# Dendritic Cell Vaccination Enhances Immune Responses and Induces Regression of HER2$^{pos}$ Ductal Carcinoma In Situ Independent of Route: Results of Randomized Selection Design Trial

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## Statement of Translational Relevance

In this randomized selection design trial, we confirmed that neoadjuvant DC1 vaccination is a safe and immunogenic treatment in ductal carcinoma in situ (DCIS) and early invasive breast cancer (IBC), inducing a tumor-specific T-cell response in the peripheral blood and the sentinel lymph nodes independent of the route of vaccination (intralesional, intranodal, or both intralesional and intranodal). The pathologic complete response (pCR) rate was similar across all three injection routes, but was higher in patients with DCIS than in patients with stage I IBC. In DCIS patients, immune responses detected in the sentinel lymph nodes, but not peripheral blood, were associated with pCR. These findings suggest that (1) vaccines are more effective in DCIS and warrant further evaluation in DCIS or other minimal disease settings, and (2) the local regional sentinel lymph node may serve as a more meaningful immunologic endpoint.

## Abstract

**Purpose:** Vaccination with HER2 peptide-pulsed DC1s stimulates a HER2 specific T-cell response. This randomized trial aimed to establish safety and evaluate immune and clinical responses to vaccination via intralesional (IL), intranodal (IN), or both intralesional and intranodal (ILN) injection.

**Methods**: Fifty-four HER2$^{pos}$ patients (42 pure DCIS, 12 early invasive breast cancer (IBC)) were enrolled in a neoadjuvant HER2 peptide-pulsed DC1 vaccine trial. Patients were randomized to IL (n=19), IN (n=19), or ILN (n=16) injection. Immune responses were measured in peripheral blood and sentinel lymph nodes by ELISPOT or in-vitro sensitization assay. Pathologic response was assessed in resected surgical specimens.

**Result**s: Vaccination by all injection routes was well-tolerated. There was no significant difference in immune response rates by vaccination route (IL 84.2% vs. IN 89.5% vs. ILN 66.7%; p=0.30). The pathologic complete response (pCR) rate was higher in DCIS patients compared with IBC patients (28.6% vs. 8.3%). DCIS patients who achieved pCR (n=12) and who did not achieve pCR (n=30) had similar peripheral blood anti-HER2 immune responses. All patients who achieved pCR had an anti-HER2 CD4 immune response in the sentinel lymph node and the quantified response was higher by response repertoire (p=0.03) and cumulative response (p=0.04).

**Conclusion**: Anti-HER2 DC1 vaccination is a safe and immunogenic treatment to induce tumor-specific T-cell responses in HER2$^{pos}$ patients; immune and clinical responses were similar independent of vaccination route. The immune response in the sentinel lymph nodes, rather than in the peripheral blood, may serve as an endpoint more reflective of anti-tumor activity.

## Introduction

Cancer progression is associated with immune dysregulation and suppression. Cancer immunotherapy aims to sensitize and restore a specific anti-tumor immune response. Our group has shown that there is a progressive loss of the anti-HER2 Th1 immune response along the continuum of HER2$^{pos}$ breast cancer; [1] and we have developed a HER2 peptide-pulsed dendritic cell (DC1) vaccine that is uniquely engineered to induce a strong anti-HER2 immune response. [2, 3] DC1s are particularly well-suited for cancer vaccines due to their potent antigen presenting capacity, sensitizing both CD4$^{pos}$ [4] and CD8$^{pos}$ [5] T-cells to specific antigens. [6-8] While CD8$^{pos}$ T-cells, and cytotoxic T-lymphocytes (CTL) in particular, have traditionally been viewed as the primary effectors of anti-tumor immunity, [9] CD4$^{pos}$ T-cells have more recently been shown to potentiate the CTL response, [5, 10] and contribute additional cytotoxicity. [11-13]

DCs migrate to, or otherwise enter, secondary lymphoid organs to interact with resident T-lymphocytes. [14, 15] DC vaccines administered intravenously, [16-18] subcutaneously, [18-20] or intranodally [18, 21, 22] have been shown to induce a specific T-cell response. Direct intranodal administration obviates migration and may, therefore, be the most efficient route of administration. In a murine model, DCs pulsed with tumor lysate and injected intranodally resulted in greater sensitization of T-cells and improved anti-tumor responses compared to intravenous or subcutaneous vaccination. [23] In our previous trial of the neoadjuvant DC1 vaccine in patients with HER2$^{pos}$ DCIS, DCs were injected into non-pathologic distant lymph nodes in the groin under ultrasound guidance. [3] In the current trial, we randomized patients to vaccination via intralesional (IL), intranodal (IN), or a combination of both intralesional and intranodal (ILN) injections and compared immune and clinical responses. The goals of this trial were to establish the safety of the various routes of DC1 vaccination, to evaluate the immune and

clinical responses induced by the various routes of vaccination, and to explore possible relationships between immune and clinical responses.

## Methods

### Study Design

This randomized selection design trial was approved by the University of Pennsylvania's Institutional Review Board and registered at www.clinicaltrials.gov (NCT02061332). All patients signed informed consent for the trial and patients were randomized to one of three different routes of vaccine administration: (1) our previously established protocol of ultrasound guided intranodal injection (IN), (2) intralesional injection (IL), and (3) both intralesional and intranodal injection (ILN).

In our previous trial, we found that a higher percentage of patients with $ER^{neg}$ tumors (4 of 10, 40.0%) had no tumor at the time of surgery (pathologic complete response, pCR) compared to patients with $ER^{pos}$ tumors (1 of 17, 5.9%). [3, 24] Because of the lower pCR rate in $ER^{pos}$ patients, the bidirectional crosstalk between the ER and HER2 signaling pathways demonstrated in preclinical studies, [25, 26] and the clinical benefits shown in dual blockade of ER and HER2, [26, 27] an amendment was approved during this trial to treat subsequent $ER^{pos}$ patients with concurrent anti-estrogen therapy. After the twenty-second patient (the seventh $ER^{pos}$ patient) was vaccinated, the subsequent 21 $ER^{pos}$ patients were prescribed anti-estrogen therapy. None of the patients, including those with invasive disease, received any other systemic treatment while enrolled in the trial.

The primary goals of this trial were to establish the safety and tolerability of the vaccine and to evaluate the immune response generated by the three different routes of vaccination. The

secondary goals of this trial were to measure the clinical response following vaccination and explore possible relationships between the immune and clinical outcomes.

