



Randomized phase II trial of a first-in-human cancer cell lysate vaccine in patients with thoracic malignancies

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Background: Although most malignancies express cancer-testis antigens (CTA), immune responses to these proteins are limited in thoracic oncology patients. This trial was undertaken to examine if a cancer cell lysate vaccine could induce immunity to CTA, and to ascertain if metronomic cyclophosphamide and celecoxib enhances vaccine-induced immune responses.

Methods: Eleven patients with primary thoracic malignancies and 10 patients with extrathoracic neoplasms metastatic to the chest rendered NED by conventional therapies were randomized to receive H1299 lung cancer cell lysates (10 mg protein/vaccine) with IscomatrixTM adjuvant via deep intradermal injection q 4 weeks ×6 with or without daily oral metronomic cyclophosphamide/celecoxib. The primary endpoint was serologic response to purified CTA assessed 1 month after the 6th vaccination. Secondary endpoints included assessment of the effects of cyclophosphamide and celecoxib on frequency and magnitude of vaccine-induced immune responses to CTA. Exploratory endpoints included evaluation of the effects of the vaccine regimens on peripheral immune subsets. Standard of care imaging studies were obtained at baseline and 1 month after the 3rd and 6th vaccinations.

Results: All patients exhibited local and systemic inflammatory responses lasting 72–96 hours following vaccinations. There were no dose limiting treatment related toxicities. Fourteen patients (67%) completed all six vaccinations. Eight of 14 patients (57%) exhibited serologic responses to NY-ESO-1. One patient developed antibodies to GAGE7; several patients exhibited reactivity to XAGE and MAGE-C2. Vaccine therapy decreased the percent of Tregs (P=0.0068), PD-1 expression on Tregs (P=0.0027), PD-L1 expression on CD14⁺ monocytes (P=0.0089), PD-L1 expression on classical monocytes (P=0.016), and PD-L1 expression on intermediate monocytes (P=0.0031). Cyclophosphamide/celecoxib did not appear to increase immune responses or enhance vaccine-induced alterations in peripheral immune subsets.

Conclusions: H1299 lysate vaccines with IscomatrixTM induce immune responses to CTA and modulate peripheral immune subsets in a manner that may enhance antitumor immunity in patients with thoracic malignancies.

Keywords: Vaccine; first-in-human; cancer-testis antigen; thoracic malignancy; pulmonary metastasis

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Introduction

Identification of serologic and cell mediated immune responses to shared as well as unique (neo) antigens on tumor cells, and impressive, durable responses following immune checkpoint blockade in cancer patients have provided unequivocal evidence supporting the theories of immune surveillance and immunoediting articulated by Burnet, and Schreiber, respectively (1,2). Transformed cells that escape elimination by immune surveillance establish a dynamic equilibrium with the host immune system. Continued selection pressure on cancer cells and the tumor microenvironment ultimately promotes “escape” of immuno-edited clonal variants and evolution of central as well as peripheral immune tolerance resulting in clinically evident malignancies (2).

Genome-wide DNA demethylation during malignant transformation leads to de-repression of a variety of genes that normally exhibit stage-specific expression during germ cell development in immunoprivileged testes or ovary (3). Aberrant activation of these cancer-testis (CT) genes (also referred to as cancer-germline genes) in somatic cells results in expression of highly restricted tumor-associated antigens that induce serologic as well as cell-mediated immune responses in cancer patients; as such, cancer-testis antigens (CTAs) have emerged as attractive targets for cancer immunotherapy (4). To date, over 200 CTAs have been identified, approximately half of which are encoded on the X chromosome (<http://www.cta.lncc.br/>). Relative to autosomal CT genes, CT-X chromosome genes often comprise extended families and are more frequently activated in cancer cells; particular gene families appear to be coordinately de-repressed in a tumor-specific manner suggesting functional oncogenic interactions (5). To date, cellular immune responses have been detected against more than 20 CT-X antigens in patients with various solid tumors (6).

Whereas most human malignancies express CT-X antigens, immune responses to these proteins are often limited in cancer patients due to low-level, heterogeneous antigen expression, epigenetic repression of genes regulating antigen processing and presentation, and local as well as systemic immunosuppression (4,7). Previously we demonstrated that epigenetic agents such as Decitabine,

Romidepsin, and DZNep can up-regulate CT-X antigen expression in thoracic malignancies (but not normal cells) *in vitro* and *in vivo* rendering them susceptible to lysis by cytotoxic T lymphocytes (CTL) recognizing NY-ESO-1 and MAGE-A3 (8-12). Furthermore, we have demonstrated that systemic administration of Decitabine followed by adoptive transfer of CTL recognizing the CTA P1A eradicates pulmonary metastases in an immunocompetent murine model (13). These observations, together with recent reports demonstrating high level CT-X gene expression in cancer stem cells (14-16), raise the possibility that pharmacologic interventions that enhance expression and presentation of CTA—particularly when administered in the context of a primed immune system may be novel strategies to eradicate dormant cancer stem cells that give rise to local as well as systemic recurrences in patients following potentially curative interventions. Herein we report results of a First-in-Human trial evaluating if a lung cancer lysate vaccine administered with or without metronomic oral cyclophosphamide and celecoxib as a strategy to inhibit suppressive activity of regulatory T (Treg) cells can induce immunity to potentially relevant CTA in patients with thoracic malignancies. We present this article in accordance with the CONSORT reporting checklist (available at <https://dx.doi.org/10.21037/tlcr-21-1>).

Methods

Ethical statement

The trial was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and the guidelines for Good Clinical Practice. The trial (NCT02054104) was approved by NCI-IRB and FDA (IND 15812), and written informed consent was obtained from all individual participants.

