Breast Cancer Research and Treatment (2022) 194:65–78
https://doi.org/10.1007/s10549-022-06562-y

CLINICAL TRIAL

The feasibility of using an autologous GM-CSF-secreting breast cancer vaccine to induce immunity in patients with stage II–III and metastatic breast cancers

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Received: 15 October 2021 / Accepted: 2 March 2022 / Published online: 28 April 2022

Abstract

Purpose The antigenic targets of immunity and the role of vaccination in breast cancer are unknown. We performed a phase I study of an autologous GM-CSF-secreting breast cancer vaccine in patients with metastatic and stage II–III breast cancer.
Methods Tumor cells from patients with metastatic ($n=15$) and stage II–III ($n=7$) disease were transduced with a replication-defective adenoviral vector encoding GM-CSF, and then irradiated. Twelve and seven patients with metastatic and stage II–III disease, respectively, received weekly vaccination for three weeks, followed by every other week until disease progression or vaccine supply was exhausted (metastatic) or until six total vaccine doses were administered (stage II–III).
Results Among those patients with metastatic disease who received vaccinations, eight had progressive disease at two months, three had stable disease for 4–13 months, and one has had no evidence of disease for 13 years. Of the patients with stage II–III disease, five died of metastatic disease between 1.16 and 8.49 years after the start of vaccinations (median 6.24 years) and two are alive as of September 2021. Toxicities included injection site reactions, fatigue, fever, upper respiratory symptoms, joint pain, nausea, and edema. Four of five evaluable patients with metastatic disease developed a skin reaction with immune cell infiltration after the fifth injection of unmodified, irradiated tumor cells.
Conclusion We conclude that tumor cells can be harvested from patients with metastatic or stage II–III breast cancer to prepare autologous GM-CSF-secreting vaccines that induce coordinated immune responses with limited toxicity.
Trial registration and date of registration clinicaltrials.gov, NCT00317603 (April 25, 2006) and NCT00880464 (April 13, 2009).

Keywords Breast cancer · Autologous cellular vaccine · GM-CSF

Introduction

The overarching goal of cancer immunotherapy is to enhance the anti-tumor immune response. Therapeutic vaccination has the potential to stimulate broad-based anti-tumor immunity by targeting tumor-associated antigens (TAAs), i.e., normal proteins that are aberrantly expressed by cancer cells. In addition, cancers amass sporadic, non-synonymous mutations that lead to the expression of altered protein sequences, neoantigens, which are specific to tumor cells and are attractive vaccine targets because they can specifically and effectively direct the immune system to eliminate cancer cells [1–3].

Allogeneic cells derived from tumor cell lines and patient-derived autologous tumor cells both serve as a rich

Prior Presentation Part of the data were presented as abstracts at the following meetings: 1. 37th Annual San Antonio Breast Cancer Symposium: December 2014. Anderson, K. et. al. A Phase Ib Study of an Adjuvant GM-CSF-Secreting Breast Cancer Vaccine. Abstract #P2-15-03 2. 31st Annual San Antonio Breast Cancer Symposium: December 2008. Anderson, K. et.al. A Phase I Study of an Autologous GM-CSF Secreting Breast Cancer Vaccine. Abstract #4124

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potential source of neoantigens [2]. Granulocyte–macrophage colony-stimulating factor (GM-CSF) promotes the maturation of dendritic cells (DCs) and activation of T cells [4]. The utility of cellular vaccines engineered to secrete GM-CSF has been demonstrated in murine models [5] and in clinical trials targeting prostate and pancreatic cancer [4, 6, 7]. Both phase II trials utilized an adenoviral vector encoding human GM-CSF (GVAX), which was well-tolerated and favorably impacted survival [6, 7]. Allogeneic GM-CSF-secreting tumor vaccines have also been tested in breast cancer patients [8, 9].

In contrast to allogeneic vaccines, autologous cancer cell vaccines are personalized to each patient. Early-phase clinical trials of vaccination with autologous tumor cells engineered to secrete GM-CSF have been tested in more than 100 patients with metastatic melanoma and non-small cell lung cancer. However, this approach has not been well studied in breast cancer [10–12]. Since breast cancer has striking molecular and antigenic heterogeneity [13], the use of autologous GM-CSF-secreting tumor vaccines provides a unique opportunity to study the antigenicity of a wide variety of breast cancer antigens in vivo. To this end, we conducted two phase I clinical trials of an autologous GM-CSF-secreting breast cancer vaccine: one conducted in patients with metastatic breast cancer, and the second in patients with stage II–III disease.

Methods

Patients

Participants were at least 18 years old with an Eastern Cooperative Oncology Group performance status of 0 or 1. Adequate hematologic and organ function was required. Participation in prior adenovirus-based trials was not allowed.

Metastatic breast cancer

Eligible patients had histologically confirmed stage IV breast cancer and had received at least one line of chemotherapy for metastatic disease. Those with human epidermal growth factor receptor 2-positive (HER2+) disease received at least one prior trastuzumab-based therapy in the metastatic setting. All anti-cancer therapy and systemic glucocorticoid therapy were completed at least 4 weeks prior to the initiation of vaccination. Surgically accessible tumor measuring at least 2 cm in greatest diameter or significant malignant effusion was required for tumor harvesting.

Stage II–III breast cancer

Eligible patients had histologically confirmed invasive breast cancer, pre-operative stage II–III, based on clinical examination and/or breast imaging. Patients were required to have a primary tumor at least 4 cm in largest diameter, or 2 cm in largest diameter after completion of neoadjuvant chemotherapy. Adjuvant therapy (chemotherapy, radiation therapy, and/or trastuzumab-based therapy) must have been completed at least 12 weeks prior to enrollment. Concurrent endocrine therapy was allowed, if it was initiated at least 4 weeks prior to the start of vaccinations.

