug (‘Both’). After approximately 72 h of incubation, 10 μL of alamar blue dye was added, the cells were incubated for at least an additional 4 h, and the optical density was read at 630 nm. Results displayed are from one representative experiment of at least four trials ±/− SEM. Letter designations represent Tukey’s HSD results.
A similar pattern was also seen with 4-Hydroxy-Tamoxifen (4HT) treatment (Fig. 1, lower panels). The AE treatment alone had no discernible effect on SKBr-3 cells ($p = 0.838$). However, there was a modest, yet statistically significant, enhancement in cytokine-induced metabolic suppression when 4HT was added ($p < 0.001$). 4HT alone did not affect metabolic activity of BT-474 cells ($p = 0.246$), but when combined with cytokines, greatly enhanced metabolic suppression compared with cytokines alone ($p < 0.001$). These results demonstrate cooperative activity between AE compounds and Th1 cytokine treatment to strongly enhance metabolic suppression in ER-expressing HER2\textsuperscript{pos} breast cancer cells.

**Clinical trial design, patient selection, and demographics**

As a result of the extremely low pCR rate in ER\textsuperscript{pos} patients treated with DC1 vaccination\textsuperscript{24,32} and the cooperative activity between Th1 cytokines and AE compounds demonstrated in vitro, we investigated combination anti-HER2 vaccination and AE therapy in patients with HER2\textsuperscript{pos}/ER\textsuperscript{pos} DCIS and T1 tumors. After approval by the Institutional Review Board of the University of Pennsylvania, 27 patients were enrolled in a clinical trial demonstrating the feasibility, safety, and immunogenicity of a neoadjuvant HER2 peptide-pulsed DC1 vaccine via injection into groin lymph nodes. Subsequently, 54 women were enrolled in a second clinical trial with subjects randomized to route of administration of the vaccine. Because all three vaccination routes were found to elicit similar immune and clinical responses (manuscript submitted), patients from both neoadjuvant trials were analyzed together in this exploratory analysis to examine the impact of AE therapy on the endpoints of safety, immune response and clinical response. After the seventh ER\textsuperscript{pos} patient in the second trial (the 25th ER\textsuperscript{pos} patient overall) completed the vaccinated, the amendment was approved to treat the subsequent 21 ER\textsuperscript{pos} patients with concurrent AE therapy and vaccination.

Of the 81 women enrolled in these two trials, median age was 55 (interquartile range (IQR) 47–60) y, median BMI was 26.0 (IQR 22.5–31.0) kg/m\textsuperscript{2}, and a majority of patients were post-menopausal ($n = 68$; 84.0%) and white ($n = 65$, 80.2%). All eligible patients were diagnosed with DCIS ($n = 76$, 93.8%) or early invasive breast cancer ($T1a$, $n = 5$, 6.2%) at the time of biopsy. All eligible patients were diagnosed with $2+$ ($n = 28$, 34.6%) or $3+$ ($n = 53$, 65.4%) HER2\textsuperscript{pos} disease, with a majority of patients having high nuclear grade (72.7%). Vaccination was administered into groin lymph nodes in 47 patients (58%), into the breast in 18 patients (22.2%), and into both lymph nodes and the breast in 16 patients (20%). Surgical resection was performed via lumpectomy in 48 patients (59.3%) or mastectomy in 33 patients (40.7%). Of those patients who underwent lumpectomy, only 37.5% ($n = 18$) received post-operative radiation therapy.

In the overall cohort, 35 patients (43.2%) had ER\textsuperscript{neg} disease and 46 patients (56.8%) had ER\textsuperscript{pos} disease. Of those patients with ER\textsuperscript{pos} disease, 25 patients (54.3%) received the DC1 vaccine alone and 21 patients (45.7%) received the DC1 vaccine plus concurrent AE therapy (Figs. 2 and 3). Demographic and clinical characteristics of these treatment groups are summarized in Table 1; no significant differences between groups were observed.

**DC1 vaccines are well tolerated**

As previously reported,\textsuperscript{24} the vaccine was well tolerated with only grade 1 and 2 adverse events. The most commonly reported adverse events associated with the vaccine were fatigue ($n = 41$, 50.6%), injection site reaction ($n = 34$, 42.0%), and chills/rigors ($n = 26$, 32.1%). These side effects did not preclude trial completion in any patient.

