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A pilot study incorporating HER2-directed dendritic cells into neoadjuvant therapy of early stage HER2+ER- breast cancer

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Type 1 dendritic cell vaccines targeting HER2 (HER2-DC1) reinvigorates antitumor immunity which correlates with neoadjuvant therapy response. A pilot trial (clinicaltrials.gov,NCT03387553,1/2/2018) using HER2-DC1 pre-neoadjuvant therapy evaluated feasibility/safety and pathologic response rates/immunogenicity. Stage II-III ER-HER2+ breast cancer patients prescribed neoadjuvant docetaxel/carboplatin/trastuzumab/pertuzumab (TCHP) were enrolled. HER2-DC1 (2×10^7 cells/vaccine) was given for 3 weeks prior to chemotherapy intranodal (IN) 1x/week (Arm A), IN 2x/week (Arm B), and 2x/week alternating intratumoral (IT) and IN (Arm C). HER2 ELISPOT counts (EHC) and immunofluorescence analysis of biopsies were performed. Six patients enrolled in Arms A and B, 18 patients in Arm C. Neoadjuvant HER2-DC1 demonstrated no unexpected safety signals. Pathologic complete response rates (pCR) across arms A, B, C were 42.8%, 66.6%, and 72.7%. Intranodal HER2-DC1 increased EHC, but IT + IN HER2-DC1 reduced EHC, possibly due to increased T cell tumor trafficking. Immunofluorescence showed increased T cell infiltration following IT + IN injections. Additional IT HER2-DC1 investigation is warranted.

Background

Prior research has shown that as breast cancers evolve from initial transformation to fully invasive tumors, the immune recognition of key tumor-associated antigens, such as HER2, decreases[1]. This is likely due to immune editing and increased tumor-mediated immunosuppression which facilitates further spread of malignancy[2]. However, some breast tumors do trigger significant influxes of T cells, and multiple studies have shown that this leads to an improved prognosis compared to immunologically "cold" tumors[3]. Also, inflamed tumors appear to respond better to preoperative systemic therapies with higher rates of pathologic complete response (pCR)[4]. The main questions that arise are how can one manipulate the tumor microenvironment of these colder tumors to promote a re-recognition of tumor associated antigens, trigger beneficial anti-tumor inflammation, and improve response rates to preoperative systemic therapy?

Dendritic cells (DCs) are potent antigen-presenting cells that play a pivotal role in initiating and regulating immune responses. Type 1 DCs (DC1s) are a specific subtype that are very proficient in antigen cross-presentation to promote Type 1 adaptive cellular immune responses against targeted antigens. Understanding the important role of DC1s in initiating and regulating immune responses led to interest in using them for cancer immunotherapy[5–7]. It has been shown that HER2 targeting DC1 vaccines can be produced by putting apheresed autologous DCs through an ex vivo process of stimulation with specific cytokines along with immunogenic HER2 peptides. These HER2 DC1 vaccines can reverse tumor mediated immunosuppression, re-establish recognition of HER2 epitopes, and lead to the regression of early breast cancers when injected into draining axillary lymph nodes[8].

Based on this information, we initiated a pilot clinical trial to test the feasibility and immune modulating effects of using a HER2 DC1 vaccine

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immediately prior to administration of standard neoadjuvant therapy for stage II-III ER- HER2+ breast cancers. The trial demonstrated that administration of DC1 vaccines during neoadjuvant therapy is feasible, and intratumoral injections led to increased immune infiltration within the tumor microenvironment.

Results

Patient accrual/demographics/disposition

From June 6th 2018 to February 14th 2023, 31 patients registered, and 30 patients were treated on this non-randomized, unblinded pilot study at the Moffitt Cancer Center. Seven patients enrolled into arm A (one unevaluable patient withdrew after consent prior to therapy), 6 patients into arm B, and 18 patients into arm C. The patient disposition as of October 17th 2023 is shown in Fig. 1. The characteristics of the treated study population are described in Table 1.

Efficacy results from neoadjuvant therapy

The overall pCR rate for evaluable patients was 20/30 (64.5%, 95% CI = 45.4–80.8) across all arms. The breakdown of pCR descriptive rates across the three arms and the breakdown of responses using residual cancer burden index (RCB) is shown in Table 2.

As of 11/10/2023, with a median follow-up of 32.8 months, 2 patients (both in arm C) have had CNS-only recurrence of their breast cancer, leading to death for a disease-free survival rate and overall survival rate of 93.3%. Of note, both patients with breast cancer recurrences had pCR responses but had higher stage disease (stage IIB and III) at presentation. Two other patients (one in arm A and one in arm C) had subsequent non-breast primaries (one lung adenocarcinoma and one pancreatic adenocarcinoma) without death, so did not count towards the recurrence-free survival secondary endpoint. With regards to neoadjuvant chemotherapy delivery 176/180 (97.8%) of planned cycles were delivered with 138/180 (76.7%) at full dose. Similarly, the majority of patients (27/30, 90%) were able to complete a year of HER2 directed adjuvant therapy. Additional data on treatment is provided as supplemental data.