Tumor harvesting and adenoviral-mediated gene transfer

Tumor samples were processed to single cell suspension by mechanical and enzymatic digestion and subsequently transduced with a replication-defective adenoviral vector encoding human GM-CSF. The adenoviral vector was manufactured by the Dana-Farber/Harvard Cancer Center Vector Core for tumor vaccine production and was approved for clinical research use by the U.S. Food and Drug Administration (BB-IND 7248). After transduction, tumor cells were washed and irradiated with 10,000 cGy. The irradiated cells were maintained in 14-day sterility cultures and tested for endotoxin and mycoplasma contamination. Individual vaccine cell dose and number varied depending on the final cell yield from vaccine production. For patients with stage II–III disease, the minimal dose was 1×10^5 cells, and maximal dose was 4×10^6 cells. For those with metastatic disease, the minimal dose was 1×10^5 cells, and maximal dose was 1×10^7 cells. The selected dose range was based upon two Phase I clinical vaccine trials testing the biologic activity of vaccination with lethally irradiated, autologous tumor cells engineered by adenoviral-mediated gene transfer to secrete GM-CSF [10, 14]. Following allocation of sufficient tumor cells for vaccine production, any additional cellular material was not transduced and was set aside for subsequent use in immunologic studies, cell line generation, and delayed-type hypersensitivity (DTH) testing. GM-CSF secretion was determined by ELISA (Endogen EH-GMSCF, Woburn, MA) according to the manufacturer's protocol.

Vaccine delivery

Immediately prior to vaccination, cells were thawed and washed in a dedicated laminar flow biosafety cabinet, then suspended in a volume of 1 ml, with one-half dose administered subcutaneously, and one-half dose administered intradermally. Injections were administered using a 23-gauge or 25-gauge needle into the patients' upper arms, thighs, or trunk on a rotating basis. Prior surgical sites were avoided.

For patients with metastatic disease, vaccines were delivered weekly for three weeks, then every other week until disease progression or supply of vaccine was exhausted. For patients with stage II–III disease, vaccines were delivered weekly for three weeks, then every other week for a total of six doses. When sufficient cells were available, injections of non-transduced, irradiated cells (1×10^6) were given on day 0 (first vaccination) and with the fifth vaccination for evaluation of baseline and vaccine-induced DTH. The DTH cells were resuspended in a volume of 0.5 ml and injected intradermally in the patients' upper arms and thighs in a rotating manner, excluding sites of prior surgery.

Clinical outcomes

The primary endpoint of both studies was to determine the feasibility of manufacturing six doses of vaccine per individual. Safety was assessed by the ability of each patient to receive six immunizations without experiencing Common Terminology Criteria for Adverse Events v3.0 grade 3 or higher toxicity. For patients with metastatic disease, the secondary objectives were to determine the time to progression and overall survival. For patients with stage II–III disease, the secondary endpoint was to monitor the rate of local or distant disease recurrence and overall survival.

Skin testing

To assess vaccination skin reactions, erythema and induration were measured 2–3 days after the first and fifth vaccinations. Four-mm punch skin biopsies of vaccine and DTH sites were obtained at the same time. Histologic analyses for macrophages, DCs, eosinophils, and lymphocytes were performed. DTH reactions were considered strong when any of the following conditions were present: mononuclear cells admixed with eosinophils and basophils accumulated around blood vessels; endothelial cells that were swollen, partially necrotic, or with vessel luminal occlusion; or the presence of dermal edema and fibrin exudation. A recall response was defined as erythema, induration, or pruritis at a prior vaccination site that occurred with subsequent vaccination.

Serum antibody detection by high density (HD) NAPPA protein microarray

Detection of serum antibodies on high density nucleic acid programmable protein arrays (NAPPA) was performed as described [15, 16]. Sequence-verified, full-length cDNA expression plasmids encoding full-length antigens as fusion proteins in pANT7_GST were obtained from the Arizona Biodesign Institute (Tempe, AZ) (http://dnasu.asu.edu/DNASU/). Plasmid DNA (100 ng/ul, 300 pl/well) corresponding to 734 unique genes and 418 control spots were co-printed with polyclonal anti-GST AB (GE Healthcare) into HD-NAPPA nano-wells sequentially using piezoelectric dispensing AU302 (Engineering Arts LLC, Tempe, AZ, USA). Specific antigens were selected for their function and expression in cancer [17, 18]. The printed DNA was transcribed and translated in situ using a human cell-free expression system (Thermo Fisher Scientific), and protein expression was detected using anti-GST Mab (Cell Signaling Technology, Danvers, MA). The 8-chamber arrays were incubated with serum at 1:100 at 4 °C overnight and bound IgG was detected by Alexa Fluor 647 goat-anti-human IgG (Thermo Scientific). Slides were scanned at 635 nm with a Tecan PowerScanner. The highly immunogenic EBV-derived antigen, EBNA-1, was included as a positive control. Negative controls included no-DNA spots, empty non-spots, and GST-only. Arrays were aligned using purified human IgG proteins. Cytokine secretion assays were performed (Supplementary Methods).

Statistical analysis

Feasibility of vaccine preparation was defined as obtaining sufficient genetically modified cells for the preparation of at least six vaccines. The threshold for feasibility was defined as the ability to prepare enough vaccine for six inoculations in 10/20 patients with metastatic disease (50%) and 20/30 patients with stage II–III disease (67%). Patients whose DTH sites were biopsied were assessable for immune infiltrates, which were graded as absent, mild-moderate, or strong. Confidence interval widths used exact binomial calculations.

Antibody responses were measured using ArrayPro Analyzer. Median spot intensities were normalized to the median intensity of the subarray:

$$\text{Spot Intensity} = \frac{\text{Median Gene Spot Intensity}}{\text{Median Spot Intensity of all Genes Printed in Sub-Array}}$$

Comparison of individual biomarkers were made to 30 gender-matched normal control sera. T cell responses were measured by ELISPOT pre- and post-treatment. Fisher's exact test was used to compare overall response according to immune response. Survival times were estimated using Kaplan–Meier methods, with survival times being censored at the time of last contact. Tests were two-sided at the 0.05 significance level and without adjustment for multiple testing.