**Patient Selection**

Female patients ≥ 18 years of age with biopsy-proven $HER2^{pos}$ DCIS, DCIS with microinvasion, DCIS with invasive disease less than 5mm, or Paget's Disease of the nipple who had not yet received definitive treatment were eligible for the trial. Patients whose DCIS was eliminated by excisional biopsy at diagnosis were not eligible. The Hercept (Dako) antibody was used to analyze HER2 expression. HER2 positivity was defined as >5% of tumor population with 2+ or 3+ staining by immunohistochemistry verified by a single pathologist (P.Z.). Each patient underwent a breast MRI scan prior to vaccination to document the initial extent of disease and exclude patients with macroscopic invasive foci. Pregnant or lactating women were excluded from the trial. Women with cardiac dysfunction, immune deficiencies, coagulopathies, or a pre-existing medical illness or medication, which might interfere with the study, were excluded from the trial. Fifty-eight women enrolled in the trial and signed informed consent; one patient's tumor was reclassified as $HER2^{neg}$ upon review, two patients voluntarily withdrew prior to the first leukapheresis, and one patient had an inadequate collection of cells by leukapheresis, yielding a final cohort of 54 patients (**Supplemental Figure 1**). Demographic and clinical data were obtained from the electronic medical record.

**Vaccination Procedure**

Vaccine preparation has been described in detail previously. [2, 3, 24, 28] Briefly, patients underwent tandem apheresis/countercurrent centrifugal elutriation to isolate monocytic dendritic cell precursors. Cells were pulsed with six HER2 major histocompatibility complex (MHC) class II binding peptides [American Peptide Corporation, Sunnyvale, CA] - three extracellular

domain (ECD) peptides (42-56, 98-114, 328-345) and three intracellular domain (ICD) peptides (776-790, 927-941, 1166-1180), and rapidly matured to a DC1 phenotype by adding IFN-gamma [Actimmune, Brisbane, CA] (1000 U/mL) and LPS [gift from Dr Anthony Suffredini, National Institute of Health (NIH), Bethesda, MD] (10 mg/mL). The monocytes of HLA-A2$^{pos}$ and HLA-A3$^{pos}$ patients were also pulsed with two HER2 MHC class I binding peptides, 369-377 and 689-697. Six weekly injections of 10-20 million HER2 peptide-pulsed DC1s were administered into the breast (IL; n = 19), into the groin lymph nodes (IN; n = 19), or half of the dose into the breast and half of the dose into the groin lymph nodes (ILN; n = 16).

Following each weekly vaccination, patients were monitored for adverse effects for a minimum of 1-2 hours. Each patient underwent cardiac evaluation including multigated acquisition (MUGA) scan or echocardiogram prior to vaccination and within two weeks of the final vaccination. All adverse events were classified by National Cancer Institute Common Toxicity Criteria (NCI-CTC version 3.0).

**Outcome Measures**

**Immune Monitoring**

Systemic anti-HER2 CD4$^{pos}$ T-cell responses were measured in 53 of 54 patients pre- and post-vaccination, systemic anti-HER2 CD8$^{pos}$ T-cell responses were measured in 22 HLA-A2$^{pos}$ patients pre- and post-vaccination, and localized anti-HER2 CD4$^{pos}$ T-cell responses were measured post-vaccination in the regional, sentinel lymph nodes (SLN) in 40 patients who underwent SLN biopsy. HER2 specific IFN-ɣ production was measured by enzyme-linked immunosorbent spot (ELISPOT) assays or by in-vitro sensitization assays, as previously described in detail. [10, 29]

Briefly, ELISPOT PVDF membrane plates [Mabtech Inc, Cincinnati, OH] were coated overnight with anti-IFN-ɣ capture antibody (1D1K). The following day, after the plates were washed with PBS [Mediatech Inc, Manassas, VA] and blocked with 10% human serum/DMEM, $2x10^5$ peripheral blood mononuclear cells (PBMC), immature or mature DC, 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, 1166-1180), or pulsed with anti-human CD3 and CD28 antibodies (0.5 µg/mL) (positive control) [BD Pharmingen, San Diego, CA]. The plates were incubated at 37$^o$C+ 5% CO2 for 24-36 hours. After the plates were washed with PBS, 100 µL of detection antibody (1mg/mL; 7 B6-1-biotin) was added to each well and the plates were incubated for 2 hours. After the plates were washed again with PBS, 100 µL of 1:1000 diluted streptavidin-HRP was added to each well and the plates were incubated for another hour. TMB substrate solution was added to reveal spot formation. Spot forming cells (SFC) were counted using an automated reader (ImmunoSpot CTL). By ELISPOT, a positive response to an individual HER2 peptide was defined as a minimum of 20 SFC/$2x10^5$ cells after subtracting the unstimulated background. Two immune response metrics based on the six class II HER2 peptides were used to quantify the immune response: (1) response repertoire (the number of peptides to which a patient responded, range: 0-6), and (2) cumulative response (the sum of the SFCs across all six class II HER2 peptides).

Alternatively, $CD4^{pos}$ or $CD8^{pos}$ T-cells were selected from the cryopreserved 120-140 lymphocyte cell fractions via negative selection [StemCell Technologies, Vancouver, Canada] for in-vitro sensitization. Autologous DC1s were suspended in serum free medium (SFM) [Invitrogen, Carlsbad, CA] with GM-CSF (10ng/ml), pulsed with one of six class II HER2 peptides (42-56, 98-114, 328-345, 776-790, 927-941, 1166-1180) or a class I HER2 peptide (10

ug/ml) (369-377), and co-cultured with $CD4^{pos}$ or $CD8^{pos}$ T-cells at a ratio of 10:1. IL-2 (30 IU/ml) [ThermoFisher, Frederick, MD] was added on day 2. On day 10, T-cells were harvested and were tested against T2 target cells pulsed with either the class II or class I HER2 peptide or irrelevant controls (p53 and colon cancer peptide). After 24 hours, the supernatant was harvested and analyzed by enzyme-linked immunosorbent assay (ELISA). By ELISA, a positive response to an individual HER2 peptide was defined as a two-fold increase in $CD4^{pos}$ or $CD8^{pos}$ HER2 peptide-specific T-cell IFN-ɣ production compared to the irrelevant peptide control. Again, the response repertoire was used to quantify the $CD4^{pos}$ immune response.

As defined in the protocol, a patient was considered to be an immune responder by meeting at least one of the following criteria: (1) the cumulative $CD4^{pos}$ response increased by two fold (post-vaccination cumulative response / pre-vaccination cumulative response > 2) and the post-vaccination cumulative $CD4^{pos}$ response was greater than 20 SFC/$10^6$ cells after subtracting the unstimulated background (by ELISPOT), or (2) the response to an individual $CD4^{pos}$ peptide increased by two fold (post-vaccination peptide response / pre-vaccination peptide response > 2) and the post-vaccination peptide response was greater than 20 SFC/$2x10^5$ cells after subtracting the unstimulated background (by ELISPOT) or (3) the post-vaccination ELISA showed a new specific response to a CD4 or CD8 HER2 peptide (HER2 peptide/control ≥ 2) that was not present pre-vaccination (HER2 peptide/control < 2).