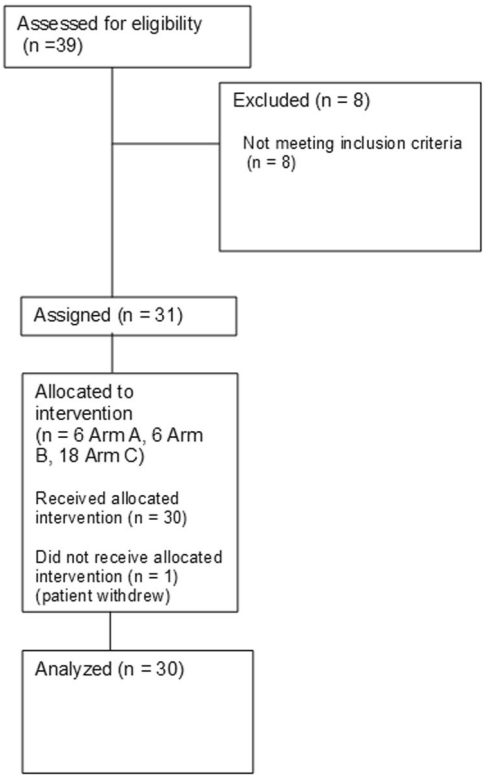

Fig. 1 | CONSORT flow diagram.

Safety data

The safety data for adverse events occurring in greater than 20% the entire study population with all arms combined is shown in Table 3. A supplemental figure (supplemental figure 1) shows common expected adverse events and distribution of grades between the three arms. The majority of adverse events were attributable to the neoadjuvant chemotherapy. The adverse events that were deemed specific to the dendritic cell vaccines, such as injection site reactions, chills, and fevers, are bolded in the table. There were four serious adverse grade 4 events and no grade 5 events. The serious adverse events were one case of pulmonary embolism in arm B, and one case each of colitis, appendicitis with perforation, and neutropenia/febrile neutropenia in arm C.

Immune correlatives

The number of activated T cells that responded to HER2 antigens at baseline and following DC1 vaccination was measured using an interferon γ ELISPOT assay that produces a visible spot for each HER2-activated T cell secreting interferon γ. The immune response data for HER2 ELISPOTs performed on blood samples drawn at baseline, week 4, 28, 56, 80, and 104 across the three arms is shown in Fig. 2. For both arms (A&B) that utilized intranodal injection there was an increase in ELISPOT counts at week 4 compared to baseline, as expected. Also, as expected, ELISPOT counts decreased because of neoadjuvant chemotherapy, and patients then had a rebound in the ELISPOT count following completion of the post-chemotherapy booster sequences. However, what was unexpected was that the week 4 ELISPOT increase was not seen in arm C following administration of the initial sequence of intratumoral and intranodal vaccines. The ELISPOT counts similarly increased over time in arm C with the post-surgical booster intranodal vaccine sequence, suggesting there was a different effect on circulating T cells attributable to the intratumoral vaccine. The immune suppressive effects of neoadjuvant chemotherapy given preoperatively have typically resolved by the 1-year mark, so the increases in ELISPOT changes within the second year are not simply a recovery of the immune system to baseline by week 104. One possibility is that the intratumoral vaccine dose caused increased trafficking of HER2-specific T cells from the circulation to the tumor site. The patients were not HLA subtyped as class II promiscuous HER2 peptides are used for DC1 pulsing and ELISPOT testing to maximize immunologic compatibility with the vaccine.

To investigate this phenomenon further, multiplex immunofluorescence (mIF) was completed on available tumor needle biopsies obtained before treatment started and after completion of the DC1 vaccine at week 4 for patients on arm C. The mIF data is shown in Fig. 3 with a representative image of a baseline and 4 week mid-treatment biopsy sample from the same patient, showing a substantial increase in stromal immune cell infiltration compared to baseline. Violin plots in Fig. 4 demonstrate the % of cells across the whole tissue section positive for CD3 (lymphocyte), CD3 + 4+ (helper T), CD3 + 45RO+ (memory T), CD3 + TCRδ + (γδ T), CD3 + 8+ (effector T), CD3 + 56+ (NKT), CD20+ (B cell), MHCII, and CD3+ interferon-γ (INFγ) + expression. The most statistically significant increases post- vaccination were noted for CD3 + 8 + , NKT, MHCII, and interferon-γ producing lymphocytes prior to the administration of neoadjuvant chemotherapy. Since there are no on-treatment biopsies from arms A and B for direct comparison, no conclusions can be drawn regarding if infiltration is greater in the tumor beds with intratumoral vaccination relative to intranodal.

Comparison of the % positive cells by mIF of the various immune cell subtypes in samples from patients who had a residual disease (non pCR) versus those with pathologic complete response (pCR) following completion of neoadjuvant chemotherapy, revealed a non-significant trend towards greater number of pCR patients with increased stromal CD3 + INFγ+ cells on their mid treatment samples compared to the RCB patient mid-treatment samples. The difference between response groups for NKT cells was not statistically significant. (Fig. 5).