Results

Patient characteristics, vaccine preparation and administration

Metastatic

Twenty-eight patients with metastatic breast cancer underwent tumor procurement for vaccine preparation, with sufficient viable tumor cells obtained from 27 patients. Harvest sites included malignant pleural effusions (23), ascites (1), lymph nodes (1), and tumor nodules (2). Thirteen patients had rapid tumor progression or were ineligible for the vaccine trial (Supplementary Table S1). From January 2006 until May 2008, 15 patients were enrolled on the vaccine trial and underwent vaccine preparation from their previously banked tumor; three patients withdrew prior to vaccine delivery due to rapid disease progression (Table 1). However, all 15 patients had sufficient tumor cells harvested to produce at least six vaccines. Based upon pre-defined criteria, the feasibility of obtaining sufficient tumor cells for six vaccinations among patients with metastatic disease was 54% (15/28).

Baseline characteristics of the 12 patients that comprise the study population who received at least one vaccination (patients M1 to M12) are shown in Table 1 and Supplementary Table S2. Patients ranged in age from 34 to 69 years, with an average of 3.45 years duration of stage IV breast cancer prior to enrollment (range: 0.4–12 years). Nine patients (67%) had hormone receptor-positive (HR+) breast cancer, six patients (43%) had HER2 + disease, and two patients (29%) had triple-negative breast cancer (TNBC; HR−/HER2−). Patients were heavily pre-treated, receiving an average of three prior chemotherapies in the metastatic setting (range: 1–7).

Vaccine dose was based on cellular yield, ranging from 10^5 to 10^7 cells per dose. Patients received a median of five vaccinations (range: 3–23). Six patients from the study population (6/12 = 50%) received six or more vaccinations. Disease progression was the reason six patients did not receive at least six vaccinations. The average yield of GM-CSF was 450 ng/10^6 cells/24 h (range: 24–1991 ng/10^6 cells/24 h) (Table 1).

Stage II–III

Eighteen patients with stage II–III breast cancer underwent tumor procurement for vaccine preparation at the time of breast surgery (Table 2). Sufficient cells for vaccination were obtained from seven patients, who made up the study population (patients A1 to A7). Based upon pre-defined criteria, the feasibility of obtaining sufficient tumor cells for the preparation of six vaccinations was 39% (7/18).

Additional characteristics of patients A1–A7 are shown in Supplementary Table S3. Patients ranged in age from 32 to 65 years, and 43% had T3, N1 tumors. Five patients (71%) had HR + tumors, one (14%) had an HER2 + tumor, and two (29%) had TNBC. Eighty-six percent of patients had a mastectomy, and 86% had a partial response to neoadjuvant therapy.

Patients A1-A7 each received six vaccinations. Tumor cell yields ranged from 9×10^5 to 5.4×10^8 cells. Vaccine dose was based on cellular yield, ranging from 10^5 to 3.98×10^6 cells per dose. The average GM-CSF yield was approximately 1061 ng/10^6 cells/24 h (range < 1 to 6081.9 ng/10^6 cells/24 h). Cell viability ranged from 56 to 100% (Table 2).

Efficacy

Metastatic

The clinical results of patients M1–M12 are shown in Table 1. Eight patients (67%) had progressive disease within 2 months of enrollment. Three patients (25%) had stable disease, with progression at 4, 4, and 13 months. One patient (M9) was surgically rendered as no evidence of disease (NED) by vaccine harvest and has remained NED for 13 years.

Stage II–III

Survival outcomes for patients A1–A7 are shown in Table 2. Five patients (71%) died of recurrent disease between 1.16 and 8.49 years after receiving the first vaccination (median 6.24 years). Two patients (29%) remain alive as of September 2021.

Adverse events

Metastatic

Treatment-related toxicities were limited to grade 1 and 2 (Table 3). At least three subjects (25%) experienced fever, fatigue, edema, nausea, leukopenia, hyperglycemia, or hyponatremia. All toxicities, except hyperglycemia, are known toxicities of GM-CSF administration and have been observed in prior autologous vaccination studies at Dana-Farber Cancer Institute [19–23]. There were no significant hepatic, renal, pulmonary, cardiac, hematologic, gastrointestinal, or neurologic toxicities attributable to vaccination. No autoimmune reactions or adenoviral infections were observed.

Table 1 Characteristics of patients with metastatic breast cancer who had at least six vaccines prepared

Study number[a]	Patient	Site of cell procurement	Vaccinated (reason)	ER	PR	HER2	Vaccine viability (%)	Vaccine doses made	Vaccine doses given	Cells per dose	GM-CSF level (ng/10^6 cells/24 h)	Response
3	M2	Ascites	Yes	Pos	–	Neg	76	14	3	4×10^6	108	PD
4	M12	Ascites	Yes	Pos	Pos	Neg	53	7	7	4×10^6	135	SD: 4 mo
6		Ascites	No-PD	Pos	Pos	Pos	70	6	0	1×10^7	5696	PD
9	M9	Breast Node	Yes	Neg	Neg	Pos	87	8	6	1×10^5	739	NED: 13 years
10	M1	Tumor	Yes	Neg	Neg	Pos	45	11	5	1×10^5	67	PD
11	M4	Ascites	Yes	Pos	Pos	Neg	95	6	5	4×10^6	1480	PD
12		Ascites	No-PD	Neg	Neg	Neg	78	7	0	4×10^6	268	PD
13	M3	Ascites	Yes	Pos	Neg	Neg	83	10	3	4×10^6	105	PD
14	M10	Ascites	Yes	Pos	Pos	Neg	49	12	10	1×10^7	1991	SD: 4 mo
15	M6	Ascites	Yes	Pos	Pos	Neg	72	6	4	1×10^7	24	PD
16	M5	Ascites	Yes	Pos	Pos	Pos	45	18	6	1×10^7	393	PD
17	M7	Lymph Node	Yes	Neg	Neg	Pos	53 55	23 8	23 4	1×10^5 1×10^6	174	SD: 13 mo
19		Ascites	No-PD	Pos	Pos	Neg	78	13	0	4×10^6	65	PD
25	M8	Ascites	Yes	Neg	Neg	Neg	81	6	4	1×10^6	25	PD
27	M11	Ascites	Yes	Pos	Pos	Pos	81	6	6	1×10^7	153	PD