In each treatment group, the five most commonly reported adverse events were identical—grade 1 and 2 fatigue, injection site reaction, chills/rigors, fever, and headache. Grade 1 and 2 vaccine-related adverse events were reported in 83% of ER\textsuperscript{neg} patients and 84% of ER\textsuperscript{pos} patients who received the vaccine alone. The addition of AE therapy did not increase the rate of adverse events—only 52% of ER\textsuperscript{pos} patients who received the vaccine and concurrent AE therapy reported grade 1 and 2 vaccine-related adverse events. The addition of AE therapy did not

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**Figure 2.** Vaccination procedure. Patients with biopsy-diagnosed HER2\textsuperscript{pos} DCIS were eligible for the trial. Patient’s monocytes were collected by leukapheresis and elutriation from which the pre-vaccine immune response was determined. The monocytes were rapidly matured into type-1 DCs and pulsed with HER2 peptides. Patients underwent 4–6 weekly vaccinations (with concurrent anti-estrogen therapy for ER\textsuperscript{pos} patients enrolled following the amendment). Patient’s monocytes were collected again by a second leukapheresis and elutriation or a simple blood draw from which the post-vaccine immune response was determined. Following vaccination, patients underwent surgical resection to cure them of residual disease. The clinical response was measured in the surgical specimen and the post vaccine immune response was also measured in the sentinel lymph nodes.
increase the rate of cardiac related toxicity; two ER<sup>pos</sup> patients who received the vaccine alone (12%) exhibited a 19% decline in EF to 50% and an 18% decline in EF to 54%, and one ER<sup>pos</sup> patient who received the vaccine with concurrent AE therapy (4.8%) exhibited an 18% decline in EF to 55%. Additionally, one ER<sup>pos</sup> patient who received the vaccine alone exhibited a 28% decrease in EF percentage on multigated acquisition (MUGA) scan, which was attributed to PVCs and was not evident when reevaluated by echocardiogram. All of the patients who experienced decreases in EF were asymptomatic, and all had repeat studies within 30 d showing that the EF subsequently returned to baseline.

![Figure 3. Patient distribution.](image)

**Table 1.** Demographic and clinical characteristics comparing patients by ER status and AE treatment

| Characteristic                      | ER<sup>neg</sup> (%) | ER<sup>pos</sup> w/o AE (%) | ER<sup>pos</sup> w AE (%) | Univariate p value |
|------------------------------------|----------------------|-----------------------------|---------------------------|--------------------|
| Number of patients                 | 35 (43.2)            | 25 (30.9)                   | 21 (25.9)                 | —                  |
| Median age (IQR)                   | 56 (48–61)           | 51 (45–60)                  | 54 (50–57)                | 0.67               |
| Race                               |                      |                             |                           |                    |
| White                              | 27 (77.1)            | 19 (76.0)                   | 19 (90.5)                 | 0.21               |
| Black                              | 5 (14.3)             | 6 (24.0)                    | 2 (9.5)                   |                    |
| Other                              | 3 (8.6)              | 0 (0)                       | 0 (0)                     |                    |
| Median BMI (IQR)                   | 27.1 (23.2–31.8)     | 25.9 (22.6–30.0)            | 23.4 (21.8–30.1)          | 0.19               |
| Menopausal status                  |                      |                             |                           |                    |
| Pre                                | 4 (11.4)             | 3 (12.0)                    | 6 (28.6)                  | 0.19               |
| Post                               | 31 (88.6)            | 22 (88.0)                   | 15 (71.4)                 |                    |
| Charlson comorbidity Score         |                      |                             |                           |                    |
| ≤2                                 | 27 (77.1)            | 21 (84.0)                   | 21 (100.0)                | 0.07               |
| ≥3                                 | 8 (22.9)             | 4 (16.0)                    | 0 (0)                     |                    |
| Stage                              |                      |                             |                           |                    |
| DCIS                               | 32 (91.4)            | 25 (100)                    | 19 (90.5)                 | 0.14               |
| Stage 1                            | 3 (8.6)              | 0 (0)                       | 2 (9.5)                   |                    |
| Nuclear grade<sup>a</sup>          |                      |                             |                           |                    |
| Low                                | 0 (0)                | 1 (4.3)                     | 1 (4.8)                   | 0.11               |
| Intermediate                       | 5 (15.2)             | 5 (21.7)                    | 9 (42.9)                  |                    |
| High                               | 28 (84.8)            | 17 (73.9)                   | 11 (52.4)                 |                    |
| Injection site                     |                      |                             |                           |                    |
| Breast                             | 9 (25.7)             | 9 (36.0)                    | 7 (33.3)                  | 0.42               |
| Node                               | 20 (57.1)            | 10 (40.0)                   | 7 (33.3)                  |                    |
| Both                               | 6 (17.1)             | 6 (24.0)                    | 7 (33.3)                  |                    |
| Surgical resection                 |                      |                             |                           |                    |
| Lumpectomy                         | 19 (54.3)            | 13 (52.0)                   | 16 (76.2)                 | 0.18               |
| Mastectomy                         | 16 (45.7)            | 12 (48.0)                   | 5 (23.8)                  |                    |
| Radiation<sup>b</sup>              |                      |                             |                           |                    |
| No                                 | 11 (57.9)            | 6 (46.2)                    | 13 (81.3)                 | 0.13               |
| Yes                                | 8 (42.1)             | 7 (53.8)                    | 3 (18.8)                  |                    |