Table 1 | Study population demographics

	Total	Arm A	Arm B	Arm C
Sex	Female 30 (100%)	6	6	18
Race/Ethnicity	White 22 (73%)	4	5	13
	Hispanic 4 (13%)	0	1	3
	Asian 2 (7%)	1	0	1
	Black 2 (7%)	1	0	1
Age	Median 59	Median 59	Median 63	Median 58
	Range (31-73)	Range (51–66)	Range (52–72)	Range (31–73)
Clinical stage	Stage II = 19 (63%)	4	4	11
	Stage III = 11 (37%)	2	2	7
	Node – = 6 (20%)	0	1	5
	Node + = 24 (80%)	6	5	13
Grade	Grade II = 9 (30%)	2	2	5
	Grade III = 21 (70%)	4	4	13
Histology	Ductal 29 (97%)	6	5	18
	Lobular 1 (3%)	0	1	0
Receptor status	ER-PR- = 30 (100%)	6	6	18
	HER2 3 + = 29 (97%)	6	6	17
	HER2 2 + = 1 (3%)	0	0	1

Table 2 | Response categories across the three arms

Arm	RCB 0 (pCR)	RCB 1	RCB 2	RCB 3
A	3 (42.8%)	1 (14.2%)	1 (14.2%)	1 (14.2%)
B	4 (66.6%)	1 (16.7%)	1 (16.7%)	0
C	13 (72.2%)	3 (16.7%)	2 (11.1%)	0

Discussion

The administration of DC1 HER2-targeting vaccines in the neoadjuvant setting was safe and feasible to perform. Apart from constitutional symptoms that were expected side effects associated with immune activation, the remainder of the side effects were due to neoadjuvant chemotherapy with no change from the expected safety profile of those agents. The incorporation of the intratumoral vaccines following a study amendment in Arm C was also feasible, with no additional safety signals. Chemotherapy treatment density, planned doses administered, and patient acceptance were not significantly altered.

Prior research into HER2-based vaccines typically either used peptide based vaccines in the adjuvant setting (i.e. GLSI-100, E75, AE37) or autologous DC vaccines (lapuleucel-T, APC8024) in the metastatic disease setting[9–12]. None of these vaccines have gained approval to date, possibly due to limitations of single antigen peptide vaccine immune activation and use of dendritic cells in the highly immunosuppressive metastatic setting. To our knowledge this is the first trial exploring differences in immune and clinical responses with intratumoral injections versus intranodal injections of HER2+ dendritic cell vaccines in the neoadjuvant setting. This trial informed the conduct of ongoing confirmatory trials using dendritic cell vaccines in the neoadjuvant setting[13].

The main limitation of this trial is that it is a small pilot study. There was no formal testing for efficacy against an a priori hypothesis so there is no firm conclusion that can be made regarding how the HER2 DC1 vaccines may affect the efficacy of neoadjuvant chemotherapy plus anti-HER2 monoclonal antibodies. In particular, the descriptive PCR rate for Arm C was in line with what TCHP combination chemotherapy would achieve in ER-HER2+ breast cancer based on multiple neoadjuvant trials[14,15]. The disease-free and overall survival of this group is similar to the outcomes seen in the KATHERINE trial. The proportion of RCB 1 cases in the arms appears similar to what is quoted in real-world data as well[16]. However, cross-trial comparisons are not definitive due to differences in populations so only a randomized trial can determine the effects of DC1 vaccines on neoadjuvant therapy response rates. The on-treatment stromal infiltration of CD3 + IFNγ+ lymphocytes appears to increase more so in pCR patients compared to those with residual cancer, which supports further investigation of how optimized delivery of DC1 vaccines can better sensitize tumors to systemic treatments. There is emerging data that other immune responses such as B cell activation and antibody-dependent cell-mediated cytotoxicity from NK cells may be important in improving immune response to intratumoral injections and should be explored in future trials[17]. Demonstrating significant changes in pCR/RCB1 response rates in highly active regimens, such as TCHP for ER- HER2+ disease, is challenging and would require larger sample sizes to detect smaller absolute effect sizes. Another factor that is under investigation is using higher doses of DC1 to increase the magnitude of T cell trafficking, which may further enhance the antitumor efficacy.

There did not appear to be any significant differences in the immunogenicity of once weekly versus twice weekly intranodal injections of the DC1 vaccines. Both schedules caused an increase in detectable HER2 ELISPOT counts relative to baseline levels. Using DC1 vaccines for intratumoral injections was based on our hypothesis that placement of the DCs near the primary tumor antigens would create a more potent trafficking signal that could recruit T cells to the tumor bed better than intranodal vaccines alone would. The unexpected drop in HER2 ELISPOT counts after the 4 weeks of intratumoral injections compared to the higher values seen in the intranodal vaccination schedules, could be explained by more HER2 recognizing T cells going to the tumor bed out of circulation and thus not being detected in peripheral blood samples. The significant increases in CD3 + 8 + T cells seen on mIF analysis of on-treatment biopsies from Arm C patients would lend support to this hypothesis. On treatment, biopsy samples were added to the protocol alongside the addition of the Arm C amendment, so we do not have week 4 samples from intranodal injection alone patients in Arms A/B to compare the T cell tumor infiltration levels. Most samples from the post neoadjuvant chemotherapy resection showed depletion of T cell infiltrates and would not be helpful in comparing effects of the different vaccine administration routes.