**Clinical Monitoring**

Subjects had a clip placed at the site of microcalcification under stereotactic guidance at the time of biopsy to serve as a marking site for future resection. Pathologic response was measured in the resected specimen - lumpectomy (n = 38) or mastectomy (n = 16). A pathologic

complete response (pCR) was defined as no DCIS or invasive breast cancer (IBC) found in the entire resected specimen. Rates of pCR were reported separately for pure DCIS and early IBC.

**Statistical Methods**

A ranking and selection design procedure [30] was employed to select the best treatment arm with regards to immune response rate. The sample size of 18 patients per arm ensured that if there was a 20% difference in true immune response rates between the best and second-best arms and a 10% difference between second-best and worst arms and assuming the best arm had a true immune response rate of 80 – 95%, then the probability of correctly selecting the superior arm exceeded 88%. The final sample sizes per arm varied slightly from the target of 18 patients, due to the introduction of a stratification by ER status after the amendment, which added concurrent anti-estrogen therapy in the treatment of $ER^{pos}$ patients.

Descriptive statistics were employed to characterize distributions of variables and of outcomes. Continuous variables were described by mean, standard error of the mean (SE), median and $25^{th}$ and $75^{th}$ percentiles. Categorical variables were described by frequencies and percentage. Normality of continuous variables was examined by normal probability plots. Comparisons of continuous variables by route of administration were conducted by ANOVA or nonparametric Kruskal-Wallis test, as appropriate. Two-group comparisons were conducted by Student's t-test or nonparametric Wilcoxon rank sum test. Pre-vaccine to post-vaccine comparisons were conducted by paired t-test or Wilcoxon signed rank test. Comparisons of categorical variables by route of administration were conducted by Fisher's exact test. Immune response rates and exact 95% confidence intervals were computed, and rates were compared by route of administration by Fisher's exact test. Rates of pCR by route of administration and associations between immune measures and pCR were reported for DCIS patients. Absolute

increases (post-vaccine – pre-vaccine) of response repertoire and fold increases (post-vaccine / pre-vaccine) of cumulative response were calculated to adjust for the pre-vaccine immune levels. All p-values were 2-sided. Statistical analyses were conducted in IBM SPSS v23.0. [30]

## Results

Between July 2009 and July 2015, 54 patients were treated on this trial. The median age was 55 years (range 35-83) and the majority of patients were post-menopausal (84.0%) and white (80.2%). Most tumors were high grade (71.7%) with 3+ HER2 expression (64.8%). Biopsy prior to vaccination diagnosed DCIS in a vast majority of patients (n = 51, 94.4%); however, 9 patients who were initially diagnosed with DCIS were found to have invasive disease in the final surgical specimen, representing a 17.6% up-staging at the time of resection, and leading to a cohort of 12 patients diagnosed with IBC. The three patients that were initially diagnosed with IBC were all HER2 positive by American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines.[31] Most patients were treated with lumpectomy (59.3%), but only a minority of patients who underwent lumpectomy received post-operative radiation (38.3%). Randomized treatment allocation was as follows: 19 patients (35.2%) received IL injections, 19 patients (35.2%) received IN injections, and 16 patients (29.6%) received ILN injections (**Supplemental Figure 1**). Demographic and clinical characteristics were similar across all three treatment groups as summarized in **Table 1**.

### Route of Vaccine Injection and ER Status Do Not Impact Vaccine Safety

Overall, the vaccine was well tolerated with only grade 1 (n = 37, 68.5%) and grade 2 (n = 15, 27.8%) adverse events reported. The most commonly reported adverse events associated

with the vaccine were fatigue (n = 22, 40.7%), injection site reaction (n = 22, 40.7%), and chills/rigors (n = 14, 25.9%). All of the patients received all six vaccine injections, and none of the patients withdrew from the trial due to the experienced side effects. The route of injection did not affect the risk of adverse events with grade 1-2 vaccine-related adverse events reported in 73.7% of patients who received IL injections, 68.4% of patients who received IN injections, and 75.0% of patients who received ILN injections. Only two patients exhibited asymptomatic declines in left ventricular ejection fraction (EF); one patient who received IL injection exhibited a decrease in EF to 55% (an 18% decrease), which subsequently returned to baseline within 30 days, and one patient who received ILN injection exhibited a decrease in EF to 49% on MUGA (a 28% decrease), which was attributed to PVCs and was not evident when immediately reevaluated by echocardiogram (EF 60-65%).

ER status and concurrent treatment with anti-estrogen therapy also did not affect the experience of adverse events. In fact, $ER^{pos}$ patients who were treated with combination anti-estrogen therapy and DC1 vaccination reported a lower rate of adverse events (61.9%) compared with $ER^{pos}$ patients who did not receive anti-estrogen therapy and were treated with vaccination alone (75.0%) or $ER^{neg}$ patients (80.0%) who were treated with vaccination alone.

**Immune Responses Increase Following Vaccination Independent of the Injection Route**

Based on 53 vaccinated patients evaluable for immune responses, 43 (81.1%) had a new or increased immune responses (either CD4 or CD8 anti-HER2 immune responses) detected in the peripheral blood following vaccination. The immune response rates (exact 95% confidence interval) by route of vaccination were: IL 84.2% (60.4 – 96.6%), IN 89.5% (66.9 – 98.7%), and ILN 66.7% (38.4 – 88.2%; **Table 2**). By the ranking and selection design, in which the arm with

the highest observed rate is selected, the IN arm could be selected; although IL and ILN arms had similar immune responses and there was no significant difference in immune response rate among the three arms (p = 0.30). The immune response rates were similar for vaccinated $ER^{neg}$ patients (83.3%) and $ER^{pos}$ patients (87.5%), but was slightly lower for the vaccinated $ER^{pos}$ patients who were treated with concurrent anti-estrogen therapy (76.2%) (**Supplemental Table 1**).