*Abbreviations: ER, estrogen receptor; AE, anti-estrogen; IQR, interquartile range; HER2, human epidermal growth factor receptor 2.

<sup>a</sup>Data not recorded for two ER<sup>neg</sup> patients, two ER<sup>pos</sup> w/o AE patients.

<sup>b</sup> Radiation following lumpectomy.
Increase in anti-HER2 Th1 immune response in the peripheral blood following DC1 vaccination

The pre-vaccination and post-vaccination systemic CD4+ immune responses were measured in the peripheral blood of 53 of the 54 patients in the second trial. Anti-HER2 Th1 responsivity to HER2-derived peptides, increased significantly from 0 (IQR 0–4) pre-vaccination to 88.7% post-vaccination (p < 0.01). Median response repertoire, defined as the number of HER2 peptides to which a patient exhibited a specific response, also increased significantly from 0 (IQR 0–2) pre-vaccination to 2 (IQR 1–4) post-vaccination (p = 0.05). Finally, median cumulative response, defined as the sum of spot-forming cells (SFC) across six HER2-derived class-II peptides as assessed by IFNy ELISPOT, increased significantly from 48.6 (IQR 23.3–96.9) pre-vaccination to 132.0 (IQR 75.6–236.9) post-vaccination (p < 0.01).

In each treatment group, the anti-HER2 Th1 immune response increased following vaccination as measured by responsivity (all p < 0.01), response repertoire (ERneg: p = 0.05; ERpos without AE treatment: p = 0.1; ERpos with AE treatment: p = 0.01), and cumulative response (all p < 0.01). However, there were no significant differences between treatment groups in pre-vaccine or post-vaccine immune responses as measured by responsivity, response repertoire, and cumulative response (Fig. 4).

Enhanced anti-HER2 Th1 immune response in the sentinel lymph node in ERpos patients who received concurrent DC1 vaccination plus anti-estrogen therapy

The post-vaccination regional immune response was measured in 40 patients, since not all patients required nor were candidates for sentinel lymphadenectomy; anti-HER2 Th1 responsivity was 80%, median response repertoire was 2 (IQR 1–5), and median cumulative response was 76.5 (IQR 23.5–209.5). The post-vaccination anti-HER2 Th1 immune responses were significantly higher in the ERpos with AE group compared to the ERpos without AE group by each metric: responsivity (ERpos with AE 92.3% vs. ERpos without AE 42.9%; p = 0.02), median response repertoire (4 (IQR 2–6) vs. 0 (IQR 0–5); p = 0.05), and median cumulative response (100.9 (IQR 67.5–174.4) vs. 0.94; p = 0.62). This disparity in anti-HER2 Th1 responses in vaccinated ERpos stratified by receipt of AE therapy was uniquely observed at the regional level, in contrast to the peripheral blood anti-HER2 Th1 responses.
Pathologic complete response rate is higher in ER<sup>pos</sup> patients receiving concurrent DC1 vaccination and anti-estrogen therapy compared with those receiving DC1 vaccination alone