Patients and protocol eligibility

Patients 18 years or older with ECOG performance status of 0–2 who had no clinical evidence of active disease (NED) detected by physical exam, CT scans of chest, abdomen, and pelvis, fused torso and if clinically indicated extremity PET/CT scans, and brain MR scans following standard of

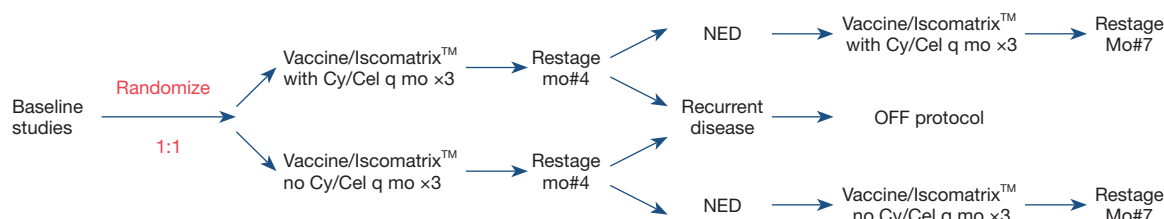


Figure 1 Protocol treatment schema.

care treatment for histologically or cytologically proven primary thoracic malignancies (localized or metastatic) or neoplasms metastatic to the chest were potentially eligible for study. Patients had to enroll within 52 weeks following definitive therapy for their malignancies, and were required to commence protocol treatment within 4 weeks of eligibility screening and enrollment. Additional eligibility criteria included resolution of all toxicities from prior therapies to \leq Grade 2, adequate bone marrow reserve, as well as hepatic and renal function, no unstable cardiovascular disease, and no decompensated pulmonary disease. Patients requiring chronic systemic steroids at supraphysiologic doses were ineligible, as were patients for whom anticoagulants could not be held for up to 24 hours. Patients with active brain metastases, as well as patients with active infections, and pregnant females were ineligible.

Vaccine production

The H1299 lung cancer line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). A master bank was established at NCI, Frederick after extensive studies confirmed that the cells were free of mycoplasma, as well as bacterial and viral contaminants. For vaccine production, H1299 cells were seeded into T-1000 flasks and cultured in RPMI media containing 10% fetal bovine serum (FBS) and Pen/Strep until confluent (approximately 7 days) without exchange of media. Cells were harvested by trypsinization, and extensively washed in HBSS with centrifugation. Cell pellets were then lysed using a freeze-thaw method and centrifuged at 1,000 rpm for 10 minutes to remove debris. Supernatants were diluted to protein concentrations of 10 mg/mL in normal saline, and then aliquoted into cryotubes (1.2 mL/tube) and frozen at -80°C . The vaccines were produced in several large batches; approximately 10% of vials from each preparation were transferred to the NIH Clinical Microbiology Lab to confirm sterility and absence of endotoxin. Immunoblot

techniques using primary and secondary antibodies listed in Table S1 were used to confirm levels of NY-ESO-1, MAGE-A1, and MAGE-A3 prior to certification and release of the final product.

Protocol design

This Phase 2.5 first-in-human trial was conducted at the Center for Cancer Research, NCI, Bethesda, MD. During registration by CRO, patients were randomized 1:1 via non-sequential computer algorithm to receive lysate (10 mg protein/vaccine) with Iscomatrix™ adjuvant via deep intradermal injection q 28 days \times 6 with or without daily oral metronomic cyclophosphamide and celecoxib (treatment schema depicted in Figure 1). No oral placebo was used; as such, neither patients nor staff were blinded to the treatment regimens. All vaccinations were administered in the same extremity for each patient. Following each vaccination, patients were monitored for approximately one hour for systemic hypersensitivity reactions. A diary was used to document compliance with protocol medications and record systemic toxicities. Each vaccination cycle was 28 days. Cyclophosphamide was administered at a dose of 50 mg PO BID for 7 days prior to the first vaccination (Day 1, cycle 1), and then on Days 8 through 14, and 22 through 28 of each treatment cycle. Celecoxib was administered at a dose of 400 mg PO BID for 7 days prior to the first vaccination, and then on Days 1 through 28 of each treatment cycle. If vaccinations were delayed, patients continued to take cyclophosphamide every other week and celecoxib daily. Contrast enhanced CT scans of the chest, abdomen and pelvis, torso PET/CT scans, and brain MR scans were obtained at baseline, and one month following the third and sixth vaccinations. Patients who experienced dose limiting toxicities possibly, probably, or definitely attributable to the lysate vaccines received no further vaccines; dose reductions of cyclophosphamide and celecoxib were permissible. Patients who experienced recurrent or new malignancies

were removed from study and followed until death. Patients who had no detectable immune responses to the vaccine regimen per criteria defined below (non-responders) were also removed from study and continued surveillance in our clinic. The first and last patients were enrolled on 9/3/2014, and 5/6/15, respectively, and date of last follow-up was May 15, 2020.

Toxicity assessment, protocol stopping rules, and off-study criteria

CTCAE version 4.0 was used to grade toxicities. Dose limiting toxicity (DLT) was defined as any Grade 2 or greater autoimmune or hypersensitivity reaction attributable to the vaccine, or Grade 3 or greater toxicity at least possibly related to the vaccine except for Grade 3 toxicities such as local injection site reactions, fatigue, fever, or local adenopathy that resolved to Grade 2 or less within 2 weeks following any vaccination. Protocol stopping rules included any autoimmune DLT at least possibly related to the vaccine that occurred in 3 of the first 10 patients who received study treatment. Criteria for removal from protocol therapy included completion of protocol therapy, vaccine-related DLT, voluntary withdrawal, disease progression, need for systemic steroids, or lack of immune response to vaccine.

Study endpoints and statistical analysis

The primary endpoint was serologic response to a panel of purified CTA one month after the six vaccination, as evidenced by the appearance of serologic reactivity in previously sero-negative patients, IgM-IgG class switch, or a two-fold or greater increase in titer of IgGs recognizing CT-X antigens. Secondary endpoints included examining if oral metronomic cyclophosphamide (CP) and celecoxib therapy increased frequency or magnitude of immune responses to the lysate vaccines. Exploratory endpoints included evaluation of immune subsets in peripheral blood before and after vaccinations with Wilcoxon matched-pairs signed rank test being used to generate p values (unadjusted for multiple variables); the Mann-Whitney U test was used to compare the two treatment groups.

Following the principles of a phase 2.5 design, the trial was intended to randomize 28 evaluable patients per arm to either receive vaccine alone or vaccine plus chemotherapy in order to have 80% power to determine if the frequency of immune responses on the combination arm exceeded

that of the vaccine alone arm, if the expected frequencies of immune responses on the two arms were 20% and 50% using a one-sided 0.10 alpha level Fisher's exact test. The accrual ceiling was set at 60 patients. Despite rapid enrollment, the trial was voluntarily suspended after accrual of only 21 patients due to a sudden permanent loss of access to Iscomatrix™ or similar adjuvant, and is formally closed as of 5/6/2021.

Survival data from 21 patients were analyzed using Kaplan-Meier curves. The difference between the two groups was determined using a log-rank test. Median overall survival with confidence intervals and p values are reported.

Evaluation of serologic responses to CTA and carbohydrate antigens

Serologic responses to purified CTA were assessed in a blinded manner by ELISA as previously described (17) using pre- and post-treatment sera at 1:100, 1:400, 1:1,600, and 1:6,400 dilutions. Antibody titers were extrapolated based on a cutoff determined from a pool of healthy donor sera and were considered significant if >100.