[a]Patients Study Number 6, 12, and 19 experienced rapid disease progression; therefore, vaccine was not administered

PD progressive disease, *SD* stable disease, *Pos* positive, *Neg* negative, *NED* no evidence of disease, *ER* estrogen receptor, *PR* progesterone receptor

Table 2 Characteristics of enrolled patients with stage II–III breast cancer

Study Number[a]	Patient	Vaccinated (reason)	ER	PR	HER2	Type of breast surgery	Number of cells recovered	Cells per dose	Number of vaccines delivered	Viability	GM-CSF level (ng/10^6 cells/24 h)	Survival (years)[b]
003	A1	Yes	Neg	Neg	Neg	Lumpectomy	5.4×10^8	3.98×10^6	6	56%	4.9	6.34
004	A2	Yes	Pos	Neg	Pos	Mastectomy	2.36×10^7	1.13×10^6	6	95%	1001.3	6.24
006		No (expansion failed)	Pos	Low Pos	Neg	Mastectomy	1.0×10^6	N/A	N/A	N/A	N/A	N/A
007	A3	Yes	Low Pos	Neg	Neg	Mastectomy	3.16×10^7	1×10^6	6	80%	222.1	Alive
008	A4	Yes	Pos	Pos	Neg	Mastectomy	3.75×10^6	1×10^6	6	100%	6081.9	8.49
009	A5	Yes	Neg	Neg	Neg	Mastectomy	2.49×10^6	4×10^5	6	90%	66.9	1.16
010	A6	Yes	Pos	Pos	Neg	Mastectomy	9×10^5	1×10^5	6	90%	<1	Alive
011		No (insufficient cells)	Pos	Pos	Neg	Mastectomy	Not done	N/A	N/A	N/A	N/A	N/A
012		No (insufficient cells)	Pos	Pos	Pos	Mastectomy	Not done	N/A	N/A	N/A	N/A	N/A
014		No (insufficient cells)	Low Pos	Low Pos	Neg	Mastectomy	6.8×10^6	N/A	N/A	N/A	N/A	N/A
015	A7	Yes	Low Pos	Neg	Neg	Mastectomy	2.4×10^6	2.85×10^6	6	89%	50.0	2.22
017		No (insufficient cells)	Pos	Pos	Neg	Mastectomy	Not done	N/A	N/A	N/A	N/A	N/A
018		No (Insufficient Cells)	Neg	Neg	Neg	Lumpectomy	Not done	N/A	N/A	N/A	N/A	N/A
019		No (insufficient cells)	Neg	Neg	Neg	Mastectomy	Not done	N/A	N/A	N/A	N/A	N/A
020		No (insufficient cells)	Pos	Pos	Neg	Mastectomy	9.4×10^5	N/A	N/A	N/A	N/A	N/A
021		No (insufficient cells)	Neg	Neg	Neg	Mastectomy	Not done	N/A	N/A	N/A	N/A	N/A
022		No (insufficient cells)	Neg	Neg	Neg	Lumpectomy	7.0×10^4	N/A	N/A	N/A	N/A	N/A
023		No (insufficient cells)	Neg	Neg	Neg	Lumpectomy	Not done	N/A	N/A	N/A	N/A	N/A

[a]Patients who were approached, but not enrolled: *Study Number 001*Did not meet disease/eligibility criteria, *Study Number 002* Patient refused consent; *Study Number 005* No tumor was banked during surgery due to inaccessible tumor location, *Study Number 013* Patient refused consent, Study Number 016: Patient refused consent

[b]From receipt of first vaccine dose

ER estrogen receptor, *PR* progesterone receptor, *Pos* positive, *Neg* negative, *N/A* not applicable

Table 3 Summary of treatment-related adverse events among all patients who received GVAX vaccine

Adverse event	Grade 1 or 2	Grade 3 or 4
Metastatic[a] ($n=12$)		
Injection site reactions		
Erythema/induration	8 (67%)	0 (0%)
Pruritis	5 (42%)	0 (0%)
Fatigue	6 (50%)	0 (0%)
Fever	3 (25%)	0 (0%)
Edema – Limbs	3 (25%)	0 (0%)
Decreased hemoglobin	2 (17%)	0 (0%)
Leukopenia	5 (42%)	0 (0%)
Neutropenia	2 (17%)	0 (0%)
Thrombocytopenia	2 (17%)	0 (0%)
Nausea	4 (33%)	0 (0%)
Hypoalbuminemia	2 (17%)	0 (0%)
Elevated ALT	2 (17%)	0 (0%)
Elevated AST	2 (17%)	0 (0%)
Hyperglycemia	6 (50%)	0 (0%)
Hyponatremia	3 (25%)	0 (0%)
stage II–III[b] ($n=7$)		
Dermatology/skin	3 (43%)	0 (0%)
Fatigue	5 (71%)	1 (14%)
Fever	2 (29%)	0 (0%)
Musculoskeletal pain	4 (57%)	0 (0%)
Headache	2 (29%)	0 (0%)
Allergic reaction	1 (14%)	0 (0%)
Cough	2 (29%)	0 (0%)
Congestion	1 (14%)	0 (0%)
Throat pain	1 (14%)	0 (0%)
Pulmonary NOS	1 (14%)	0 (0%)
Infection	2 (29%)	0 (0%)
Nausea	1 (14%)	0 (0%)
Elevated AST or ALT	1 (14%)	0 (0%)

[a]Toxicity present in ≥2 patients; possibly, probably, or definitely related to vaccine

[b]Toxicity present in ≥1 patient; possibly, probably, or definitely related to vaccine

ALT alanine transaminase, *AST* aspartate transaminase, *NOS* not otherwise specified

Stage II–III

The observed toxicities attributed to vaccination are shown in Table 3. The most common treatment-related toxicities included fatigue (85%), musculoskeletal pain (57%), and dermatological manifestations (43%). One patient developed grade 2 upper respiratory tract infection, and one patient experienced grade 3 fatigue.