In conclusion, the results from this pilot study demonstrate that the administration of neoadjuvant DC1 vaccines is safe and feasible when given

Table 3 | Adverse events by CTCAE term and grade with all grade incidence >20%

CTCAE Term	G1	%	G2	%	G3	%	G4	%	Total	%
Diarrhea	3	9.7	16	51.6	10	32.3	0	0.0	29	93.6
Fatigue	16	51.6	13	41.9	0	0.0	0	0.0	29	93.5
Nausea	10	32.3	13	41.9	3	9.7	0	0.0	26	83.9
Anemia	11	35.5	9	29.0	5	16.1	0	0.0	25	80.6
Chills[a]	**12**	**38.7**	**11**	**35.5**	**1**	**3.2**	**0**	**0.0**	**24**	**77.4**
Injection site reaction[a]	**11**	**35.5**	**9**	**29.0**	**0**	**0.0**	**0**	**0.0**	**20**	**64.5**
Lymphocyte count decreased	11	35.5	2	6.5	5	16.1	0	0.0	18	58.1
Fever[a]	**13**	**41.9**	**4**	**12.9**	**0**	**0.0**	**0**	**0.0**	**17**	**54.8**
Platelet count decreased	13	41.9	3	9.7	0	0.0	0	0.0	16	51.6
Anorexia	10	32.3	4	12.9	1	3.2	0	0.0	15	48.4
Dehydration	2	6.5	10	32.3	3	9.7	0	0.0	15	48.4
Peripheral sensory neuropathy	10	32.3	5	16.1	0	0.0	0	0.0	15	48.4
Abdominal pain	7	22.6	3	9.7	4	12.9	0	0.0	14	45.2
Rash maculo-papular	4	12.9	6	19.4	3	9.7	0	0.0	13	41.9
Weight loss	7	22.6	6	19.4	0	0.0	0	0.0	13	41.9
Pruritus	8	25.8	4	12.9	1	3.2	0	0.0	13	41.9
White blood cell decreased	6	19.4	6	19.4	1	3.2	0	0.0	13	41.9
Alanine aminotransferase increased	12	38.7	1	3.2	0	0.0	0	0.0	13	41.9
Myalgia	9	29.0	4	12.9	0	0.0	0	0.0	13	41.9
Hypokalemia	4	12.9	6	19.4	1	3.2	1	3.2	12	38.7
Sinus tachycardia	7	22.6	5	16.1	0	0.0	0	0.0	12	38.7
Hypertension	3	9.7	4	12.9	5	16.1	0	0.0	12	38.7
Vomiting	7	22.6	3	9.7	1	3.2	0	0.0	11	35.5
Headache	7	22.6	3	9.7	1	3.2	0	0.0	11	35.5
Edema limbs	6	19.4	5	16.1	0	0.0	0	0.0	11	35.5
Dyspnea	7	22.6	3	9.7	1	3.2	0	0.0	11	35.5
Neutrophil count decreased	5	16.1	4	12.9	0	0.0	1	3.2	10	32.3
Dizziness	9	29.0	1	3.2	0	0.0	0	0.0	10	32.3
Aspartate aminotransferase increased	9	29.0	1	3.2	0	0.0	0	0.0	10	32.3
Dysgeusia	9	29.0	1	3.2	0	0.0	0	0.0	10	32.3
Alopecia	10	32.3	0	0.0	0	0.0	0	0.0	10	32.3
Pain	8	25.8	1	3.2	0	0.0	0	0.0	9	29.0
Hypoalbuminemia	8	25.8	1	3.2	0	0.0	0	0.0	9	29.0
Mucositis oral	6	19.4	3	9.7	0	0.0	0	0.0	9	29.0
Generalized muscle weakness	3	9.7	4	12.9	1	3.2	0	0.0	8	25.8
Rash acneiform	6	19.4	2	6.5	0	0.0	0	0.0	8	25.8
Palmar-plantar erythrodysesthesia	1	3.2	6	19.4	1	3.2	0	0.0	8	25.8
Hypotension	4	12.9	3	9.7	0	0.0	0	0.0	7	22.6
Weight gain	7	22.6	0	0.0	0	0.0	0	0.0	7	22.6
Dry skin	7	22.6	0	0.0	0	0.0	0	0.0	7	22.6
Skin hyperpigmentation	6	19.4	1	3.2	0	0.0	0	0.0	7	22.6
Palpitations	5	16.1	2	6.5	0	0.0	0	0.0	7	22.6
Hot flashes	7	22.6	0	0.0	0	0.0	0	0.0	7	22.6
Bone pain	6	19.4	0	0.0	1	3.2	0	0.0	7	22.6
Arthralgia	5	16.1	2	6.5	0	0.0	0	0.0	7	22.6
Epistaxis	7	22.6	0	0.0	0	0.0	0	0.0	7	22.6

[a]Attributed to the DC1 vaccine.

prior to neoadjuvant chemotherapy. The HER2 DC1 vaccine caused a significant influx of T cell infiltrates into treated tumors, and this could potentially be greater in patients who were pCR upon completion of therapy. Additional investigation of intratumoral DC1 vaccines with higher doses of DC1 cells, de-escalated chemotherapy backbones such as the ongoing NATASHA study (NCT05325632), and using other antigens is warranted.

Methods

Study design and participants

This pilot clinical trial was conducted at a single center (H. Lee Moffitt Cancer Center, clinicaltrials.gov, NCT03387553 1/2/2018).