Analysis of peripheral immune subsets

Cryopreserved, viable PBMCs were thawed and stained for immune subset analysis as previously reported (18). The following immunophenotypic markers were used to define immune subsets: CD4⁺ T-cells were CD8⁻CD4⁺; CD8⁺ T-cells were CD4⁻CD8⁺; Tregs were CD45⁺CD8⁻CD4⁺CD25^{hi}Foxp3⁺; monocytes were CD14⁺, classical monocytes were CD14⁺⁺CD16⁻; non-classical monocytes were CD14⁺, CD16⁺⁺ and intermediate monocytes were CD14⁺⁺CD16⁺. Antibodies used for these assays are listed in Table S1. Samples were run on a MACSQuant flow cytometer (Miltenyi Biotec, Bergisch Gladbach, DE), and analyzed using FlowJo software version 10.6.1 (FlowJo LLC, Ashland, OR). Wilcoxon matched-pairs signed rank test was used to generate p values (unadjusted for multiple variables) for analysis of pre/post treatment assays combining all patients eligible for evaluation;

Results

CT antigen expression in H1299 lysates

While screening a large panel of lung cancer lines for NY-ESO-1 and HLA expression, we identified the H1299

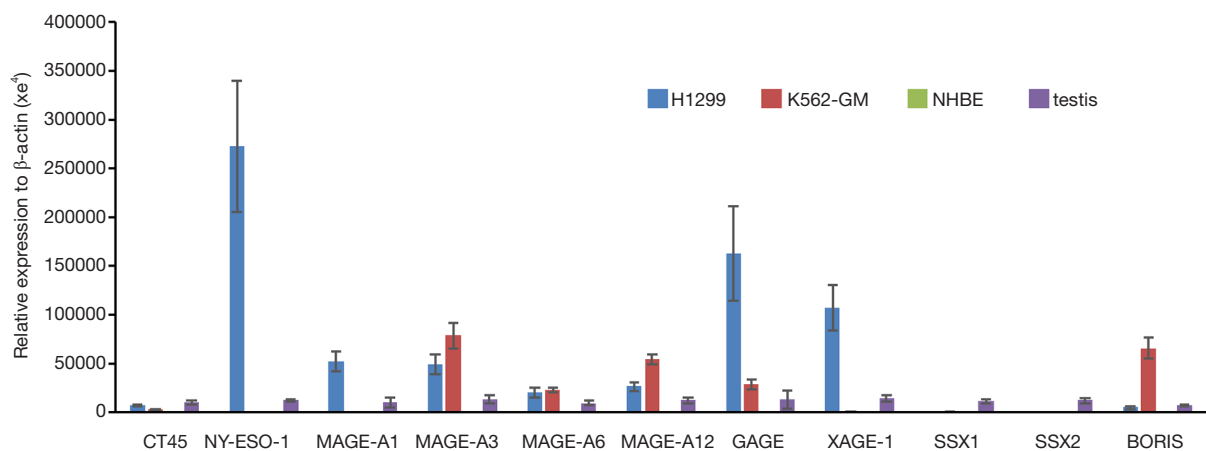


Figure 2 qRT-PCR analysis demonstrating elevated CT-X gene expression in H1299 cells.

large cell lung cancer line as having high level expression of NY-ESO-1 and other CT-X genes typically upregulated in epithelial malignancies (7). Representative qRT-PCR values for a panel of CT genes in H1299 cells are depicted in *Figure 2*. Notably, H1299 cells express numerous CT-X genes (except for SSX family members that are activated in synovial sarcomas) at levels exceeding those observed in normal testes, and in general at higher levels than K562-GM cells that were used for a previous live cell vaccine targeting MAGE-A3 in patients with thoracic malignancies at the NCI (D. Schrupp; unpublished). These genes are not expressed in normal respiratory epithelial cells. IHC analysis demonstrated that in contrast to focal CTA expression typically observed in epithelial cancer cells (10), H1299 cells exhibit homogeneous CT-X antigen expression (*Figure 3A*) due in part to stable amplification of the X-chromosome (19).

H1299 cells express class I as well as class II MHC antigens (data available upon request); consequently, when used as live, irradiated cell vaccines, these cells could induce allo-reactivity that might limit responses to CT-X antigens. As such, experiments were performed to examine if freeze-thaw lysate techniques could be used to deplete HLA proteins while preserving CT-X antigen levels. As shown in *Figure 3B*, total protein yields were not significantly different in freeze-thaw lysates compared to conventional RIPA buffer lysates; Decitabine treatment did not significantly increase yields, presumably due to endogenous maximal demethylation of the respective CT-X gene promoters. Immunoblot analysis (*Figure 3C*) demonstrated that freeze-thaw lysis markedly decreased levels of class I MHC proteins while retaining levels of CT-X antigens such as NY-ESO-1, MAGE-A1 and

MAGE-A3 relative to RIPA lysates.

Patient demographics

Of 25 patients screened, 4 were deemed ineligible due to detection of active disease ($n=3$) or withdrawal prior to randomization ($n=1$; *Figure 4*). 11 patients with primary thoracic malignancies and 10 patients with extra-thoracic neoplasms metastatic to the chest were enrolled in the study (*Table 1*). The median age of all patients was 51 years (range, 18.6–73.3); of note, median age of patients with primary thoracic malignancies was significantly higher than that of patients with cancers metastatic to the chest (61 *vs.* 36 years; $P<0.05$). Twelve (57%) of the patients were male. 8 patients (38%) had lung cancer; another 8 patients had sarcomas. 8 patients had locally advanced or metastatic disease at diagnosis and were rendered NED by surgery, chemotherapy, CRT, or combined modality treatment, whereas 7 developed metachronous metastases that were resected to render them NED prior to protocol enrollment. Fourteen patients (67%) received prior chemotherapy, whereas 12 (57%) received prior XRT. The melanoma patient had received several prior immunotherapy regimens. Three patients had previously participated in a live-irradiated K562-GM vaccine trial in our Branch (DSS; unpublished); none of these patients had exhibited an immunologic response to purified MAGE-A3 (the primary endpoint of that trial).