Clinical responses were available for all trial patients (n = 81). Overall, 18 of the 81 immunized patients (22.2%) had no evidence of residual disease identified in the resected surgical specimen; these patients were considered to have pCR. The pCR rate in the ER<sup>neg</sup> group (n = 11, 31.4%) and the ER<sup>pos</sup> group receiving AE therapy (n = 6, 28.6%) were similar (p = 1.00); however, the pCR rate in the ER<sup>pos</sup> group not receiving AE therapy (n = 1, 4.0%) was significantly lower than the rate in both the ER<sup>neg</sup> group (p = 0.01) and the ER<sup>pos</sup> group receiving AE therapy (p = 0.04; Fig. 6).

Overall, there was no difference in systemic immune responses in patients achieving pCR and those with residual disease, as measured by anti-HER2 Th1 responsivity, response repertoire, and cumulative response. Further comparison of regional immune responses in the sentinel lymph nodes was limited due to the relatively small sample size. Nevertheless, in ER<sup>pos</sup> patients receiving combination anti-HER2 vaccination and concurrent AE therapy those achieving pCR (n = 3) demonstrated higher immune responses in the sentinel lymph nodes by all three metrics compared with those failing to achieve pCR (n = 10): responsivity (100% vs. 90%), response repertoire (6 vs. 2.5), and cumulative response (354.0 vs. 85.5).

Outcomes for subjects treated with DC1 vaccines

pCR following neoadjuvant treatment of invasive breast cancer has been shown to correlate with a decreased risk of recurrence; however, there is no similar published data assessing the risk of recurrence in DCIS patients who achieved pCR following neoadjuvant treatment. In our institutional experience, although the numbers are small, none of the patients who achieved pCR experienced an additional breast event at a median follow-up of 42 mo (Fig. 7A). Subsequent breast events, defined as either DCIS or invasive breast cancer identified in either breast, occurred in seven vaccinated patients (8.6%). One HER2<sup>pos</sup>/ER<sup>neg</sup> DCIS patient developed a contralateral T1 HER2<sup>neg</sup>/ER<sup>pos</sup> invasive breast cancer more than 5 y after completion of vaccination, and two HER2<sup>pos</sup>/ER<sup>neg</sup> DCIS patients developed ipsilateral HER2<sup>pos</sup>/ER<sup>neg</sup> Paget’s disease with DCIS. The remaining four patients who developed subsequent breast events had ER<sup>pos</sup> DCIS, but received vaccination without concurrent AE therapy—one patient developed T1a triple negative invasive breast cancer in the contralateral breast presenting with new microcalcifications after one year, and the remaining three patients developed HER2<sup>neg</sup> lesions, including one with ER<sup>pos</sup> ipsilateral nodal disease who had refused initial sentinel node biopsy. Notably, ER<sup>pos</sup> patients enrolled later in the trial were treated with concurrent vaccination and AE therapy, and, therefore, median follow up is shorter (38 mo) for these patients. Nevertheless, none of the patients who had ER<sup>pos</sup>...
tumors and received concurrent vaccination and AE therapy have experienced a subsequent breast event (Fig. 7B). Additionally, three treated patients have died. Two ERneg patients died of unrelated comorbidities 1.5 and 4 y after vaccination, and one ERpos patient treated with vaccination and concurrent AE was found to have a contralateral invasive lobular carcinoma recurrence of her previously treated contralateral breast. The lesion was picked up on post-vaccine MRI of the breast and the patient was found to have been harboring extensive Stage IV lobular cancer. She subsequently died of metastatic disease.

Although, the rate of lumpectomy was similar across the three groups, as shown in Table 1; the rate of radiation following lumpectomy was lower in the group of patients with ERpos tumors who received AE therapy (18.8%) than in the group of patients with ERpos tumors who did not receive AE therapy (53.8%, \( p = 0.06 \)). Nevertheless, patients with ERpos tumors who received AE therapy had a decreased rate of SBE despite the lower rate of radiation.