The eligibility criteria for the trial included females, ≥18 years of age, diagnosed with their first case of clinical stage T2-3N0-2M0 estrogen

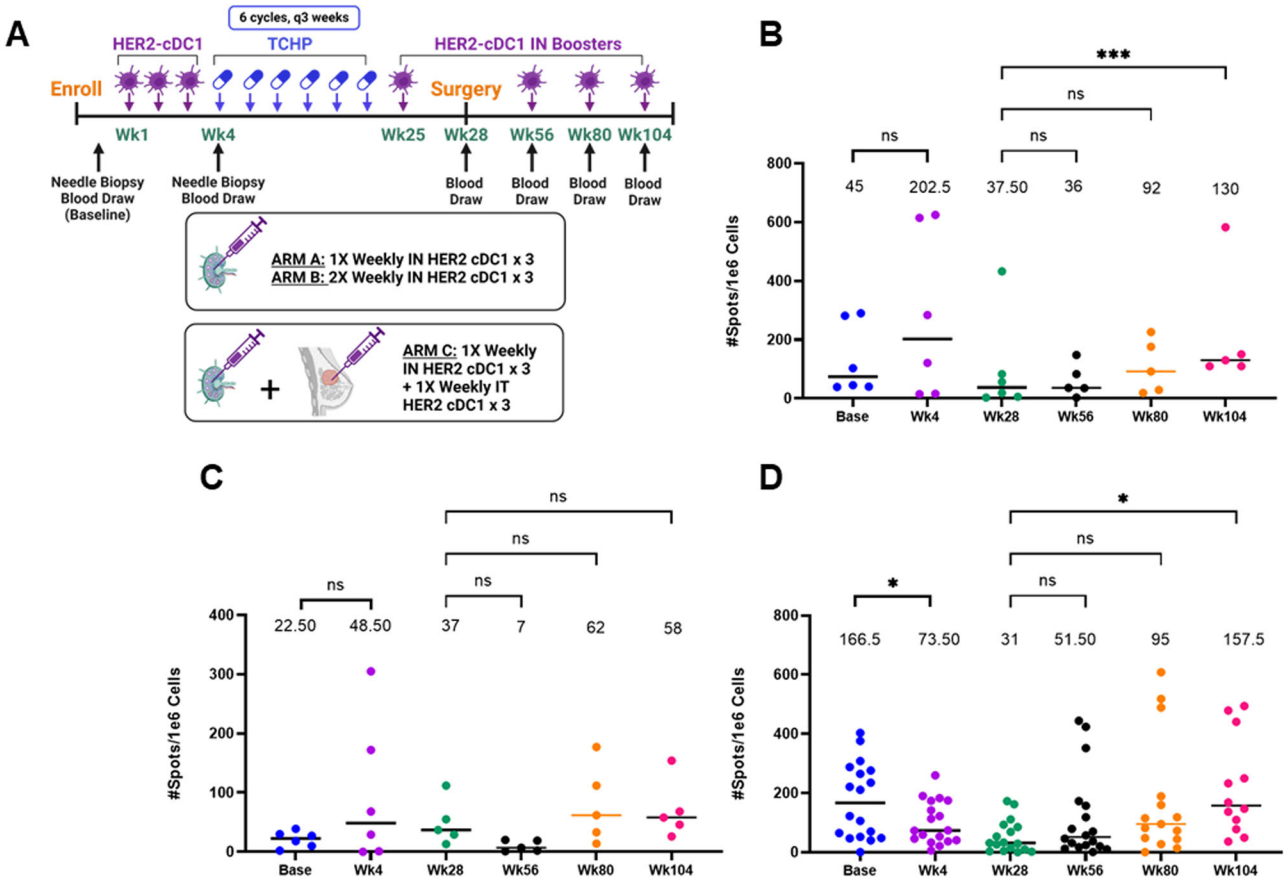

Fig. 2 | HER2 ELISPOT counts across different timepoints with medians for the timepoints above each column. A The trial schema is illustrated to clarify the timing of treatments and sampling. The figures show the ELISPOT counts for Arms **A**, **B**, and **C** (**B**, **C**, **D**, respectively). Both Arms **A** and **B** experience a statistically nonsignificant increase in median ELISPOT counts at week 4, followed by a drop during chemotherapy, and an increase over time with booster intranodal vaccination post chemotherapy particularly in arm **A**. Arm **C** differs in that there is an initial drop in circulating ELISPOT levels at week 4 followed by an increase over time during the boosters.