Clinical responses in vaccinated patients

All patients exhibited local inflammatory responses

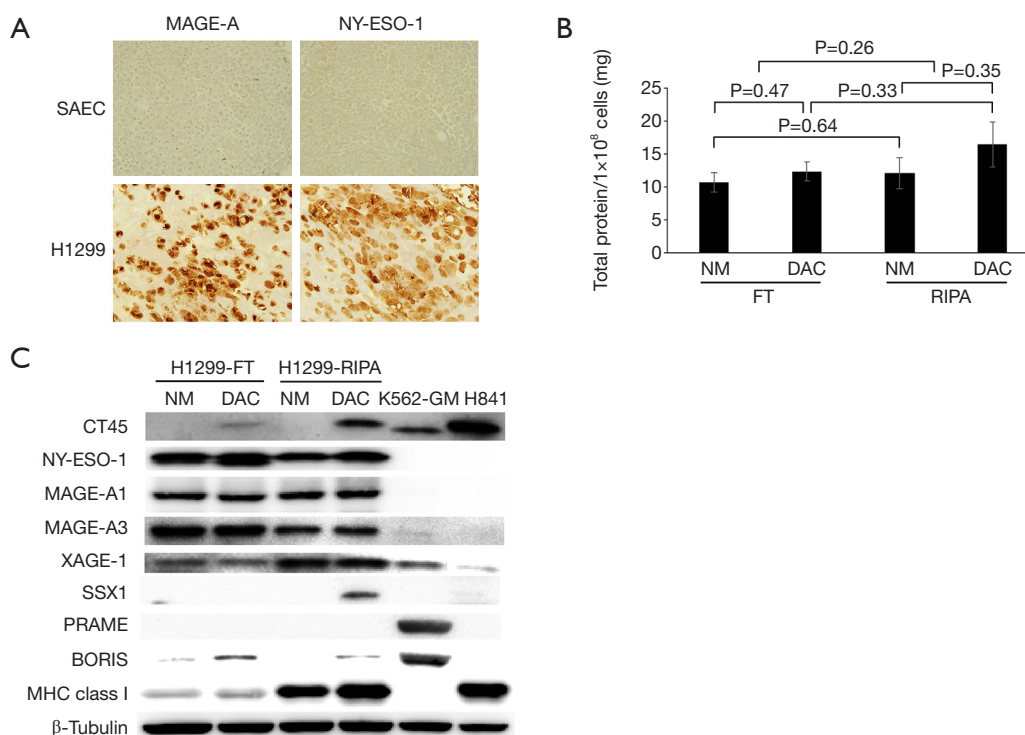


Figure 3 Cancer-testis antigen expression in cultured H1299 lung cancer cells and cell lysates. (A) IHC analysis ($\times 20$) showing increased CT-X antigen expression in H1299 cells in a homogeneous manner compared to normal respiratory epithelial cells (SAEC). (B) Total protein yield in lysates from 1×10^8 H1299 cells by freeze-thaw (FT) vs. RIPA buffer. (C) Immunoblot analysis demonstrating that freeze-thaw lysis decreased levels of MHC class I proteins compared to RIPA lysates while retaining levels of CT-X antigens. Levels of NY-ESO-1 and MAGE proteins are much higher in H1299 lysates relative to HLA-deficient K562-GM cells or H841 lung cancer cells.

(Figure 5A) and flu-like symptoms lasting 72–96 hours following vaccinations. There were no dose limiting treatment related toxicities. 14 patients (67%) including 9 with primary thoracic malignancies completed all six vaccinations and were evaluable for treatment response (Figure 4). Four patients (one with melanoma, 3 with sarcomas) were removed from study early due to disease recurrence. An additional sarcoma patient voluntarily withdrew from study due to college commitments. One lung cancer patient was removed from study early due to second primary NSCLC, and one NSCLC patient was removed from study due to difficulty assessing disease status in an irradiated mediastinum and was deemed to have progressive disease.

Molecular responses in vaccinated patients

Eight of 14 patients (57%) exhibited unequivocal serologic responses to NY-ESO-1; 6 of these 8 responders (75%)

had primary thoracic malignancies. Six of 9 patients (67%) with primary thoracic malignancies compared to two of five patients (40%) with pulmonary metastases who completed the vaccine regimen exhibited unequivocal serologic responses to NY-ESO-1. One patient developed antibodies to GAGE7; several patients exhibited reactivity to XAGE and MAGE-C2. Results of the analysis are summarized in Table 2, and Figure 5B. Vaccine therapy reduced the percent of Tregs ($P=0.0068$), and decreased intensities of PD-1 expression on Tregs ($P=0.0027$), PD-L1 expression on monocytes ($P=0.0089$), PD-L1 expression on classical monocytes ($P=0.016$), and PD-L1 expression on intermediate monocytes ($P=0.0031$) (Figure 6). Metronomic cyclophosphamide/celecoxib did not increase immune responses or enhance vaccine-induced alterations in peripheral immune subsets (results pertaining to peripheral blood Tregs are depicted in Figure S1). Additional analysis revealed no associations between metrics of immunologic responses and survival, possibly due to the limited number

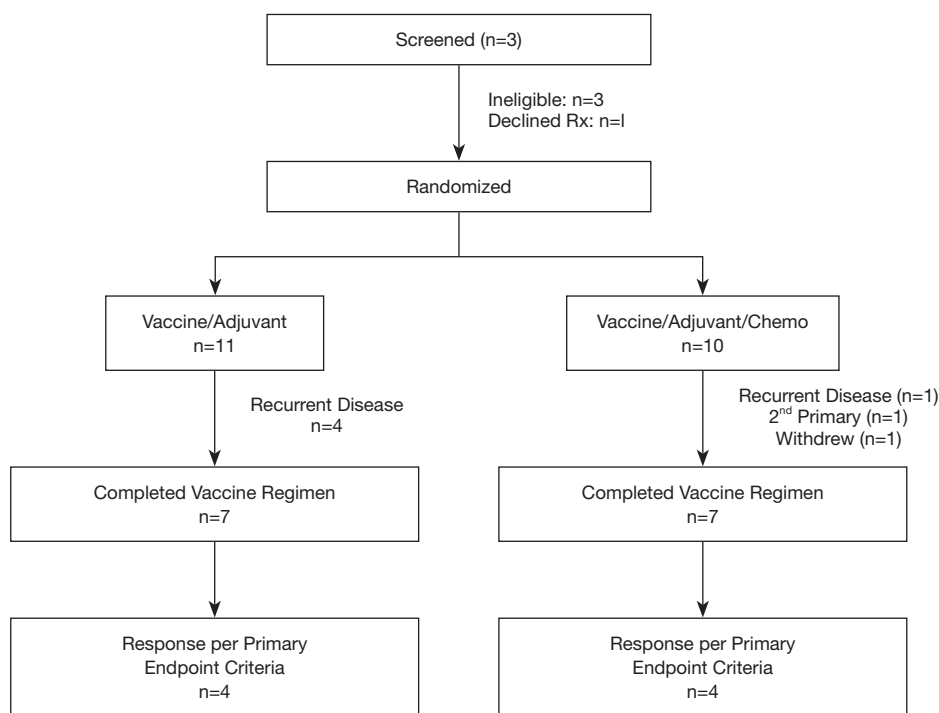


Figure 4 Trial participant flow.

of patients on this trial.