We also examined the $CD4^{pos}$ Th1 immune response in a quantified manner using two metrics: response repertoire and cumulative response. Overall, the quantified $CD4^{pos}$ Th1 immune response in the peripheral blood significantly increased from pre-vaccination to post-vaccination, in response repertoire (median ($25^{th}$ – $75^{th}$ percentile), 0 (0-2) to 2 (1-4), p < 0.001; **Figure 1A**), and cumulative response (48.6 (18.5-102.5) to 132.2 (72.5-238.5), p = 0.0001; **Figure 1B**). By each injection route, the quantitative response significantly increased from pre-vaccination to post-vaccination by response repertoire (IL 0 (0-1) to 2 (1-3), p = 0.001; IN 1 (0-3) to 4 (3-5), p < 0.001; ILN 0 (0-2) to 2 (1-3), p = 0.007; **Figure 1C**) and cumulative response (IL 38.1 (11.8-60.1) to 100.8 (44.2-203.9), p < 0.001; IN 82.8 (18.6-158.1) to 176.7 (131.7-338.1), p < 0.001, ILN 39.2 (23.3-108.3) to 93.1 (67.5-209.7), p = 0.002; **Figure 1D**). The absolute increase in response repertoire was similar across all three injection routes (IL 1 (1-2), IN 2 (1-3), ILN 1 (0-2); p = 0.16; **Figure 1E)**, and the fold increase in cumulative response was also similar across all three injection routes (IL 2.2 (1.6-4.4), IN 2.3 (1.6-7.9), ILN 2.2 (1.2-5.7); p = 0.81; **Figure 1F**). The quantified immune responses for each patient are shown in **Supplemental Figure 2**.

In the HLA–$A2^{pos}$ patients, the peripheral blood $CD8^{pos}$ T-cell response rate increased from 3 of 22 patients (13.6%) exhibiting a HER2-specific response pre-vaccination to 16 of 22

patients (72.7%) post-vaccination (p = 0.0002, McNemar's test). The 3 patients who exhibited a pre-vaccination anti-HER2 $CD8^{pos}$ T-cell response maintained the response, and an additional 13 patients developed a new anti-HER2 $CD8^{pos}$ T-cell response. There was no significant difference in the $CD8^{pos}$ T-cell response across all three injection routes, although the rate was lowest for the IN group (p = 0.17; **Table 2**)

Of 40 patients evaluable for quantified $CD4^{pos}$ Th1 immune response in the SLN, 32 (80%) had a HER2-specific response; median response repertoire was 2 ($25^{th}$ – $75^{th}$ percentile, 1-5) and median cumulative response was 76.5 (23.5-209.5). Although the post-vaccination SLN immune response showed the highest immune response rate in the IN group (91.7%), it was not significantly higher than the rates in the IL (71.4%; p = 0.33) or ILN (78.6%; p = 0.60) groups (**Table 2**). The quantified SLN response repertoire (p = 0.60; **Figure 1G**) and cumulative response (p = 0.56; **Figure 1H**) were also similar across all three injection routes. By definition, evaluation of the SLN immune response was limited to the post-vaccination response at the time of lymphadenectomy and could not be compared to pre-vaccination immune levels. Additionally, the limited nodal specimens were only examined for the $CD4^{pos}$, but not the $CD8^{pos}$, responses.

We compared the immune responses in patients with DCIS (n = 42) and patients with stage I IBC (n = 12). DCIS and IBC patients had similar immune response rates (**Table 3**) and similar increases in the quantified response repertoire (**Supplemental Figure 3A**) and cumulative response (**Supplemental Figure 3B)**.

**Clinical Responses are Associated with Increased SLN Immune Response, but not Peripheral Immune Response**

Clinically, 13 patients had no disease in the surgical specimen at the time of surgical resection, achieving a pCR. Patients with DCIS achieved a higher rate of pCR (12/42, 28.6%.) compared with patients with invasive disease (1/12, 8.3%). We further investigated the pCR rate in DCIS patients. Patient variables (age, BMI, race, menopausal status, and comorbidities) and tumor variables (grade, ER, PR, and HER2 status) were not different between DCIS patients who achieved a pCR and DCIS patients who did not achieve a pCR. In DCIS patients, the rate of pCR was similar by route of injection (IL 23.1%, IN 31.3%, ILN 30.8%; **Supplemental Table 2**). The pCR rate was similar for $ER^{neg}$ DCIS patients who underwent vaccination alone (31.5%) and $ER^{pos}$ DCIS patients who underwent vaccination with concurrent anti-estrogen therapy (33.3%). Interestingly, none of the five $ER^{pos}$ DCIS patients who underwent vaccination alone and did not receive concurrent anti-estrogen therapy achieved a pCR. Finally, the pCR rate was higher in the 21 HLA-$A2^{neg}$ DCIS patients compared to the pCR rate in the 21 HLA-$A2^{pos}$ DCIS patients (38.1% vs. 19.0%); however, this difference was not statistically significant ($p = 0.3$). Furthermore, comparing the patients who underwent vaccination with both class I and class II peptides (HLA-$A2^{pos}$ or HLA-$A3^{pos}$ patients; $n = 30$) with the patients who underwent vaccination with the class II peptides alone (HLA-$A2^{neg}A3^{neg}$; $n = 12$) showed more similar rates of pCR (26.7% vs. 33.3%; $p = 0.7$). Since vaccination led to similar immune and pathologic responses regardless of the injection route, ER status, or HLA typing in patients with DCIS, we further explored the relationship between the immune response and the clinical response.

In the peripheral blood, DCIS patients who achieved a pCR and DCIS patients who did not achieve a pCR had similar overall immune response rates (**Table 4**) and similar increases in the quantified CD4 response repertoire (**Supplemental Figure 4A)** and cumulative response (**Supplemental Figure 4B)** following vaccination. However, the DCIS patients who achieved a pCR started with a slightly higher quantified CD4 immune response in the peripheral blood, as

measured by both median response repertoire (1 vs. 0) and median cumulative response (94 vs. 34).

In contrast, a more robust response was detected in the SLN in the DCIS patients who achieved a pCR compared with the DCIS patients who failed to achieve a pCR. All seven of the DCIS patients who achieved a pCR had an anti-HER2 immune response detected in the SLN (**Table 4**). Furthermore, the post-vaccination quantified immune response in the SLN was higher in the pCR group than in the non-pCR group as measured by both response repertoire (median 5.0 vs. 1.0, p = 0.03; **Figure 2A**) and cumulative response (243.0 vs. 52.0, p = 0.04; **Figure 2B**). Regardless of the route of vaccination, the response repertoire in the SLN was higher in the DCIS patients who achieved pCR (median IL 3.5 vs. 1.0; IN 3.5 vs. 2.5; ILN 5.0 vs. 1.0; **Figure 2C**) and the cumulative response in the SLN was higher in the patients who achieved pCR (IL 217.5 vs. 24.5; IN 187.0 vs. 66.0; ILN 243.0 vs.58.0; **Figures 2D**).

## Discussion

This study confirmed that neoadjuvant DC1-based vaccination in early breast cancer is safe and immunogenic. Furthermore, the immune and clinical responses induced by DC1 vaccination were similar regardless of the route of vaccine administration. However, a higher rate of pCR was achieved in the DCIS patients compared with the IBC patients. Finally, the clinical response in DCIS patients correlated with the immune response detected in the SLN, but not the immune response detected in the peripheral blood.