**Discussion**

In this study, both preclinical and clinical data indicate efficacy of combination treatment with anti-HER2 and AE treatment. In vitro, Th1 cytokines IFN\( \gamma \) and TNF-\( \alpha \) combined with either of two AE drugs (that work via distinct mechanisms) significantly suppress metabolic activity in HER2pos/ERpos breast cancer cell lines. These studies provided rationale for adding AE drugs to vaccine therapy for HER2pos/ERpos early breast cancer in ongoing phase-I clinical trials. In such trials, the addition of AE therapy to DC1 vaccination increased the anti-HER2 CD4+ Th1 immune response and the rate of pCR in patients with HER2pos/ERpos tumors.

Cancer vaccine trials have routinely utilized peripheral blood immune responses as an endpoint; however, such responses are frequently discordant with clinical outcomes.\(^{15,36,37}\) In one study, vaccination-induced immune responses detected in the peripheral blood without leading to clinical tumor regression and, conversely, tumor regression was frequently observed in the absence of peripherally detected immune responses.\(^{38}\) In the present study, we similarly found that immune responses measured in the peripheral blood did not correspond with clinical responses. Anti-HER2 DC1 vaccination increased CD4+ T-cell immune responses in the peripheral blood similarly in the three treatment groups, but rates of pCR differed. In contrast, both the anti-HER2 immune responses measured in the sentinel lymph nodes and the pCR rate were significantly higher in ERpos patients who received anti-HER2 vaccination and concurrent AE therapy compared with the ERpos patients who received anti-HER2 vaccination alone. Furthermore, ERpos patients who received anti-HER2 vaccination and concurrent AE therapy and achieved pCR had a more robust immune response detected in the sentinel lymph nodes than those who failed to achieve pCR.

We have previously demonstrated that DC1 vaccination induces a predominantly CD4+ lymphocytic infiltrate into the tumor region\(^{23}\) and that tumor destruction following vaccination is due to the activity of the Th1 cytokines, IFN\( \gamma \), and TNF-\( \alpha \).\(^{31}\) The CD4+ Th1 lymphocytes that are critical to the antitumor activity may be sensitized at the sentinel lymph nodes that drain the tumor or they may gain access to the tumor via the sentinel lymph nodes.\(^{39}\) The unique position of the sentinel lymph nodes, situated at the portal into and out of the diseased area, may explain the increased tumor-specific immune response found in the sentinel lymph nodes in patients with better clinical responses; however, the results of this study do not distinguish whether the increased tumor-specific immune response is caused by the exposure to destroyed tumor or induces tumor destruction. In either case, the antitumor Th1 immune response measured in the sentinel lymph nodes may better reflect antitumor activity and serve as a predictive biomarker for response to immunotherapy. Because sentinel lymphadenectomy is frequently a component of standard surgical management of breast cancers,
immune monitoring in the sentinel nodes could be performed without exposing patients to additional interventions. In patients for whom sentinel lymphadenectomy is not otherwise required, or long-term monitoring is advantageous, core biopsy may be preferable.

Of equal importance, vaccination plus AE therapy was safe and well tolerated. All patients completed treatment and the immune and clinical responses were measured after only 4–6 weeks of combination treatment, without relying upon long-term AE therapy. The latter is relevant as vaso-motor and gynecologic symptoms and concern for thrombotic events and endometrial cancer often lead to reduced compliance with AE therapy. Even under optimal conditions, 11–31% of patients in NSABP trials did not complete the 5 y of recommended tamoxifen treatment. In our clinical trials, only a minority of patients (38.1%) continued taking AE beyond one year; most patients stopped taking AE due to the intolerability of the side effects. Moreover, although most patients in the study had high-grade lesions, most did not undergo post-lumpectomy radiation. Despite this, ipsilateral breast recurrence and the overall rate of subsequent breast events remained low.