Clinical outcomes

Although not a stated objective in the protocol, outcome data are available for all patients enrolled on the trial. With a median follow-up of 62.1 months (range, 59–68.7), 12 patients (2 SCLC, 3 NSCLC, 2 EsC, 4 sarcoma, 1 RCC) are alive and NED, and 2 patients (both with NSCLC) are alive with new, but controlled malignancies (one DLBCL; one second primary NSCLC). 11 of the 14 surviving patients (79%) received all six vaccinations. In contrast, of the 7 patients (4 sarcoma, 1 melanoma, 1 TET, 1 NSCLC) who succumbed to their malignancies, only 3 (42%) received all six vaccinations.

Ten patients randomized to vaccine with metronomic cyclophosphamide/celecoxib. Seven of these patients received all six vaccinations. Two of these 7 patients were removed from study one month following the 6th vaccination due to disease recurrence; these two individuals and one more of the seven patients (43%) subsequently died of disease. Eleven patients randomized to vaccine alone. Seven of these patients completed all six vaccinations; none of these patients (0%) have died (*Figure 7*). There were no

obvious associations between histology or stage of tumors and outcomes in these patients.

Discussion

The highly restricted expression, potential oncogenic activities, and immunogenicity of CTA makes them attractive targets for cancer immunotherapy (4,5). However, limited information is available regarding the potential efficacy of vaccines targeting these proteins in patients with thoracic malignancies. In general, immunologic responses to these vaccines are more evident in patients with no evidence of disease or minimal disease burden relative to patients with extensive, bulky disease (20); this was our rationale for evaluating the H1299 lysate vaccine in the adjuvant setting. Atanackovic *et al.* (21) observed that a vaccine containing recombinant whole protein MAGE-A3 with a proprietary immunostimulant (AS15 or AS02B) induced CD4⁺ and CD8⁺ immune responses in lung cancer patients. Furthermore, a Phase II trial suggested that this vaccine might prolong survival in certain subsets of lung cancer patients undergoing definitive resection (22). Immunologic responses in lung cancer patients coincided with an 84 gene signature identified in tumor tissues of

Table 1 Patient demographics

Patient #	Gender	Age, years	Histology	Stage	Prior surg	Prior chemo	Prior XRT	Prior immuno
1	F	33.9	TET	IVA	Y	Y	Y	N
2	M	43.2	SCLC	LS	N	Y	Y	N
3	M	55.1	NSCLC	IA	Y	N	N	N
4	M	45.9	SAR	IV	Y	Y	Y	N
5	M	23.9	SAR	IV	Y	Y	Y	N
6	M	40.6	ACC	III	Y	N	N	N
7	M	39.5	SAR	IV	Y	Y	Y	N
8	F	68.3	NSCLC	IB	Y	N	N	N
9	M	35	SAR	IV	Y	N	Y	N
10	F	59.5	NSCLC	IA	Y	N	N	N
11	F	21.7	SAR	IV	Y	Y	Y	N
12	M	63.8	NSCLC	IIA	Y	Y	N	N
13	F	65.1	ESC	IV	N	Y	N	N
14	M	18.6	SAR	IV	Y	Y	N	N
15	F	58.3	SAR	IV	Y	Y	Y	N
16	M	51.2	ESC	IIIA	Y	Y	Y	N
17	F	63.5	NSCLC	IA	Y	N	N	N
18	M	65.2	NSCLC	IIIA	Y	Y	Y	N
19	F	21.8	SAR	IV	Y	Y	Y	N
20	M	73.3	MEL	IV	Y	Y	N	Y
21	F	51.9	SCLC	LS	N	Y	Y	N

TET, thymic epithelial tumor; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; SAR, sarcoma; ACC, adrenocortical carcinoma; ESC, esophageal cancer; MEL, melanoma; LS, limited stage.

melanoma patients responding to MAGE-A3 vaccines (22); the majority of genes predictive of response to vaccines were immune-related. In another trial, NY-ESO-1 DNA vaccines mediated CD4⁺ T cell responses in 5 of 5 patients with advanced stage NSCLC, three of whom developed CD8⁺ T cell responses to purified NY-ESO-1 peptides (23). Two lung cancer patients with measurable disease at the start of vaccination exhibited stable disease for 16 and 23 months; two additional lung cancer patients and one esophageal cancer patient with Stage IV disease who were NED at the time of protocol entry remained free of disease.

One potential strategy to enhance immune responses to cancers exhibiting low-level, heterogeneous expression of CTA and immune-related genes (24-26) involves vaccination with autologous cells genetically engineered

to express high-levels of these potential targets. Fontana *et al.* (27) observed immunologic responses to MAGE-A3 in 3 of 10 (30%) melanoma patients vaccinated with autologous PBL genetically-modified to express this CT-X antigen. Russo *et al.* (28) observed immunologic responses to MAGE-A3 in 6 of 23 melanoma patients receiving autologous lymphocytes genetically engineered to express this antigen. Of 19 patients with measurable disease, one had an objective response, and four patients exhibited durable stabilization of disease.