In this study, HER2 peptide-pulsed DC1 vaccination was well-tolerated with only mild adverse events reported. Fatigue, injection site reaction, and chills/rigors were the most commonly reported adverse events, without evidence of immunotherapy-induced-autoimmunity

or irreversible cardiac toxicity. We specifically compared three routes of vaccination – intralesional, intranodal or combined intralesional and intranodal. Previous studies have shown that migration of DCs to the lymph nodes is required for induction of immune response, [32] and imaged the migration of DCs injected intradermally, subcutaneously, or intranodally to the draining lymph node regions. [32-34] Theoretically, direct intranodal injection may be expected to be more effective because the DCs would be placed directly at the site of T-sensitization; however, in these studies, despite fewer DCs arriving at the lymph nodes following intradermal injection than intranodal injection, similar immune responses were detected in the peripheral blood regardless of the route of vaccination. [34, 35] Similarly, in this study, our analysis of the immune response did not show any significant differences by route, but may be limited by small sample sizes. Specifically, the CD8 immune response was only measured in the peripheral blood and was only measured in 22 patients, and, therefore, the lower rate of CD8 responders in the IN group (n=7) did not achieve statistical significance. Additionally, in the ILN group, we hypothesize that the rate of immune responders may be lower because the vaccination dose was split in half with half of the dose injected into the breast and half of the dose injected into the lymph node. Nevertheless, despite the half doses delivered at each injection site and the lower immune response rate in the ILN group, the clinical response in the ILN group was similar. Overall, the current study demonstrated that the vaccine was equally tolerated with similar adverse events by all routes of injection, and the immune and clinical response rates following vaccination were similar regardless of the route of vaccination.

The main goal of DC vaccination, and consequently the main outcome measured in clinical trials, is stimulation of antigen-specific T-cells that recognize and eliminate tumor cells. In a meta-analysis of twenty-nine trials involving over 900 patients using DCs as cellular

adjuvants, antigen-specific cellular immunity was induced in 77% of patients with prostate cancer and 61% of patients with renal cell carcinoma. [36] In this trial, 81% of patients developed a HER2-specific immune response following vaccination. Despite the favorable safety profile and the high immunogenicity, DC-based vaccination has been heavily criticized for the disappointing and variable clinical responses.[37-39] In a systematic review of all published clinical trials, the objective clinical responses following DC vaccination were much more limited: 7.1% in patients with prostate cancer, 8.5% in patients with melanoma, 11.5% in patients with renal cell carcinoma, and 15.6% in patients with malignant glioma. [39] These rates are similar to the pCR rate we found in patients with IBC (8.3%), and, in this context, the 28.6% rate of pCR in DCIS patients is even more impressive. Of note, in this study, we chose to analyze patients based on the final pathology found in the resected specimen, rather than the pathology found in the diagnostic biopsy. Diagnostic biopsy caries a risk of sampling error and the rate of upstaging found in this study (17.6%) is similar to rates reported in the literature. [40-42] On the other hand, progression from DCIS to IBC over the course of the 6 weeks of vaccination is very unlikely. Therefore, we chose to include those patients who were diagnosed with IBC in the definitive surgical specimen in the group of IBC patients. Alternatively, if we had grouped the patients based on the initial biopsy results, there would have been only 3 patients in the IBC group and 51 patients in the DCIS group. Of the fifty-one patients initially diagnosed with DCIS on biopsy, twelve achieved a pCR leading to a pCR rate of 23.5%, only slightly less than the 28.6% pCR rate in the group of DCIS patients diagnosed by final pathology.

We have previously argued that DC-based vaccines may be more effective in patients with pre-invasive/early stage disease with a smaller tumor burden and preserved anti-HER2 Th1 immunity; [1, 2] and similar studies using vaccination in pre-invasive cervical lesions (cervical

intraepithelial neoplasia 2/3) have also led to similar rates of regression (30%). [43] Although the DCIS patients and the IBC patients had similar immune response rates in this study, the rate of pCR was higher in DCIS patients, suggesting that immunotherapy may be more effective in DCIS. We have previously shown that there is a progressive loss of the anti-HER2 Th1 immune response along the continuum of HER2$^{pos}$ breast cancer – DCIS patients have a diminished immune response and the immune response is nearly absent in patients with IBC. [1] The anti-HER2 CD4 Th1 response prior to vaccination may distinguish patients with pure DCIS from those with DCIS and IBC, as the DCIS patients have a higher CD4 Th1 response than the patients with IBC. Measuring the pre-vaccination immune response may allow us to successfully identify and treat DCIS patients with preserved immune responses. Additionally, in future studies, response rates in both DCIS and IBC may be further improved by prolonged vaccination regimens to boost responses, the addition of targeted therapies, such as trastuzumab and pertuzumab, to decrease tumor proliferation, or modification of the tumor microenvironment to enhance migration of activated lymphocytes to tumor regions. [44]

Criticisms of DC-based vaccination point to the discordance between the immune and clinical responses. Vaccination has been shown to induce an immune response in the peripheral blood without leading to clinical tumor regression and, conversely, lead to tumor regression without inducing an immune response. [45] In this study, both the CD4$^{pos}$ and the CD8$^{pos}$ immune responses in the peripheral blood increased following vaccination; however, neither was associated with the pathologic response rate. On the other hand, the anti-HER-2 CD4 Th1 response measured in the SLN was significantly higher in the patients who achieved pCR compared with the patients who did not achieve a pCR, suggesting that the SLN immune response better reflects the pathologic response.

In our comparison of DCIS patients who achieved a pCR with DCIS patients who failed to achieve a pCR, both the rate of CD4 immune responders and the rate of CD8 immune responders in the peripheral blood were lower in the group of patients who achieved a pCR. Despite the lack of statistical significance, we can speculate that the lower rate of immune responders in the pCR group may be due to the congregating of the immune response in the SLN. In patients who achieved a pCR, the circulating immune response rate may be lower because the T-cells home to the SLN in order to gain access to the tumor. The higher CD4 immune response rate in the SLN in the group of patients who achieved a pCR further supports this theory that the immune response concentrated in the SLN successfully attacks the tumor. An analogous study of the immune response in patients with acute bacterial soft tissue infections identified activated $CD4^{pos}$ and $CD8^{pos}$ T-cells in the peripheral blood and at the site of infection, but found that only $CD4^{pos}$ and $CD8^{pos}$ T-cells harvested from the infected site (but not $CD4^{pos}$ or $CD8^{pos}$ T-cells in the peripheral blood of the same patients) expressed CXCR6 and produced IFN-ɣ, suggesting a uniquely local role for CD4 and CD8 T-cells in host defense. [46]