Injection site reactions

Metastatic

Skin site reactions to vaccine were measured 48–72 h after the first and fifth vaccination on all evaluable patients. Seven of the patients developed injection site reactions to vaccine at baseline (dose 1), and five evaluable patients had injection site reactions to the vaccine after the fifth dose (mean 1.4 vs. 4.1 cm, $p=0.13$) (Fig. 1a). Average baseline erythema was positively correlated to increasing vaccine dose ($p<0.005$) (Fig. 1b). There was no correlation between erythema and GM-CSF secretion rate (data not shown).

A separate DTH dose, consisting of 1×10^6 non-transduced, irradiated tumor cells, was injected in the contralateral thigh at the same time as the first and fifth vaccinations in seven patients (patients at the 10^5–10^6 dose levels did not receive DTH due to cell yield). The DTH injection was used to assess immune reactivity to tumor cells independent of GM-CSF secretion and adenoviral infection. There was no DTH reactivity at baseline for any patient ($n=7$). Three patients at the 10^7 dose level and one patient at the 4×10^6 dose level developed a DTH response after the fifth vaccine (mean 0 vs. 1.5 cm, $p=0.11$), suggesting induction of anti-tumor immunity with vaccinations (Fig. 1c). The specific level of immunity (DTH, B cell, or T cell immunity) by vaccine dose was not assessed. Skin biopsies of vaccination sites (Fig. 2a) and DTH injection sites (Fig. 2b) revealed mild-to-moderate infiltration of lymphocytes, granulocytes, and macrophages, indicative of inflammation.

Stage II–III

Skin site reactions to vaccine were measured 48–72 h after the first and fifth vaccination on all evaluable patients. No skin reactions were observed after the first dose. Five of seven patients developed grade 1–2 erythema and induration after the fifth dose. Erythema ranged from 1.5×1.4 cm to 7.0×5.0 cm (Supplementary Figure S1), and induration ranged from 0.1×0.1 cm to 2.5×2.3 cm (Supplementary Table S4). Tumor cells were not recovered in sufficient number to perform DTH analysis in this cohort of patients.

Correlative studies: metastatic breast cancer cohort

Induction of B cell immunity to multiple tumor antigens post-vaccination

To measure antigen-specific B cell immunity, we designed custom protein microarrays for detecting antibodies in patient sera to 734 selected tumor antigens [15] (Supplementary Table S5). Baseline (both pre-enrollment and Day = 0) and post-vaccination sera from patients from the metastatic

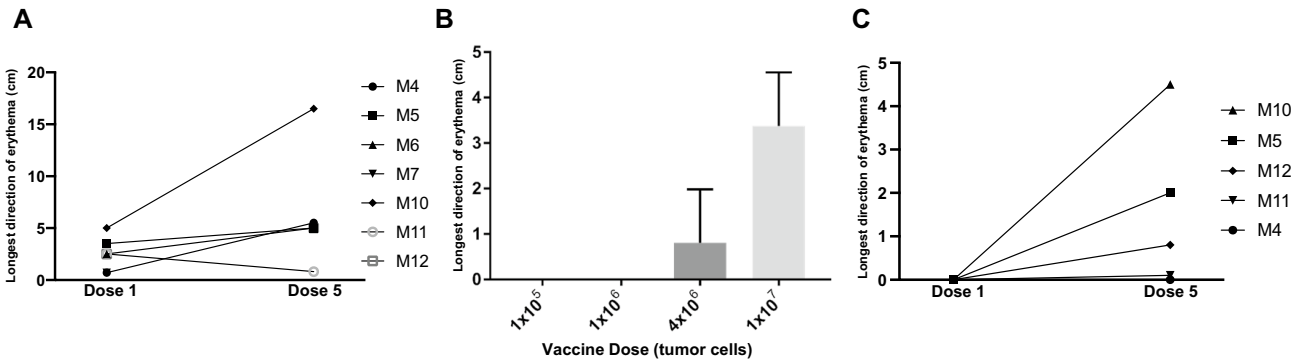

Fig. 1 Vaccination induces local inflammation in patients with metastatic breast cancer. **a** GM-CSF-modified tumor cells were injected in contralateral limbs in patients with metastatic breast cancer. Skin site reactions were observed in seven patients 48–72 h after the first vaccine dose and in five patients after the fifth vaccine dose, and the longest dimension of erythema (cm) is shown, with patient numbers noted. Other patients did not exhibit any skin reaction. **b** Average baseline erythema at each vaccine dose level. **c** Unmodified cells at 10^6 cells/dose (DTH) were injected in contralateral limbs of seven patients with metastatic breast cancer. Skin site reactions were measured 48–72 h after the first and fifth injection. No responses were observed after the first injection. Four responses were observed after the fifth injection, and the longest dimension of erythema (cm) is shown, with patient numbers noted

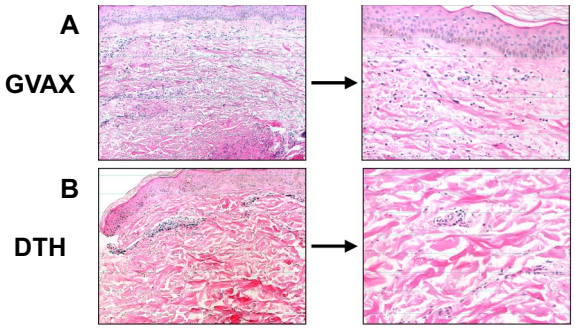

Fig. 2 Vaccination induces leukocyte infiltration. Representative skin biopsies from a patient with metastatic breast cancer showing inflammatory cellular infiltration following **a** injection of GVAX vaccine, and **b** following the fifth dose of 1×10^6 non-transduced, irradiated cells (DTH). Inflammatory infiltrate included lymphocytes, granulocytes, and macrophages

group were added to the arrays, bound IgG was detected with secondary antibodies, and the results compared to sera from 30 healthy women.