An alternative strategy is to use allogeneic cells to immunize cancer patients against multiple CTA that potentially can be up-regulated specifically in tumor cells by aforementioned systemic gene induction regimens (7). A recent report by Safavi *et al.* (29) demonstrating that

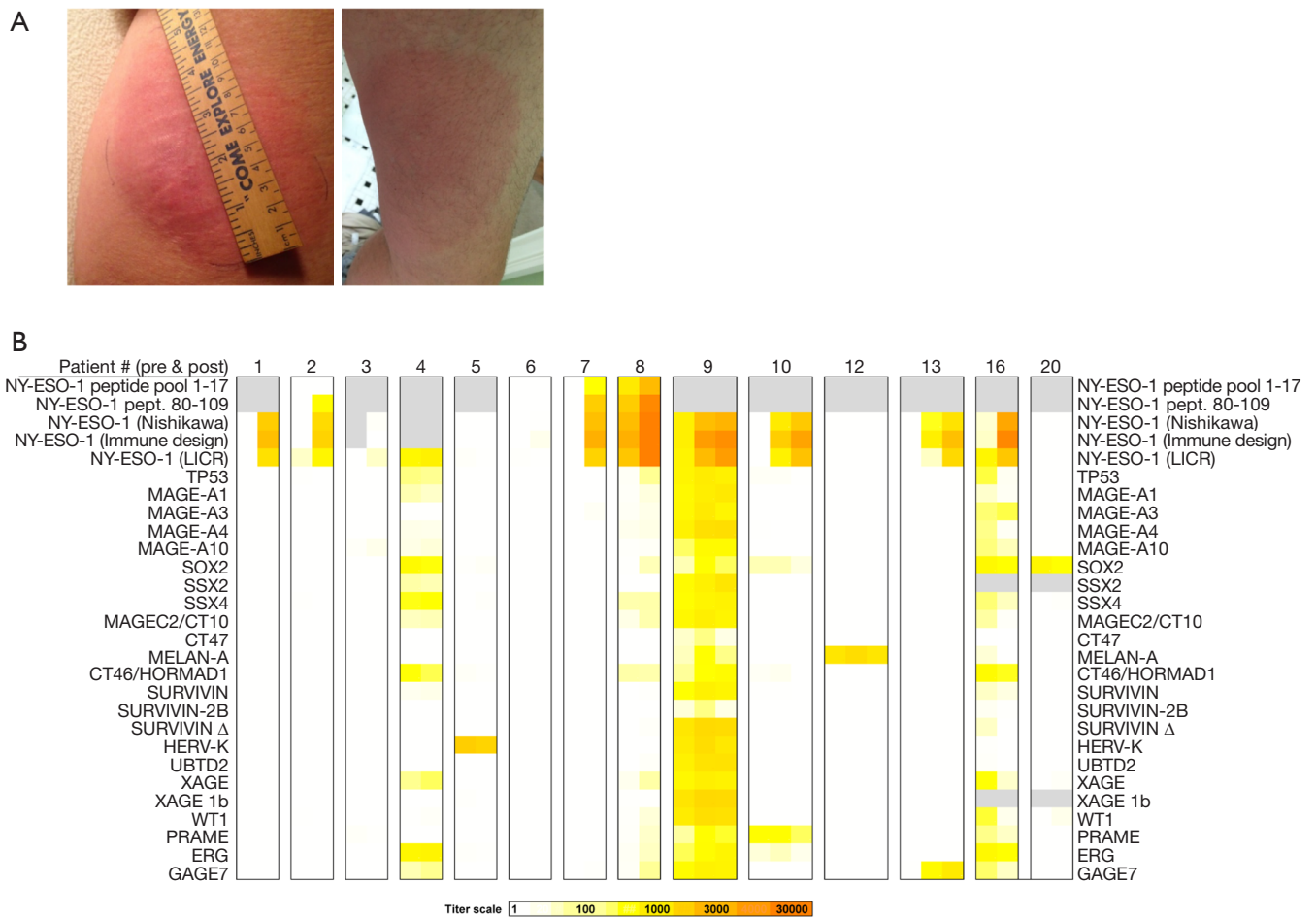


Figure 5 Local and systemic effects of H1299 lysate vaccine with Iscomatrix™ adjuvant in cancer patients. (A) Representative photos from two patients demonstrating local inflammatory responses at the injection sites approximately 48 h following vaccinations. (B) Analysis of serologic responses to a panel of potential tumor associated antigens including CT-X and non-CT-X antigens in pre- and post-vaccination patient sera.

a testicular germ cell vaccine administered with BCG significantly reduces growth and lethality of 4T1 breast cancer cells in a murine cancer model supports our approach in humans. In the present study we utilized the H1299 lung cancer line to immunize thoracic oncology patients against potentially relevant CTA focusing primarily on responses to CT-X antigens. We observed a relatively high percentage of patients with objective responses to NY-ESO-1. Despite these encouraging results, we did not observe significant responses to MAGE family members which are among the most commonly expressed CT-X antigens in thoracic malignancies (30,31). This may be due to amounts and relative immunogenicities of CT-X antigens in the vaccine lysates, as well as the stringency regarding what was defined

as antibody responses in our trial. Pujol *et al.* (32) observed variable antibody responses in all lung cancer patients who received MAGE-A3 immunotherapeutic (300 µg purified protein with proprietary adjuvant) q3 weeks x8 weeks. The lack of toxicities associated with the immunization regimen in our trial suggests that the protein content per vaccination can be safely increased to possibly broaden immune responses to CT-X antigens.

CD4⁺ CD25⁺ T cells expressing the forkhead box P3 (Fox P3) transcription factor mediate immune tolerance in cancer patients (33,34). Chronic inflammation within tumors facilitates conversion of FoxP3⁺ T_H cells to a suppressive phenotype (35). Absolute numbers and percentages of Tregs within the tumor microenvironment and peripheral blood

Table 2 Summary of treatment responses

Patient #	Histology	ECOG	Cohort	# Vaccines	Toxicity	Response	Off study reason	Status	OS, months
1	TET	0	VC	6	N	Y	PD	DOD	56.6
2	SCLC	0	V	6	N	Y	on study	NED	67.7
3	NSCLC	0	V	6	N	N	NR	NED	64.7
4	SAR	0	V	6	N	INC	NR	NED	64.6
5	SAR	0	VC	6	N	N	NR	DOD	21.6
6	ACC	0	VC	6	N	N	NR	NED	65.8
7	SAR	0	VC	6	N	Y	on study	NED	65.8
8	NSCLC	0	VC	6	N	Y	PD	DOD	25.4
9	SAR	0	V	6	N	Y	on study	NED	64.8
10	NSCLC	0	V	6	N	Y	DLBCL	DLBCL	64.8
11	SAR	1	V	3	N	N/A	PD	DOD	27.2
12	NSCLC	0	VC	6	N	N	NR	NED	64.6
13	ESC	0	V	6	N	Y	On study	NED	64.6
14	SAR	0	V	3	N	N/A	PD	DOD	22.2
15	SAR	0	VC	3	N	N/A	PD	DOD	20.9
16	ESC	0	VC	6	N	Y	On study	NED	62.1
17	NSCLC	0	VC	3	N	N/A	NSCLC*	NED	62.1
18	NSCLC	0	V	3	N	N/A	PD	NED	61.2
19	SAR	0	VC	2	N	N/A	Withdrew	NED	59.8
20	MEL	1	V	2	N	N/A	PD	DOD	7.4
21	SCLC	0	V	6	N	N	NR	NED	60.3

*, 2nd primary. TET, thymic epithelial tumor; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; SAR, sarcoma; ACC, adrenocortical carcinoma; ESC, esophageal cancer; MEL, melanoma. NR, No response.

of cancer patients correlate with advanced stage of disease and poor survival in these individuals (34,36).