This study is subject to several limitations that warrant emphasis. The described trials were not designed or powered to evaluate treatment with combination anti-HER2 DC1 vaccination and AE therapy. ERpos patients were not randomized to treatment with or without AE; rather, the amendment to the trial protocol to treat subsequent ERpos patients with combination anti-HER2 vaccination and AE therapy was introduced after initiation of the trial. As a result, the follow up time is shorter for patients who were treated with concurrent vaccination and AE therapy. Additionally, some of our analyses of the immune responses were limited by small numbers. Different immune monitoring standards in two distinct trials limited our ability to consider all data in a combined analysis. Finally, the frequently small tumor volume precluded routine measurement of the immune response at the tumor itself. Larger randomized studies evaluating the combination of anti-HER2 DC1

Figure 7. Subsequent breast events comparing patients by pathologic complete response and ER status and AE treatment. None of the patients who had a pathologic complete response experienced a SBE (A), and none of the ERpos patients who received concurrent DC1 vaccination and anti-estrogen therapy experienced a SBE (B).
vaccination and AE with comprehensive immune monitoring, which would include measurements of the immune response in (1) the peripheral blood, (2) the locoregional lymph nodes, and (3) the tumor, and long-term clinical follow-up are warranted.

The seemingly exponential growth of the targeted and immunotherapeutic armamentarium in recent years provides opportunities for rational combined multi-targeted approaches in high-risk patients. The present study demonstrates the merit of such approaches. Targeted combination therapy is safe and effective and may present an alternative to current regimens requiring long-term AE use and radiotherapy in high-risk HER2pos/ERpos patients. Given the current interest in reducing overtreatment of DCIS, vaccination may be of particular appeal, allowing a reduction in the duration of AE therapy or omission of post-operative radiation, and limiting the extent of surgical resection.

**Method**

**In vitro cell culture**

Human breast cancer cell lines, SKBr-3 (HER2pos 3+/ERneg) and BT-474 (HER2pos 3+/ERpos), were obtained from American Type Culture Collection (ATCC; #HTB-30, #HTB-20). The cells were cultured in McCoy’s 5A media (Gibco #16600108) or HybriCare media (ATCC #46-X), respectively, supplemented with 10% v/v fetal bovine serum (FBS) (Atlanta Biologicals #S11150), 100 units/mL of penicillin and 100 µg/mL of streptomycin sulfate (BioWhittaker #17-602E). Cells were incubated at 37°C and 5% CO2 for the duration of the experiments.

**Alamar blue assay**

SKBr-3 and BT-474 cells were harvested with trypsin (Bio-Whittaker #CC-5012), counted, seeded in 96-well culture plates (8 × 10³ cells/50 µL per well), and incubated overnight. The next day, additional media was added to untreated controls and cells were treated with Th1 cytokines (20 ng/mL recombinant human IFNγ and 20 ng/mL recombinant human TNF-α) (Peprotech #300-02, #300-01A), either a tamoxifen metabolite (5 µM; 4HT) (Sigma-Aldrich #94873), or the ERα antagonist Fulvestrant (0.5 µM; ICI 182,780) (Selleckchem #S1191), or a combination of both cytokines plus AE. Cells were incubated at 37°C and 5% CO2 for approximately 72 h after treatment. On the third day post-treatment, cell viability was measured by adding 10 µL of stock resazurin sodium salt (5.6 mM; Sigma-Aldrich #R7017) to each sample well (100 µL/well final volume), which is equivalent to a working concentration of 560 µM resazurin sodium salt. The reduction of resazurin to resorufin was measured via optical density at 630 nm with a Bio-Tek ELx800 absorbance reader.

**Clinical trial design**

After approval by the Institutional Review Board of the University of Pennsylvania, we conducted two neoadjuvant clinical trials of a HER2 peptide-pulsed DC1 vaccine (NCT001070211 and NCT02061332). The primary objectives were to evaluate the feasibility, safety, and efficacy of DC1 vaccination. The secondary objective was to assess clinical and immune responses. Preliminary results of these trials showed that the vaccine was safe, well tolerated, and induced an increase in the anti-HER2 Th1 immune response and a decline or eradication of HER2 expression. Further review of the preliminary results showed that vaccination was more effective in hormone-independent (ERneg) patients. Based on the preclinical data and the preliminary results of the clinical trial, an addendum was approved to treat subsequent ERpos patients with hormone dependent (ERpos) tumors with concurrent AE therapy. Rather than a randomized control trial, after the 25th ERpos patient (the seventh ERpos patient in the second trial) completed the vaccinated, the subsequent 21 ERpos patients were treated with concurrent AE therapy and vaccination.