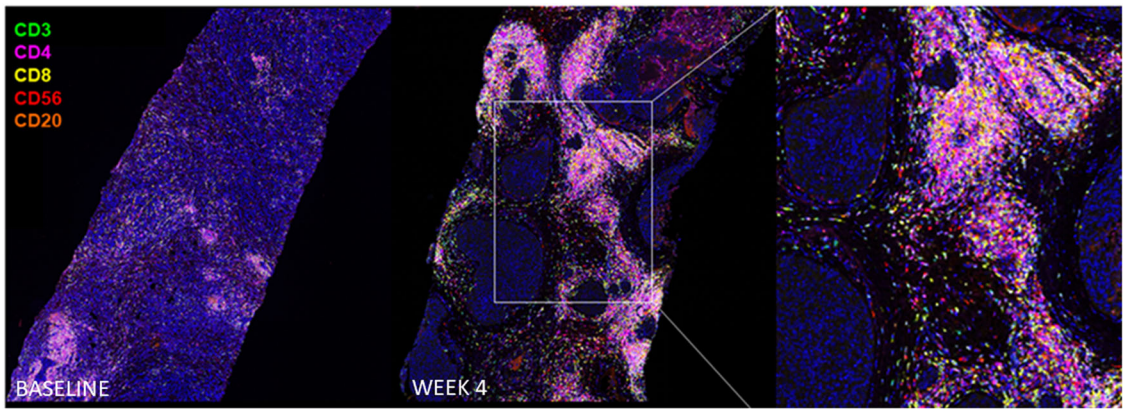

Fig. 3 | Immunofluorescence images of baseline and on treatment biopsies for Arm C patients with markers for CD4+ helper T cells, CD8+ effector T cells, CD56 + NK/NKT cells, and CD20 + B cells. The left most panel is a zoomed in section to better demonstrate the distribution of cell types within the stroma around tumor islands.

receptor negative HER2+ invasive breast cancer by College of American Pathologists (CAP) criteria, who were suitable candidates for neoadjuvant docetaxel/carboplatin/trastuzumab/pertuzumab (TCHP) chemotherapy. Clinical staging of patients utilized clinical exam, mammography, breast ultrasonography, breast magnetic resonance imaging, computed image tomography, and nuclear medicine bone scans as per standard of care. Patients must also have an Eastern Cooperative Group performance status of 0-1, adequate organ function/hematologic counts, and an echocardiogram within institutional normal limits. Patients presenting with inflammatory/bilateral/multicentric breast cancers, active herpes simplex virus infections, on anticoagulation, immunodeficiency, or autoimmune disease were excluded.

All study procedures were carried out after informed consent was obtained in accordance with all regulatory requirements including the

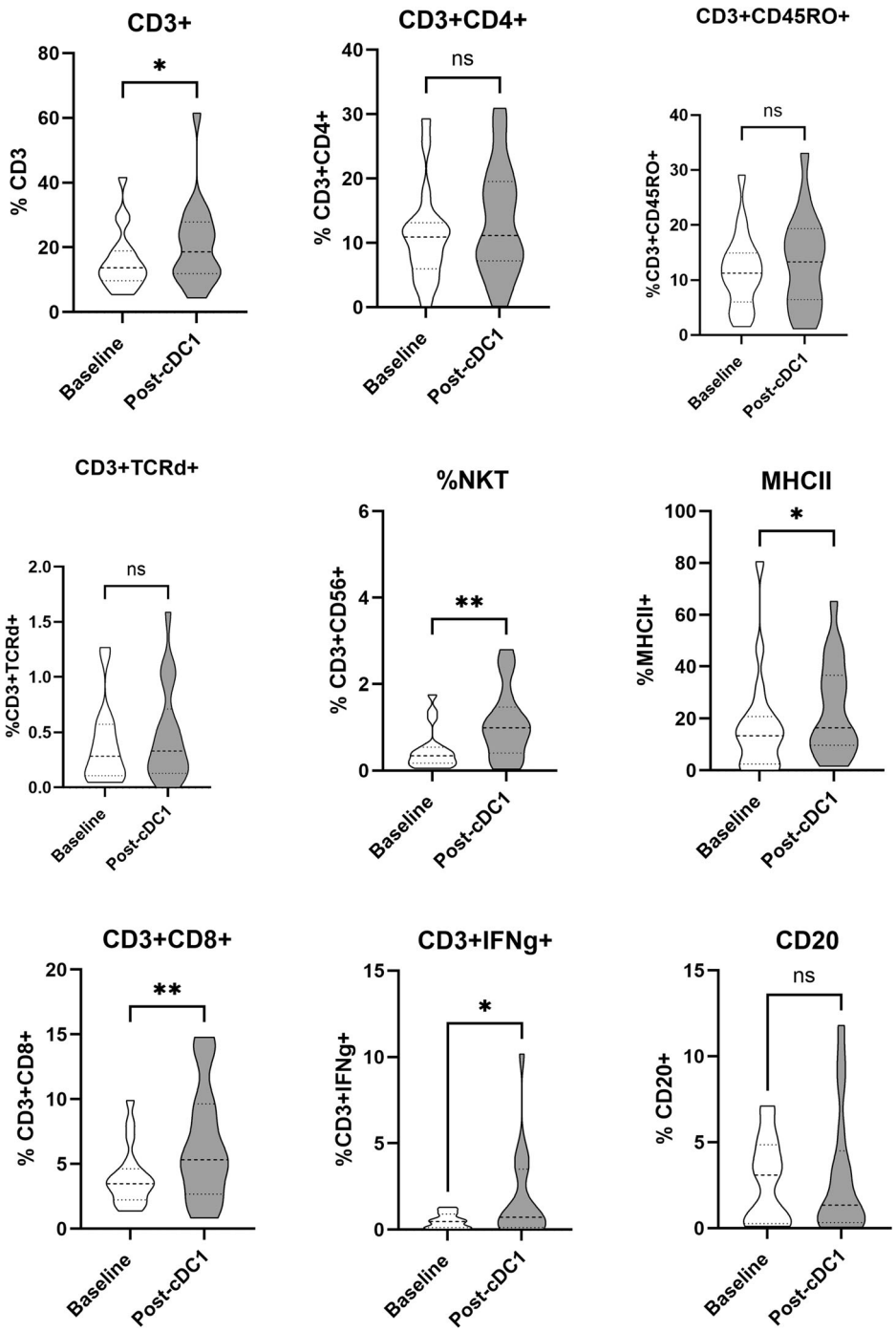

Fig. 4 | Violin plots showing the frequency of different cell subtypes within analyzed tissue sections from baseline and week 4 biopsies from Arm C patients. Statistically significant increases were noted in CD8+ effector T cells, interferon-gamma secreting lymphocytes, and NKT cells.