Several studies have suggested that the capacity of cancer vaccines to induce effective antitumor immunity may be abrogated by simultaneous expansion of vaccine-induced immunosuppressive Tregs (37,38). Of particular relevance regarding induction of immunity to CT-X antigens are observations that Tregs attenuate immune responses to NY-ESO-1 vaccines in patients with melanoma as well as prostate, lung, and esophageal carcinomas (20,23). In the current study we utilized oral metronomic cyclophosphamide and celecoxib as a strategy to target activity of Tregs (39,40) yet did not observe any significant impact of the regimen with respect to frequency or magnitude of serologic responses to the lysate vaccines,

possibly due to the limited number of patients on the trial. Interestingly, the vaccine regimen did modulate peripheral immune subsets in a manner which theoretically could enhance antitumor immunity. Rather than expand Tregs, the vaccine, with or without cyclophosphamide and celecoxib resulted in a significant decrease in peripheral Tregs and a decrease in the expression of PD-1 on Tregs, which is consistent with a decrease in Treg immunosuppressive activity (41). This was accompanied by a potentially favorable modulation of innate immunity, as shown by a decrease in PD-L1 expression on total monocytes and monocyte subsets (42). Whether this was related to the lysate vaccine, the potent adjuvant, or both is unknown at present.

Limitations of the present study include early termination

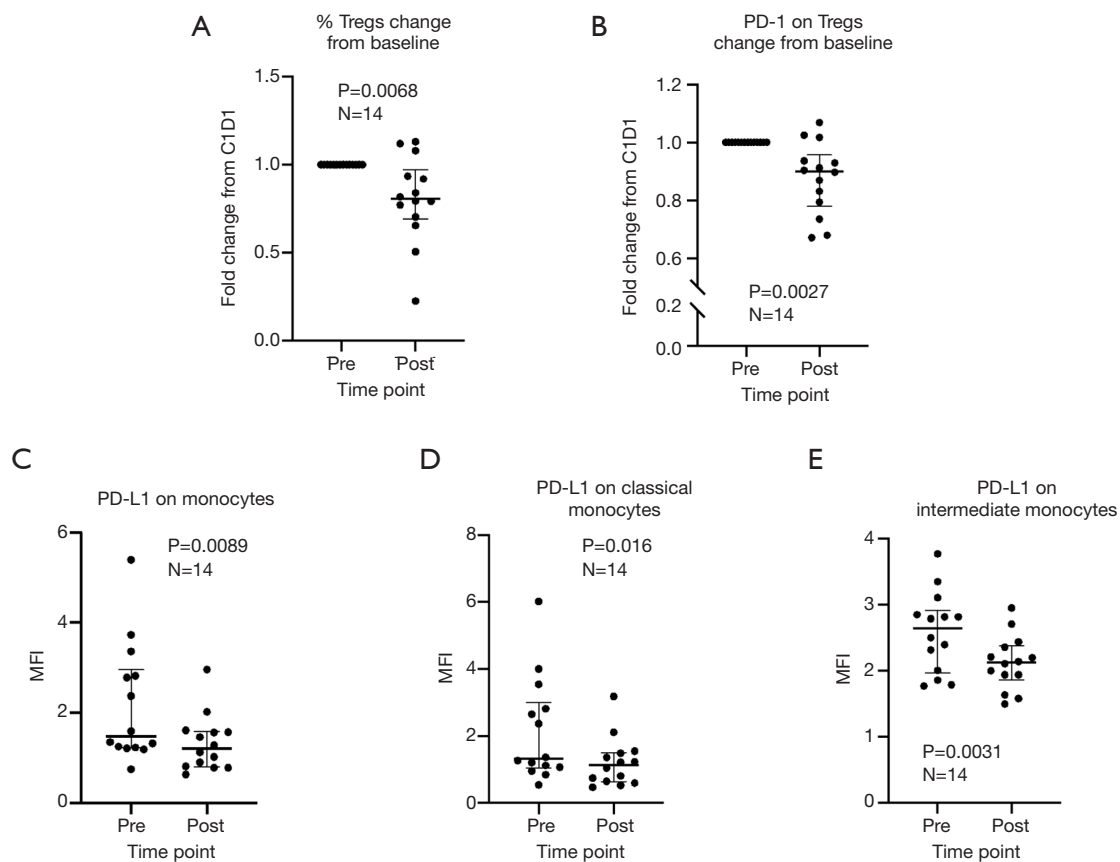


Figure 6 Flow cytometry analysis of peripheral immune subsets in cancer patients before and after H1299 lysate and iscomatrix™ treatment regimen. (A) Changes in percent Tregs among CD4⁺ T cells (B) Changes in intensities of PD-1 expression [median fluorescence intensity (MFI)] on Tregs. (C,D,E) Changes in intensities of PD-L1 expression (MFI) on total monocytes, classical monocytes, and intermediate monocytes, respectively. Wilcoxon matched-pairs signed rank test was used to generate P values (unadjusted for multiple variables).

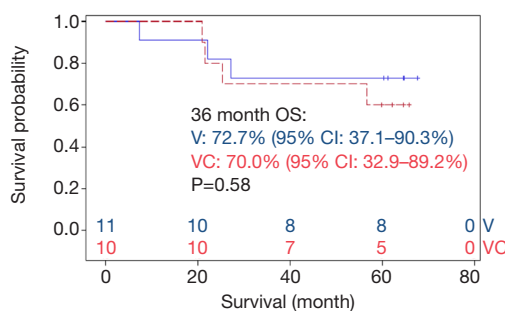


Figure 7 OS of patients randomized either to vaccine with adjuvant or vaccine with adjuvant and metronomic cyclophosphamide/celecoxib.