In Fig. 3, heatmaps of the antibody responses (defined as normalized signal > 1.5) are shown for eight vaccinated patients with metastatic disease. Autoantibodies that were present in > 5% of healthy women are shown in grey. All patients had strong antibody responses to the EBV viral antigen EBNA-1, which did not change following vaccination. The baseline variability of antibody reactivity is shown by comparing "Pre" and "D0" timepoints for patients M5, M7, and M10, who had sera obtained prior to enrollment. As this variability was only observed for a few tumor antigens (of 734) and not for the viral antigen EBNA-1, it may reflect an active variation of serologic response to tumor in these heavily pretreated patients. Known tumor antigens, such as p53 [15], CTAG1A, CTAG2 [24], and NUDT11 [18] were detected at baseline for patient M7, although CTAG2 immunity waned over the 9-month period (Fig. 3A). Patient M6 also had strong induction of CTAG1A, CTAG2, as well as the known autoantigen PRL [25] by D22, but this waned completely by D56, suggesting that there may a transient induction of antibodies by GVAX in this patient. Isolated late (D56 or later) serologic responses were also observed, as were loss of immunity after vaccination (Fig. 3b). There was no clear association between antibody breadth or specificity with disease response (M7, M10, M12) in this limited analysis (Fig. 3a).

Induction of T cell immunity to multiple tumor antigens post-vaccination

To measure antigen-specific T cell immunity in the peripheral blood, we selected nine HLA-A2-restricted epitopes corresponding to immunogenic tumor antigens that have been identified in the literature (Supplementary Table S6) [26–31]. Using the MULTIPRED HLA-binding algorithm [32], we also predicted four novel HLA-A2-binding epitopes derived from two immunogenic tumor antigens, TPD52 and S100A7, that are strongly overexpressed in DCIS and in high-risk breast cancers [33, 34]. The HLA-A2-restricted EBV BMLF1 epitope [35] was used as a positive control. Three vaccinated patients with metastatic breast cancer were HLA-A2+ and were evaluable for immune reactivity after 2 months of vaccinations.

To determine if autologous vaccination is associated with an increase in antigen-specific T cells in the peripheral blood, the frequency of T cells was measured by IFN-γ

A

Gene	M7_Pre	M7_D0	M7_D27	M7_D55	M7_D83	M7_D112	M7_D139	M7_D167	M7_D195	M7_D278
EBNA	3.7802	4.0672	4.7380	4.4138	5.3019	4.4199	5.1793	4.8790	4.9819	4.2248
CTAG1A	54.9866	54.3408	62.8935	55.6797	64.3446	70.9253	62.8332	54.7761	58.3651	30.6571
CTAG2	10.2248	24.0813	24.9959	17.7842	9.8429	18.2933	12.2617	5.2304	12.1323	4.9664
TP53	18.0448	20.2919	21.6622	24.1147	20.9180	23.1797	20.5024	22.4663	20.5080	16.1777
NUDT11	4.5520	6.3035	4.7774	5.4877	7.0633	8.2868	6.9291	4.6065	5.6126	2.6715
LOXL4	1.5177	1.6567	1.6727	1.6262	1.6259	1.6710	1.6692	1.4034	1.4141	1.3718
CT45A3	1.5007	1.5564	1.6094	1.5956	1.6190	1.6742	1.6155	1.3618	1.3906	1.4678
TMEM171	0.9975	1.4403	1.5624	1.4206	1.0290	1.5292	1.3634	1.2666	1.3266	1.3593
MGC3262	1.0333	1.6998	1.1248	0.9524	1.0191	1.0584	1.0594	1.1626	0.9701	1.3055
SCARB1	13.9598	1.0265	1.0835	1.0170	1.0565	1.0303	1.0134	0.9787	0.9221	1.0231
BST2	0.9567	1.0124	1.0336	1.0586	0.9828	0.9610	3.5475	0.8703	0.9904	0.9693
TUBE1	1.0273	1.0348	0.9482	1.0170	0.9416	1.0173	2.1774	0.9371	0.7834	1.0259

Gene	M12_D0	M12_D56
EBNA	6.1675	5.0551
KRCC1	1.1369	1.6858
ZDHHC7	0.9823	1.6680
RAD51AP1	1.5008	1.5234
SEC14L4	1.5040	1.0895

Gene	M10_Pre	M10_D0	M10_D28	M10_D58
EBNA	3.4394	3.8281	5.1373	4.1192
RUNDC3A	1.2227	1.4289	2.1213	2.4417
PIK3CD	1.2864	1.9506	1.9725	2.3360
ELOVL2	1.0712	1.5237	1.5744	1.4661
LRP12	2.1182	1.1364	1.1419	1.1626
DBH	1.0015	1.5830	1.0206	1.0217
PLEKHA9	33.8864	0.8972	0.3822	0.7154

B

Gene	M3_D0	M3_D7
EBNA	2.9740	3.5213
TIMP3	1.7756	1.7092
RP13-36C9.1	1.0619	1.5390
ESAM	1.0006	1.5035

Gene	M4_D0	M4_D29	M4_D57
EBNA	3.3808	4.2533	3.0700
MSF	3.6496	4.4837	1.9990
TIMP3	1.8841	1.9824	1.9505
ARMCX1	1.0826	1.0651	1.8106
PRL	1.6458	1.1609	0.9929