The discrepancy between the peripheral blood immune response and the clinical response is also particularly apparent when examining the groups by ER status. The $ER^{pos}$ patients who were treated with anti-estrogen had a noticeably lower proportion of immune responders measured in the peripheral blood, but a higher rate of pCR. In the SLN, the immune response was higher in the $ER^{pos}$ patients who received concurrent DC1 vaccination and anti-estrogen therapy compared to the $ER^{pos}$ patients who received DC1 vaccination alone, supporting the importance of the immune response in the SLN. This study was not powered to evaluate the addition of anti-estrogen therapy to vaccine therapy, and the lack of statistical significance is

likely due to the small numbers in this study. Combination anti-HER2 vaccination and anti-estrogen therapy is more completely examined in our overall institutional experience. [13]

This study is subject to limitations that warrant emphasis. First, the anti-HER2 immune responses were not measured at the level of the tumor. We have previously reported an increase in periductal lymphocytic infiltration at the site of residual DCIS following DC1 vaccination. [10] However, given that DCIS lesions are small, most patients did not have sufficient tissue to assess the pre and/or post anti-HER2 specific lymphocytic infiltrate and there were no immunohistochemical or immunofluorescent signs of the former DCIS lesion in those patients who achieved a pCR. In order to overcome this obstacle, we investigated the closest pathologic sample, the regional draining SLN. The SLN was an appealing media to measure the immune response since SLN biopsy is frequently part of standard treatment and would not add any additional morbidity or mortality. Furthermore, the SLN immune response could be compared between DCIS patients who achieved pCR and DCIS patients who failed to achieve pCR. Second, the findings of this study also leave the cause and effect of the association between the SLN immune response and the clinical response open to question. By definition, the SLN immune response cannot be examined pre and post vaccination to measure the immune response induced by vaccination. Furthermore, 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 anti-tumor Th1 immune response measured in the SLN may better reflect anti-tumor activity and serve as a marker for response to immunotherapy. Further studies of immunotherapy may more thoroughly evaluate the immune response in the peripheral blood, the SLN, and the tumor to correlate more meaningfully with the clinical response.

In summary, this trial showed that DC1 vaccination remained safe and well-tolerated independent of the route of vaccination. DC1 vaccination was also equally effective in inducing

immune and clinical responses independent of the route of vaccination. The clinical response was much higher in DCIS patients compared to IBC patients, and DCIS patients who achieved a pCR showed a higher immune response measured in the SLN. The SLN has been extensively evaluated for its prognostic and therapeutic capacity with respect to tumor progression. We suggest that the SLN may also be evaluated for its immunologic properties, and that future trials may further explore the immune response detected in the SLN as an endpoint to better evaluate immune based therapies.

Author Contributions: Conception and Design: BJC, RM, SW, KF, AD, PZ, SX, EF; Development of Methodology: LL, RM, JD, BJC; Acquisition of Data: LL, JD, SX, EF, BJC; Analysis and Interpretation of Data: LL, RM, BJC; Writing, Review, and Revision of Manuscript: LL, RM, JD, SX, RR, CF, EF, KF, AD, PZ, SW, BJC

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## FIGURE LEGENDS

**Figure 1:** The $CD4^{pos}$ Th1 immune response in the peripheral blood pre-vaccination and post-vaccination quantified by (**A**) response repertoire (**$p < 0.001$**) and (**B**) cumulative response (**$p = 0.0001$**), in all 53 patients. The $CD4^{pos}$ Th1 immune response in the peripheral blood pre-vaccination and post-vaccination by injection route, quantified by (**C**) response repertoire (IL: **$p = 0.001$**; IN: **$p < 0.001$**; ILN: **$p = 0.007$**) and (**D**) cumulative response (IL: **$p < 0.001$**; IN: **$p < 0.001$**; ILN: **$p = 0.002$**), and similar increases across injection routes displayed by (**E**) the absolute increase in response repertoire ($p = 0.16$) and (**F**) the fold increase in cumulative response ($p = 0.81$). The $CD4^{pos}$ Th1 immune response in the SLN by injection route, quantified by (**G**) response repertoire and (H) cumulative response.

**Figure 2:** The $CD4^{pos}$ Th1 immune response in the SLN by clinical response in DCIS patients, quantified by (**A**) response repertoire (**$p = 0.03$**) and (**B**) cumulative response (**$p = 0.04$**); and the $CD4^{pos}$ Th1 immune response in the SLN by clinical response for each injection route, quantified by (**C**) response repertoire and (**D**) cumulative response.