Declaration of Helsinki and approval by Advarra institutional review board application # Pro00023642.

Procedures

Patients underwent leukapheresis in the Moffitt Apheresis Unit. The product was transported immediately to the Moffitt Cell Therapies Core lab for DC1 production under good manufacturing practice (GMP) conditions. To separate the lymphocyte and monocyte fractions, the pheresis pool obtained was subjected to countercurrent centrifugal elutriation on a Model J-6M centrifuge (Beckman Instruments) equipped with a JE-5.0 elutriation rotor. The transitional (150 cc/minute) and monocyte-rich (180 and 190 cc/minute) fractions were pooled and cultured in serum free medium with 50 ng/ml (250 IU/ml) rhGM-CSF and IL-4 1000 IU/ml. The next morning 20 μg/ml HER-2/neu peptides (3 ECD p42-56 (HLDMLRHLYQGCQVV), p98-114 (RLRIVRGTQLFEDNYAL), p328-345(TQRCEKCSKPCARVCYGL) and 3 ICD p776-790 (GVGSPYVSRLLGICL), p927-941 (PAREIPDLLEKGERL) and p1166-1180 (TLERPKTLSPGKNGV)) were added to the culture. The peptides were pulsed in separate wells to avoid competition for binding in the MHC. The evening of day 2, IFN-γ (1000 u/ml) was added, and the next morning, LPS (10 ng/ml) was added, and the cells were cultured an additional 2–8 hours until activated. The cells were then harvested from the plates, and cell count, viability, gram stain, BacTAlert sterility testing, endotoxin testing and FACS analysis were conducted for quality assurance prior to release. Meanwhile, the cells were resuspended in cryopreservation media (5% DMSO, 57% plasmalyte, and 38% human albumin) for cryopreservation. Vials were frozen in a controlled rate freezer and stored in monitored LN2 tanks until administration.

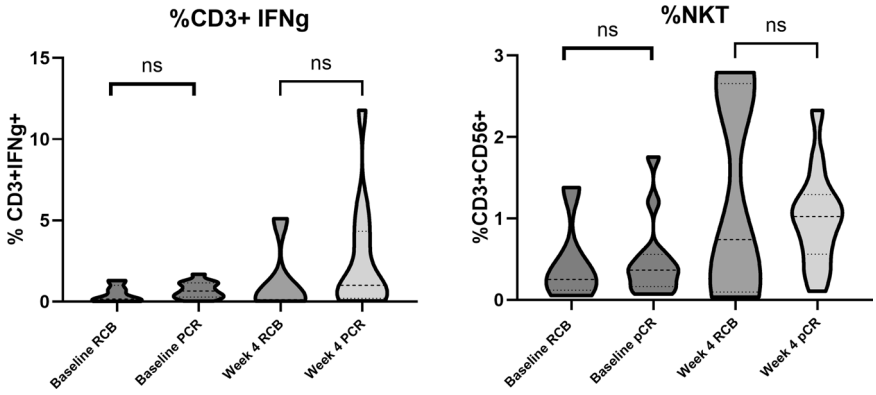

Fig. 5 | Comparison of mIF samples from residual cancer patients (Non pCR) and those who eventually attained a pathology complete response (pCR). There was a trend for pCR patients to have greater elevations in CD3 + IFNγ+ cells within their stroma after 4 weeks of DC1 vaccination compared to Non pCR patients while NKT cells appeared similar between the two groups. T cell and NKT infiltration was increased in both groups relative to baseline.

The study treatment consisted of dendritic cell type 1 (DC1) injections given for 3 weeks. The schedule of ultrasound-guided injections during the first three weeks varied across the three arms. Arm A patients got once a week intranodal injections, arm B patients got twice a week intranodal injections, and the arm C patients got twice a week injections with one injection given intratumoral while the second one was intranodal. Patients enrolled initially into arms A and B in an alternating fashion until they were completed, then patients began enrolling into arm C. The introduction of intranodal injections in arm C occurred as an amendment to the original protocol design following the completion of arms A and B. Each intratumoral and intranodal injection used a target dose of 2×10^7 DC1 cells/vaccine across all arms. Patients would then undergo standard six cycles of TCHP chemotherapy every 3 weeks followed by surgical resection and adjuvant radiation as medically indicated. Patients would also get intranodal booster DC1 vaccines at weeks 56, 80, and 104 after completion of any standard adjuvant anti-HER2 therapy.