Gene	M5_Pre	M5_D0	M5_D7	M5_D14	M5_D28	M5_D55
EBNA	3.7139	4.0835	3.4430	4.3274	3.9325	3.4291
TIMP3	1.7778	1.7170	1.5742	1.9948	2.0566	2.0640
CT45A3	1.6903	1.5934	1.0471	1.8676	1.5919	1.8366
LOXL4	1.3893	1.3674	0.8570	1.8551	1.6157	1.8287
TMEM171	1.0339	1.6019	1.1178	1.6392	1.3297	1.7254
DKFZP547N043	1.3018	1.3887	1.1343	1.5401	1.2383	1.2520
GJA5	1.6714	1.0162	1.0432	1.0469	1.0953	1.1171
MSF	1.0284	1.0503	1.5350	1.0709	0.9513	1.0285
PRL	1.1308	1.0392	1.6300	1.1022	0.9841	0.9941
DBH	1.0032	1.5823	0.9199	0.9009	1.0397	0.9341

Gene	M11_D0	M11_D56
EBNA	5.1226	4.0000
KPNA4	1.1213	2.7628
LOXL4	2.3768	2.5392
CT45A3	2.5198	2.2531
GAS2	0.9068	2.1169
CNOT2	1.0932	1.8593
TIMP3	2.0179	1.8082
DOM3Z	0.9515	1.6095
CCND1	0.4432	1.5721
TFB1M	1.1277	1.5585
CRELD1	1.1201	1.5119
ADA	0.9502	1.5096

Gene	M6_D0	M6_D22	M6_D56
EBNA	4.3553	4.2259	3.8956
DLD	2.7379	0.8481	0.8644
CSNK1E	2.1781	0.7765	0.7631
PLEKHA9	2.1144	3.1965	3.1628
EPHX4	2.0769	1.0859	1.0142
UBE2B	1.9899	1.0652	1.0494
PTPRA	1.7055	1.0955	1.1224
ST8SIA4	1.5800	0.8727	0.8839
SSR4	1.5719	1.1018	1.0683
CCDC104	1.5314	1.3142	1.2400
DDR1	1.5091	0.9228	1.0299
CTAG1A	1.0658	10.3058	1.0280
CTAG2	1.0283	5.2711	0.8795
PRL	0.9433	2.0670	1.1972
BACE2	0.9443	1.9029	1.2897
BACH1	1.0101	1.8687	0.9198
TP53	1.0294	1.8401	1.0431
FBXO44	1.1427	1.8218	1.2306
WWP2	0.9960	1.7446	1.0362
ANP32A	0.7682	1.6913	0.9494
AQP3	0.9049	1.6675	1.3545
HIST1H2AD	1.1204	1.6317	1.2111
SF3B4	0.9322	1.6221	0.9840
PSMC1	1.2591	1.5895	0.9047
RLN1	1.0658	1.5879	1.2444
ESAM	1.0101	1.5823	1.0915
ARSA	1.1569	1.5418	1.3891
GK	0.9342	1.5402	0.9311
NUDT11	1.0840	1.5267	1.0746
OXCT	1.1447	1.5243	1.0085
CT45A3	1.4494	1.5187	1.3652
GP9	0.6883	1.5147	0.8839
GQ129254	0.8998	1.5123	2.0893
MPP2	1.1872	1.4558	1.5816
TIMP3	1.2126	1.4145	1.5351
CNOT6L	1.0435	1.4097	1.5697
STK12	0.8775	1.3405	1.8641
FLJ10378	1.0395	1.2649	1.7251
TNFRSF21	0.9980	1.2124	1.5244
PLAGL2	0.6609	1.2100	1.5577
ITK	0.8664	1.1933	1.5351
PTPLA	0.9180	1.1583	1.8660
GJC3	0.7581	1.0565	1.5653
KCNK6	0.7075	0.9045	1.5439
SP2	1.0010	0.8894	1.6590

Fig. 3 Immune profile for selected patients with metastatic breast cancer who received GVAX vaccine. **a** Immune profile among patients with stable disease or disease response following vaccination. **b** Immune profile among patients with disease progression following vaccination. Heatmaps of antibody response (normalized signal > 1.5) in vaccinated patients compared with healthy controls. D = Day number post-first GVAX vaccination

ELISPOT assay at 0- and 2-months post-vaccination to the panel of peptides listed in Supplementary Table S6. A representative ELISPOT from patient M11 is included in Supplementary Figure S2, which shows an increase in TPD52_70 (from 0 to 17.5 spots/10^5 PBMC; $p = 0.06$) and Survivin-specific T cells (from 0 to 33 spots/10^5 PBMC; $p = 0.02$).

By ELISPOT analysis, patient M10 developed an increase in CEA (from 0 to 54.5 spots/10^5 PBMC, $p < 0.001$) and

Survivin-specific T cells (from 0 to 12.5 pots/10^5 PBMC, $p=0.02$), but no other epitopes. All three patients had strong reactivity to the positive control epitope EBV BMLF-1 at all-time points (data not shown).

Discussion

Neoantigens generated by tumor somatic mutations can induce T cell activation. Neoepitope-specific vaccines have been tested in animal models [36] and early phase clinical trials [37–39]. Strong neoepitope-specific immunity has been detected after vaccination and sustained progression-free survival observed, leading to multiple ongoing clinical trials [37–39]. In comparison, autologous tumor cell vaccines represent a complex mixture of self and tumor neoantigens. Complex cellular vaccines, which possess a rich diversity of tumor antigens, could potentially overcome adaptive resistance caused by antigenic loss during treatment [40].

Unfortunately, for many solid tumor types, the theoretical promise and preclinical efficacy of therapeutic vaccines have not translated to success in clinical trials. This is particularly true for breast cancer, in which several vaccines against TAAs such as MUC-1 [41], Her2/neu [42], and hTERT [43] have been tested in early phase clinical trials, with limited efficacy. Several mechanisms of immune evasion, including immunoediting [44], clonal heterogeneity within tumors [45], and the immunosuppressive tumor microenvironment [46] likely contribute to the poor response to TAA-based vaccines.