due to sudden, permanent loss of access to Iscomatrix and inability to obtain a similar type of adjuvant to continue the trial. Production of the lysate was halted for several

years due to restrictions imposed by the NIH following the Red Team Report (<https://www.sciencemag.org/news/2016/06/nih-uproar-over-report-slammng-clinical-center-leadership-shakeup>), which among other issues was highly critical of methods of production of cell based products for early phase clinical trials; extensive renovations and additional oversight of all production facilities imposed by the NIH resulted in prolonged voluntary suspension of the trial; this issue has now been resolved. Additionally, the trial included a heterogeneous group of patients (one cohort of 11 patients with primary thoracic malignancies of whom 9 (82%) completed the vaccine regimen, and another cohort of 10 patients with pulmonary metastases from extra-thoracic primaries, of whom only 5 (50%) completed the investigational treatment). These findings suggest that the vaccine regimen is not feasible in these latter patients, possibly due to histology and burden of

subclinical metastatic disease, as well as underlying immune status. The lack of primary tumor tissues from most of the patients prevented analysis of baseline CTA expression and correlation with serum antibody titers. Given the prolonged voluntary suspension related to adjuvant availability and new lysate production, this trial has been closed. A new trial will be initiated in the near future utilizing a higher lysate dose and Montanide® ISA 51 VG adjuvant with or without the IL-15 superagonist, N-803 in an attempt to induce immunity to more CT-X antigens in lung cancer patients from whom baseline tumor tissues are available for assessment of antigen expression and evaluation of serologic as well as cell mediated immune responses to autologous tumor cell lysates and lung cancer stem cells.

Conclusions

H1299 cell lysate vaccines can induce immunity to potentially relevant CTA in thoracic malignancies, and modulate peripheral immune subsets in a manner that may enhance anti-tumor immune responses. Lack of systemic toxicities suggests that the vaccine dose can be safely increased. Despite premature suspension of the trial, our findings support further analysis of the lysate vaccine in combination with either cytokines or immune checkpoint inhibitors as a strategy to target dormant cancer stem cells in thoracic malignancies.

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Footnote

Reporting Checklist: The authors have completed the CONSORT reporting checklist. Available at <https://dx.doi.org/10.21037/tlcr-21-1>

Trial Protocol: Available at <https://dx.doi.org/10.21037/tlcr-21-1>

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The trial was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and the guidelines for Good Clinical Practice. The trial (NCT02054104) was approved by NCI-IRB and FDA (IND 15812), and written informed consent was obtained from all individual participants.

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Table S1 Reagents used for this study

Antibodies		
Name	Company	Catalogue #
CT45	United States Biological	34313
NY-ESO-1	Millipore Sigma	N2038
MAGE-A1	AbCam	ab21472
MAGE-A3	AbCam	ab38496
XAGE-1	AbCam	ab134805
SSX1	AbCam	ab206839
PRAME	AbCam	ab219650
BORIS	AbCam	ab187163
MHC Class I	AbCam	ab134189
b-Tubulin	AbCam	ab15568
CD45	BioLegend	HI30
CD4	BioLegend	RPA-T4
CD8	BioLegend	SK1
CD25	BioLegend	BC96
Foxp3	BioLegend	206D
CD14	BioLegend	HCD14
CD16	BioLegend	3G8
PD-1	BioLegend	29F
CTLA-4	BioLegend	L3D10
TIM-3	BioLegend	F38-2E2
HLA-DR	BioLegend	L243
PD-L1	BioLegend	29E.2A3
TaqMan primers and probes		
Name	Company/sequence	Catalogue #
MAGE-A1	Thermo Fisher	Hs00607097_m1
MAGE-A6	Thermo Fisher	Hs00602508_m1
MAGE-A12	Thermo Fisher	Hs04176236_m1
GAGE	Thermo Fisher	Hs00275620_m1
XAGE-1	Thermo Fisher	Hs00220764_m1
SSX1	Thermo Fisher	Hs00846692_s1
SSX2	Thermo Fisher	Hs00817683_m1
CT45 F	5'-CTCTGCCATGTCCAAAGCAA-3'	
CT45 R	5'-AAGTCATCAATCTGAGAATCCAATTG-3'	
CT45 probe	6FAM-AAGCTTATGACAGGACATGCTATTCCACCCA-TAMRA	
NY_ESO-1 F	5'-TGCTTGAGTTCTACCTCGCCAT-3'	
NY-ESO-1 R	5'-GCTCCTGCGGGCCAG-3'	
NY-ESO-1 probe	6FAM-TTTCGCGACACCCATGGAAGCAG-TAMRA	
MAGE-A3 F	5'-TCCTGTGATCTTCAGCAAAGCTT-3'	
MAGE-A3 R	5'-GGGTCCACTTCCATCAGCTC-3'	
MAGE-A3 probe	6FAM-CAGTTCCTTGACAGCTGGTCTTTGGCAT-TAMRA	
BORIS F	5'-CCCATTGTGCCACCATCA-3'	
BORIS R	5'-AGCATGCAAGTTGCGCATAT-3'	
BORIS probe	6FAM-TGCACGGAAAAGCGACCTACGTGTG-TAMRA	
b-actin F	5'-GCGAGAAGATGACCCAGATC-3'	
b-actin R	5'-CCAGTGGTACGGCCAGAGG-3'	
b-actin probe	6FAM-CCAGCCATGTACGTTGCTATCCAGGC-TAMRA	
Cell lines		
Name	Company	Catalogue #
H1299	ATCC	CRL-5803
H841	ATCC	CRL-5845
NHBE	Lonza	CC-2541
SAEC	Lonza	CC-2547S
K562-GM	Barry Kobrin, Johns Hopkins University	

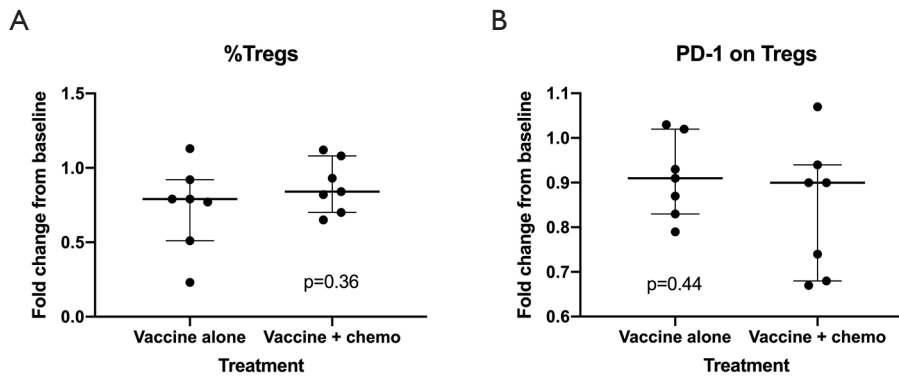


Figure S1 Comparison of fold change from baseline of percent Tregs (A) and intensity of PD-1 expression on Tregs (B) between vaccine alone and vaccine with chemotherapy. The Mann-Whitney U test was used to compare the two groups.