Outcomes

The primary endpoints of the pilot trial were the immune response by HER2 ELISPOT of the arm C and the pathologic complete response rate. Pathologic complete response (pCR/RCB0) rates (defined as no residual invasive disease in the breast or sampled nodes) after completion of neoadjuvant therapy. Secondary endpoints were residual cancer burden index (RCB) response category, toxicity using CTCAE v4.03, recurrence-free survival (RFS) in months, and other immune correlates. An early stopping continuous monitoring rule for unacceptable toxicities (grade 3 or higher events related to the vaccine) was included in the protocol.

Human IFN-γ ELISPOT Assay

To evaluate anti-HER2 peripheral immune response, IFN-γ production from PBMCs was analyzed using the human IFN-γ ELISPOT kit (cat. #HIFNgp-1M/10, Cellular Technologies Limited). ELISPOT plates precoated with human IFN-γ capture antibody were incubated with the six HER2 class II peptides that the DC1s were originally pulsed with (4 μg/well), media only (untreated/negative control), or anti-human CD3 (Orthoclone OKT3, cat. #73337989, Johnson and Johnson, treated/positive control, 15 ng/mL). Cryopreserved PBMCs were plated (2×10^5 cells/well) in CTL-Test medium supplemented with 1% L-glutamine and incubated at 37 °C, 5% CO_2 for 48 hours. Plates were then processed per manufacturer's protocol and as previously described[18]. Spot-forming cells (SFC) were counted using an automated reader (Immunospot Cellular Technology Limited) and the number SFU/1e6 PBMCs was calculated following subtraction of untreated background values.

Multiplex Immunofluorescence (mIF) Procedure

Formalin-fixed and paraffin-embedded (FFPE) tissue samples were immunostained using the AKOAYA Biosciences OPAL TM 7-Color Automation IHC kit (Waltham, MA) on the BOND RX autostainer (Leica Biosystems, Vista, CA). The OPAL 7-color kit uses tyramide signal amplification (TSA)-conjugated to individual fluorophores to detect various targets within the multiplex assay. Sections were baked at 65°C for one hour then transferred to the BOND RX (Leica Biosystems). All subsequent steps were performed using an automated OPAL IHC procedure (AKOYA). OPAL staining of each antigen occurred as follows: heat induced epitope retrieval (HIER) was achieved with Citrate pH 6.0 buffer for 20 min at 95 °C before the slides were blocked with AKOYA blocking buffer for 10 min. Then slides were incubated with primary antibody, CD56 (CST, E7X9M, 1:50, dye 570) at RT for 60 min followed by OPAL HRP polymer and one of the OPAL fluorophores during the final TSA step. Individual antibody complexes were stripped after each round of antigen detection. This was repeated five more times using the following antibodies; CD8 (DAKO, C8/144B, HIER-EDTA pH 9.0, 1:100, dye520), CD4 (CM, EP204, HIER- EDTA pH 9.0, 1:100, dye540), CD20 (Dako, L26, HIER-EDTA pH 9.0, 1:300, dye 620), CD3 (Abcam, Rb poly, HIER- EDTA pH 9.0, 1:200, dye650), and PCK (DAKO, AE1/AE3, HIER- Citrate pH 6.0, 1:200, dye690). After the final stripping step, DAPI counterstain was applied to the multiplexed slide and was removed from BOND RX for coverslipping with ProLong Diamond Antifade Mountant (ThermoFisher Scientific). All slides were imaged with the Vectra®3 Automated Quantitative Pathology Imaging System.

mIF quantitative image analysis

Multi-layer TIFF images were exported from InForm (AKOYA) and loaded into HALO (Indica Labs, New Mexico) for quantitative image analysis. A trained classifier identified areas of tumor, stroma, or non-tissue regions. Pan-cytokeratin staining was used to identify tumor. The classifier was created and tested on various images in the image set. The tissue was segmented into individual cells using the DAPI marker, which stains cell nuclei. For each marker, a positivity threshold within the nucleus or cytoplasm was determined per marker based on published staining patterns and intensity for that specific antibody. After setting a positive fluorescent threshold for each staining marker, the entire image set was analyzed with the created algorithm. The generated data includes positive cell counts for each fluorescent marker in the cytoplasm or nucleus and the percent of cells positive for the marker. Along with the summary output, a per-cell analysis was exported to provide the marker status, classification, and fluorescent intensities of every individual cell within an image.

Statistical analysis

All statistical analysis was carried out using Prism Graphpad 9.3.1 (San Diego, CA). Since this was a pilot trial, there was no formal a prior hypothesis testing to determine the sample size in the arms regarding immune or clinical responses.

Descriptive methods were used to tabulate frequencies of patient characteristics, adverse events, and pathologic response rates. Patients were

evaluable for toxicity if they received any study treatment but must have received at least 2 doses of chemotherapy to be evaluable for efficacy per protocol. Median recurrence-free survival was analyzed using the Kaplan-Meier method.

A paired t-test was used to compare the baseline and week 4 post-DC1 pre-chemotherapy mIF and ELISPOT data. For comparison of week 28, 56, 80, and 104 ELISPOT booster shot data post chemotherapy a mixed methods analysis with correction for multiple testing was performed to account for missing values. Statistical significance was set at $<p = 0.05$.

Data availability

Deidentified data can be provided following approved data sharing agreement with investigators upon request.

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Author contributions

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Competing interests

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Additional information

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