Figure 1

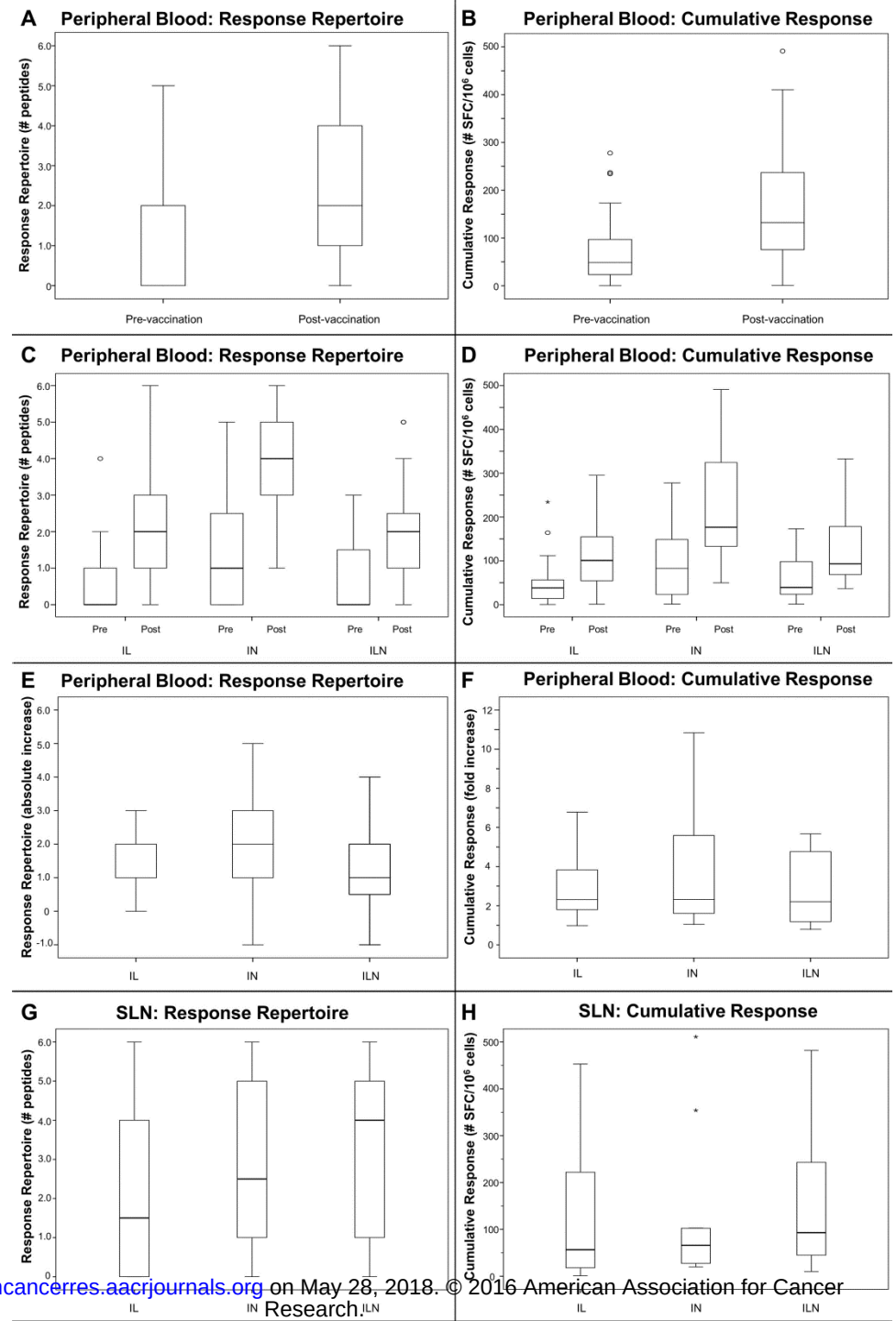

Figure 2

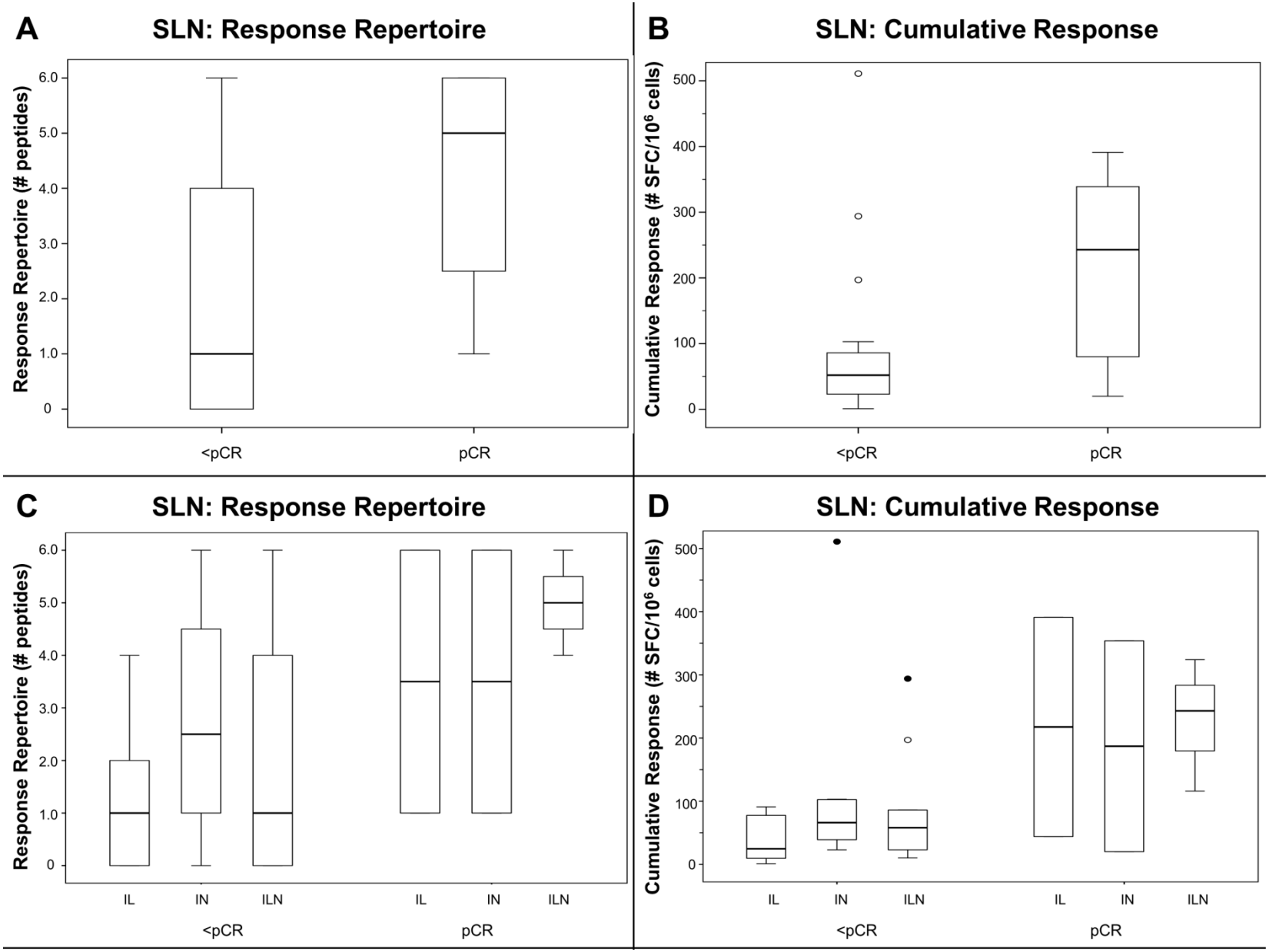

**Table 1**: Patient, tumor, and treatment characteristics by route of administration

| | Route of vaccine administration | | | | | |
|---|---|---|---|---|---|---|
| | Intralesional, n=19 | | Intranodal, n=19 | | Intralesional and Intranodal, n=16 | |
| | mean±SE | range | mean±SE | range | mean±SE | range |
| Age, years | 54.8±1.5 | 36-68 | 52.0±2.1 | 37-63 | 56.6±2.9 | 35-83 |
| BMI | 25.9±1.5 | 19-39 | 27.2±1.3 | 20-38 | 28.7±1.8 | 22-51 |
| | n | % | n | % | n | % |
| Race | | | | | | |
| Caucasian | 15 | 78.9 | 14 | 73.7 | 14 | 87.5 |
| African-American | 2 | 10.5 | 3 | 15.8 | 2 | 12.5 |
| Hispanic | 0 | 0.0 | 2 | 10.5 | 0 | 0.0 |
| Asian | 2 | 10.5 | 0 | 0.0 | 0 | 0.0 |
| Menopausal status | | | | | | |
| Pre-menopausal | 3 | 15.8 | 5 | 26.3 | 3 | 18.8 |
| Post-menopausal | 16 | 84.2 | 14 | 73.7 | 13 | 81.3 |
| Charlson Comorbidity Score | | | | | | |
| ≤2 | 17 | 89.5 | 17 | 89.5 | 14 | 87.5 |
| ≥3 | 2 | 10.5 | 2 | 10.5 | 2 | 12.5 |
| Current medications | | | | | | |
| None | 1 | 5.3 | 2 | 10.5 | 2 | 12.5 |
| <5 | 12 | 63.2 | 11 | 57.9 | 11 | 68.8 |
| >5 | 6 | 31.6 | 6 | 31.6 | 3 | 18.8 |
| Grade[a] | | | | | | |
| Low | 1 | 5.3 | 0 | 0.0 | 0 | 0.0 |
| Intermediate | 4 | 21.1 | 7 | 38.9 | 3 | 18.8 |
| High | 14 | 73.7 | 11 | 61.1 | 13 | 81.3 |
| Stage | | | | | | |
| Ductal carcinoma in situ | 13 | 68.4 | 16 | 84.2 | 13 | 81.3 |
| Invasive breast cancer[b] | 6 | 31.6 | 3 | 15.8 | 3 | 18.8 |
| ER status | | | | | | |
| Negative | 8 | 42.1 | 11 | 57.9 | 6 | 37.5 |
| Positive | 11 | 57.9 | 8 | 42.1 | 10 | 62.5 |
| PR status | | | | | | |
| Negative | 11 | 57.9 | 13 | 68.4 | 7 | 43.8 |
| Positive | 8 | 42.1 | 6 | 31.6 | 9 | 56.3 |
| HER2/neu status | | | | | | |
| 2+ | 6 | 57.9 | 6 | 26.3 | 7 | 43.8 |
| 3+ | 13 | 42.1 | 13 | 73.7 | 9 | 56.3 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Surgery & Radiation | | | | | | |
| Lumpectomy, no radiation | 9 | 47.4 | 10 | 52.6 | 7 | 43.8 |
| Lumpectomy, radiation | 4 | 21.1 | 2 | 10.5 | 6 | 37.5 |
| Mastectomy | 6 | 31.6 | 7 | 36.8 | 3 | 18.8 |

[a] Tumor grade missing for 1 patient who received an intranodal injection

[b] Prior to vaccination, 3 patients were diagnosed with IBC by biopsy; following vaccination, an additional 9 patients who were initially diagnosed with DCIS were upstaged to IBC in the final surgical specimen (IL: 2 patients diagnosed by biopsy, 4 patients upstaged; IN: 1 patient diagnosed by biopsy, 2 patients upstaged; ILN: 0 patients diagnosed by biopsy, 3 patients upstaged)

**Table 2**: CD4 and CD8 immune outcomes in the peripheral blood and in the sentinel lymph nodes by route of vaccine administration.

| | Route of vaccine administration | | | | | | | | | p-value |
|---|---|---|---|---|---|---|---|---|---|---|
| | Intralesional, n=19 | | | Intranodal, n=19 | | | Intralesional and Intranodal, n=16 | | | |
| | n | % | exact 95% CI | n | % | exact 95% CI | n | % | exact 95% CI | |