**Patient selection**

Female patients older than 18 y with biopsy-proven HER2pos DCIS or T1a tumors and an Eastern Cooperative Oncology Group (ECOG) Performance Status Score of 0 or 1 were eligible for the trial. All tissue specimens were reviewed by a single pathologist (P.Z.) for eligibility; HER2 positivity was defined as >5% of cells expressing 2+ or 3+ intensity of the HER2 protein. Women of childbearing age were required to have a negative serum pregnancy test and were required to use a medically acceptable form of birth control. Women with cardiac dysfunction, HIV, Hepatitis C, known coagulopathies, or a pre-existing medical illness or medications that might interfere with vaccination were excluded. Women who had received definitive treatment or whose DCIS was eliminated by excisional biopsy at diagnosis were not eligible for the trial. Eighty-one women were enrolled in the trial and completed the vaccination treatment. Demographic and clinical data were obtained from the electronic medical record. Immune responses were measured in patients enrolled in the second trial (NCT02061332)—53 of 54 patients had pre and post-vaccination CD4+ immune responses measured in the peripheral blood; and 40 patients had post-vaccination CD4+ immune responses measured in the sentinel lymph nodes. All 81 patients underwent surgical resection with pathologic examination of the resected specimen.

**Vaccination procedure**

Vaccine preparation and delivery have been described in detail previously. Briefly, monocytic DC precursors were obtained from patients via tandem leukapheresis/countercurrent centrifugal elutriation. Monocytes were cultured at 37°C in serum-free medium (SFM, Invitrogen #12065-74) with granulocyte-macrophage colony-stimulating factor (GM-CSF Leukine®) and IL-4 (R&D Systems #204-IL-050). The following day, cells were pulsed with six HER2-derived major histocompatibility complex (MHC) class II binding peptides—three extracellular domain (ECD) peptides (42–56, 98–114, and 328–345) and three intracellular domain (ICD) peptides (776–790, 927–941, and 1166–1180) (Bachem Americas Inc. #4098340, #4100281, #4100278, #4098341, #4100280, #4100279). After 8–12 h, 1,000 U/mL of IFNγ (Actimmune®) was added, and 6 h
before harvest, 10 mg/mL of clinical-grade LPS [gift from Dr Anthony Suffredini, National Institute of Health (NIH), Bethesda, MD] was added to complete the rapid maturation to a type-1 polarized DC (DC1). For HLA-A2pos patients, the monocyte pool was pulsed with MHC class I binding peptides 369–377 and 689–697. Two hours later, the cells were harvested, washed, counted and assessed for viability and contamination. The vaccine was administered in the General Clinical Research Center at the Hospital of the University of Pennsylvania. Four to six weekly injections of 10–20 × 106 HER2-peptide pulsed DCs in 1 mL of sterile saline were administered into the breast, groin lymph nodes, or both with half of the dose administered into the breast and half of the dose administered into the groin. As previously described, vaccines were evaluated for IL-12p70 production by ELISA and functional activity by flow cytometry. Patients were monitored for adverse effects for a minimum of 1–2 h following each weekly vaccination. All adverse events were classified by National Cancer Institute Common Toxicity Criteria (NCI-CTC version 3.0), were assessed at least weekly during vaccination, and were monitored until their resolution.

**Immune monitoring**

Systemic anti-HER2 CD4+ T-cell responses were generated from autologous peripheral blood mononuclear cells (PBMC) pulsed with HER2 peptides. Localized anti-HER2 CD4+ T-cell responses were measured in the locoregional sentinel lymph node(s) (SLN) in 40 patients who underwent SLN biopsy. IFNγ production was measured by enzyme-linked immunosorbent spot (ELISPOT) assays or by in vitro sensitization assays, as previously described.