The results of the present study demonstrate that the preparation of autologous GM-CSF-secreting tumor cell vaccines from patients with metastatic breast cancer is feasible, with 54% of patients enrolled having at least six vaccines developed from harvested tumor. However, the success rate was considerably lower among patients with stage II–III disease (39%). We specifically designed these studies to harvest treatment-resistant cells after chemotherapy; however, improvements in neoadjuvant therapies are likely to impact the feasibility of harvesting viable tumor cells post-treatment. Even still, patients most likely to derive benefit from autologous GM-CSF-secreting tumor cell vaccines include those with high-grade early-stage HR + disease and TNBC with at least 2 cm of residual disease after neoadjuvant chemotherapy [47]. Encouragingly, the average GM-CSF yield in both cohorts of patients was higher than the average yields for prior studies of lung cancer and melanoma [10, 14], and vaccination among our cohort of patients was associated with minimal toxicity.

Using high density NAPPA microarrays, we identified multiple patterns of serologic responses in vaccinated patients with metastatic breast cancer. First, vaccination did not impact the serologic response to the viral antigen EBNA-1, which remained strong for all patients at all time-points. We compared the serologic response to 734 antigens among our patients to the response among 30 healthy control women. Both known [48–51] and novel autoantibodies were detected, suggesting that the vaccinated stage IV patients generated novel antibodies, at least transiently, to multiple target antigens. Our feasibility study was too small to allow us to perform functional assays to determine whether the antibodies produced were pro-tumor or anti-tumor. However, the high variability of the tumor antigen-specific IgG response suggests that proteome-based serologic profiling is needed for monitoring complex immunotherapies such as cellular vaccines in larger trials.

Identification of the antigenic T cell targets of cellular vaccination is challenging since potential antigens include the entire tumor proteome. In our metastatic cohort, we predicted 12 potential target HLA-A2-restricted epitopes and performed IFN-γ ELISPOT analysis to measure T cell immunity in peripheral blood from three patients that were HLA-$A2^+$ and evaluable for immune responses. All three patients developed T cell immunity to 1–2 antigens, and each patient developed different patterns of immunity. These included the known tumor antigens survivin, CEA, and HER2/neu, as well as the novel antigen TPD52, which is strongly overexpressed in many solid tumors and induces protective T cell-mediated immunity in a murine breast cancer model [33]. This heterogeneity of immune responses could be due to underlying variation of breast cancer tumor antigen expression, or to host variation of immune recognition. Further interpretation of these results is limited by the small number of patients available for IFN-γ ELISPOT analysis, therefore we view these data as hypothesis generating. This study had several limitations, including the technical challenge of obtaining sufficient tumor cells from each patient to produce autologous vaccines. The criteria for feasibility in these studies was defined as production of vaccine allowing for six inoculations for 10/20 (50%) patients with metastatic disease and was determined to be feasible (15/28; 54%). This number was not as robust as anticipated due to the onset of rapid disease progression among 46% of patients accrued, which precluded subsequent enrollment onto the vaccine trial after successful tumor harvesting among 28 patients. In addition, the small number of patients treated on our study prevented an assessment of therapeutic efficacy.

We planned to enroll 30 patients with stage II–III breast cancer and anticipated that 20 (67%) would have sufficient modified tumor cells for six vaccinations. Unfortunately, the intended accrual was not reached, and only 7/18 (39%) received vaccine. Over time, accrual became challenging with the advent of more effective preoperative systemic therapy which reduced the amount of residual disease available for vaccine generation. Thus, many of the planned analyses of feasibility and therapeutic efficacy

in this cohort of patients with earlier stage disease could not be performed. Nevertheless, the recovery of sufficient cells to deliver six vaccinations to each patient in the stage II–III group suggests that this methodology can be used for vaccine generation and should be studied further.

In conclusion, tumor cells can be harvested after chemotherapy from patients with breast cancer in sufficient number for the preparation of autologous GM-CSF-secreting vaccines. Autologous vaccination can induce tumor-specific delayed-type hypersensitivity reactions, as well as antigen-specific T and B cell immune responses with limited toxicity. This supports further investigation of autologous vaccination as a therapeutic option for breast cancer resistant to conventional treatment. Our feasibility study was too small to identify any difference in vaccine preparation among the various subtypes of breast cancer. Given the benefits of adding immune checkpoint blockade (ICB) to chemotherapy in the treatment of TNBC [52, 53], further exploration of the addition of autologous vaccination to ICB is intriguing. Our feasibility study supports the role of autologous GM-CSF-secreting vaccines as potential candidates for future cancer vaccine development.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10549-022-06562-y.

Acknowledgements We would like to thank Nabihah Tayob PhD for her statistical assistance and William Johns for technical assistance with immune profiling. We would like to thank Christine Canning and Keri Hannagan for their support during the conduct of the clinical trial and thank Kaitlyn Bifolck and Valerie Goldstein and the Whiteley Center for their assistance in manuscript preparation and submission.

Author contributions KSA designed the study and correlative science, conducted the trial, and wrote the manuscript. TE wrote the manuscript and participated in study analysis. MC, MM and LZ performed the immunohistochemistry. MC, YC and HD assisted in the conduct of the clinical trial. EW and GD participated in study design and analysis. WB participated in the statistical analysis. BO participated in the conduct of the clinical trial, study analysis and wrote the manuscript. All authors contributed to manuscript review and approval.

Funding This work has been supported by NCI/Avon Foundation P30 CA006516 (K.S.A.), the Breast Cancer Research Foundation (K.S.A.), NCI/Early Detection Research Network U01CA214201 (K.S.A.) and the Dana-Farber/Harvard Cancer Center Breast SPORE program 5 P50 CA89393 (E.W.).

Data and materials availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Conflict of interest GD is an employee of Novartis with stock ownership.

Ethical approval The clinical trial was approved by the Dana-Farber/Harvard Cancer Center (DF/HCC) Institutional Review Board (DF/HCC #05-110; DF/HCC #08-217; DF/HCC #05-111/NCT00317603; DF/HCC #08-216/NCT00880464).

Consent to participate All patients signed written informed consent for tissue harvesting and subsequent vaccinations.

Consent for publication Consent for publication was included in the patient consent forms for the study.

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