| Overall CD4 PB Immune Responders[a] | 16 | 84.2 | 60.4-96.6 | 17 | 89.5 | 66.9-98.7 | 10 | 66.7 | 38.4-88.2 | 0.30 |
| Overall CD8 PB Immune Responders[b] | 6 | 85.7 | 42.1-99.6 | 3 | 42.9 | 9.9-81.6 | 7 | 87.5 | 47.3-99.7 | 0.17 |
| Overall CD4 SLN Immune Responders[c] | 10 | 71.4 | 41.9-91.6 | 11 | 91.7 | 61.5-99.8 | 11 | 78.6 | 49.2-95.3 | 0.54 |

CI (confidence interval), PB (peripheral blood), SLN (sentinel lymph node)
[a] one patient (ILN) not evaluable for overall CD4 PB immune response
[b] 22 total HLA-A2+ patients evaluated for CD8 PB immune response (7 IL, 7 IN, 8 ILN)
[c] 40 total patients evaluated for SLN CD4 immune response (14 IL, 12 IN, 14 ILN)

**Table 3**: CD4 and CD8 immune outcomes in the peripheral blood and in the sentinel lymph nodes by disease stage

| | Disease Stage | | | | | | p-value |
|---|---|---|---|---|---|---|---|
| | DCIS, n = 42 | | | IBC, n = 12 | | | |
| | n | % | exact 95% CI | n | % | exact 95% CI | |
| Overall CD4 PB Immune Responders[a] | 33 | 80.5 | 65.1-91.2 | 10 | 83.3 | 51.6-97.9 | 1.00 |
| Overall CD8 PB Immune Responders[b] | 12 | 66.7 | 50.0-86.7 | 4 | 100 | 47.3-[*] | 0.29 |
| Overall CD4 SLN Immune Responders[c] | 25 | 78.1 | 60.0-90.7 | 7 | 87.5 | 47.3-99.7 | 0.67 |

DCIS (ductal carcinoma in situ), IBC (Invasive breast cancer), CI (confidence interval), PB (peripheral blood), SLN (sentinel lymph node)
[a] one patient (DCIS) not evaluable for overall CD4 peripheral immune response
[b] 22 total HLA-A2+ patients evaluated for CD8 peripheral immune response (18 DCIS, 4 IBC)
[c] 40 total patients evaluated for SLN CD4 immune response (32 DCIS, 8 IBC)
[*] lower bound of one-sided exact 95% CI

**Table 4:** CD4 and CD8 immune outcomes in the peripheral blood and in the sentinel lymph nodes by pathologic response in 42 DCIS patients

| | Pathologic Response | | | | | | p-value |
|---|---|---|---|---|---|---|---|
| | Non-pCR, n = 30 | | | pCR, n = 12 | | | |
| | n | % | exact 95% CI | n | % | exact 95% CI | |
| Overall CD4 PB Immune Responders[a] | 25 | 86.2 | 68.3-96.1 | 8 | 66.7 | 34.9-90.1 | 0.20 |
| Overall CD8 PB Immune Responders[b] | 11 | 73.3 | 44.9-92.2 | 1 | 33.3 | 0.8-90.5 | 0.51 |
| Overall CD4 SLN Immune Responders[c] | 18 | 72.0 | 50.6-87.9 | 7 | 100 | 65.2-[*] | 0.17 |

DCIS (ductal carcinoma in situ), CI (confidence interval), PB (peripheral blood), SLN (sentinel lymph node)
[a] one patient (non-pCR) not evaluable for overall CD4 peripheral immune response
[b] 18 total HLA-A2+ patients evaluated for CD8 peripheral immune response (15 non-pCR, 3 pCR)
[c] 32 total patients evaluated for SLN CD4 immune response (25 non-pCR, 7 pCR)
[*]lower bound of one-sided exact 95% CI

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# Dendritic Cell Vaccination Enhances Immune Responses and Induces Regression of HER2 $^{pos}$ DCIS Independent of Route: Results of Randomized Selection Design Trial

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