Briefly, ELISPOT PVDF membrane plates were coated overnight with anti-IFNγ capture antibody (Mabtech Inc. #3420-2H, 1D1K). The following day, after the plates were washed with PBS (Mediatech Inc. #21-040-CMX12) and blocked with 10% human serum/DMEM, 2 × 107 PBMCs or SLN cells were plated in each well either unstimulated, pulsed with HER2-derived class II peptide (4 μg) (42–56, 98–114, 328–345, 776–790, 927–941, and 1166–1180), or pulsed with anti-human CD3/CD28 antibodies (0.5 μg/mL) (positive control, BD Pharmingen #555337, #555726), and incubated at 37°C with 5% CO2 for 24–36 h. After the plates were washed with PBS, 100 μL of detection antibody (1 mg/mL; 7-B6-1-biotin) was added to each well and the plates were incubated for 2 h. After the plates were washed again with PBS, 100 μL of 1:1,000 diluted streptavidin-HRP was added to each well and the plates were incubated for another hour. TMB substrate solution (Mabtech Inc. #3651-10) was added to reveal spot formation. Spot-forming cells (SFC) were counted using an automated reader (ImmunoSpot CTL).

A positive response to an individual HER2 class-II peptide was defined as a minimum of 20 SFC/2 × 106 cells after subtracting the unstimulated background. Three metrics were used to quantify the CD4+ Th1 response: (1) responsivity (the proportion of patients responding to ≥1 peptide), (2) response repertoire (the number of peptides to which a patient responds), and (3) cumulative response (the sum of the SFCs across all 6 class-II HER2 peptides). For in vitro sensitization, CD4+ T cells were selected from the cryopreserved lymphocyte cell fractions via negative selection (StemCell Technologies #14052). Autologous DC1 were suspended in SFM with GM-CSF (10 ng/mL) pulsed with one of the six class-II HER2 peptides, and co-cultured with the CD4+ T cells at a ratio of 10:1. IL-2 (30 IU/mL; ThermoFisher #PHC0026) was added on day 2. On day 10, T-cells were harvested and were tested against T2 target cells pulsed with one of the six class-II HER2 peptide or irrelevant controls (p53 and colon cancer peptide). After 24 h, the supernatant was harvested and analyzed by enzyme-linked immunosorbent assay (ELISA). A positive response to the HER2 peptide was defined as a 2-fold increase in CD4+ T-cell IFNγ production compared to the irrelevant peptide controls. Again, responsivity and response repertoire metrics were used to quantify the CD4+ immune response.

**Clinical monitoring**

Pathologic response was examined at the time of surgical resection—lumpectomy (n = 48) or mastectomy (n = 33). A pCR following immunization was defined as absence of residual DCIS or invasive breast cancer at the time of surgical resection.

Patients were monitored after surgical resection for the development of subsequent breast events. A subsequent breast event was defined as DCIS or invasive breast cancer identified in either the ipsilateral or contralateral breast.

**Statistical analysis**

In vitro experiments were repeated at least three times with consistent results. Values were processed using Microsoft Excel 2010 software and are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using one-way ANOVA; p values <0.05 were considered statistically significant. The Shapiro–Wilk test was used to verify the assumption of the normality of errors. To identify statistical differences between treatment groups, the Tukey’s Honest Significant Difference test was used. Statistical analyses were conducted with SigmaPlot v12.5 statistical software (Systat Software Inc., San Jose, CA).

For the clinical trials, descriptive statistics were performed to evaluate demographic, immunologic, and clinical data. Continuous variables were described by median and IQR, and compared by ANOVA or nonparametric Kruskal–Wallis test, as appropriate. Categorical variables were described by frequencies and percentage, and compared by Fisher’s exact test. Two-group comparisons were conducted by Student’s t-test or nonparametric Wilcoxon rank sum test, as appropriate. A p value of less than 0.05 was considered statistically significant; all tests were two sided. Development of subsequent breast events was compared using Kaplan–Meier analysis. Analyses were performed with STATA 12.0/IC statistical software (STATA Corp, College Station, TX) and SPSS 22.0 (IBM Corporation, Armonk, NY).

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.
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