

Folate Receptor Alpha Peptide Vaccine Generates Immunity in Breast and Ovarian Cancer Patients

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Abstract

Purpose: Folate receptor alpha (FR) is overexpressed in several cancers. Endogenous immunity to the FR has been demonstrated in patients and suggests the feasibility of targeting FR with vaccine or other immune therapies. CD4 helper T cells are central to the development of coordinated immunity, and prior work shows their importance in protecting against relapse. Our previous identification of degenerate HLA-class II epitopes from human FR led to the development of a broad coverage epitope pool potentially useful in augmenting antigen-specific immune responses in most patients.

Patients and Methods: We conducted a phase I clinical trial testing safety and immunogenicity of this vaccine, enrolling patients with ovarian cancer or breast cancer who completed conventional treatment and who showed no evidence of disease. Patients were initially treated with low-dose cyclophosphamide and then vaccinated 6 times, monthly. Immunity and safety were examined during the vaccine period and up to 1 year later.

Results: Vaccination was well tolerated in all patients. Vaccine elicited or augmented immunity in more than 90% of patients examined. Unlike recall immunity to tetanus toxoid (TT), FR T-cell responses developed slowly over the course of vaccination with a median time to maximal immunity in 5 months. Despite slow development of immunity, responsiveness appeared to persist for at least 12 months.

Conclusions: The results demonstrate that it is safe to augment immunity to the FR tumor antigen, and the developed vaccine is testable for therapeutic activity in most patients whose tumors express FR, regardless of HLA genotype. *Clin Cancer Res*; 24(13); 3014–25. ©2018 AACR.

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Introduction

Despite advances in surgery and adjuvant systemic therapy, distant relapse and subsequent mortality from breast and ovarian cancers remain considerable public healthcare problems with an estimate of greater than 40,000 deaths from breast cancer and 14,000 deaths from ovarian cancer annually (1). Strategies for

adjuvant therapy that complement current modalities are needed to decrease the recurrence rates for breast cancer and ovarian cancer. Two shared features of breast cancer and ovarian cancer that may be exploited in the adjuvant setting are as follows: (i) the majority of patients without overt distant metastatic disease at diagnosis achieve an initial complete remission from their cancers; and (ii) women with breast cancer and ovarian cancer often generate endogenous immune responses to their tumors. Generation of antitumor immunity has been consistently associated with better outcomes (2–4), leading to the hypothesis that improving antitumor immune responses with strategies such as vaccination may improve disease-free and overall survival.

We have identified the folate receptor alpha (FR α) as a candidate therapeutic vaccine antigen for patients with breast cancer and ovarian cancer (5). FR α is normally expressed in areas of the body that need to retrieve folic acid prior to excretion (kidney proximal tubules) or to concentrate it in the cerebrospinal fluid (choroid plexus). In addition, FR α is expressed in the lung, although its function there is unknown (6, 7). In contrast to this relatively restricted expression in healthy tissues, FR α is frequently highly overexpressed in several epithelial tumors, including breast cancer and ovarian cancer (8–12). We have previously demonstrated that many women with breast cancer and ovarian cancer have evidence of spontaneous T-cell and antibody-immune responses to FR α at levels higher than observed in women who had not experienced breast cancer or ovarian cancer (13). Importantly, spontaneous immunity to FR α was not associated with paraneoplastic syndrome or other symptoms of autoimmunity in our cases or controls (13).

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Translational Relevance

Folate receptor alpha (FR α), in its native form, has recently been identified as a potential tumor antigen due to its aberrant expression in a variety of cancers, such as ovarian, lung, and breast cancer. Patients with these diseases often demonstrate preexisting immunity to the protein, indicating it is a natural target of the immune response. This preexisting immune response and its previously defined role in tumor aggressiveness suggest that it may be useful in vaccine preparations designed to augment preexisting immunity to levels that may be therapeutic. In this first report, we demonstrate that immune responses to the FR α can safely be generated in patients previously diagnosed with cancer. Our findings illustrate the potential for the safe use of vaccines targeting the FR α to prevent disease recurrence. Additional uses to consider would be incorporating newer adjuvant and use of the vaccine in combination with new immunotherapeutic strategies, such as immune checkpoint blockade.

Specifically, we have identified five putative HLA class II-binding peptides from the FR α sequence that elicited T-cell immune responses from breast cancer and ovarian cancer patients (13). We estimated that >88% of women will express HLA proteins that would recognize at least one of the peptides, such that the majority of unselected HLA diverse patients would have the potential to respond to vaccine (13). This is significantly higher than vaccines incorporating class I-binding peptides, which would be presented to CD8⁺ cells. The use of HLA class II-binding peptides specifically targets activation of CD4⁺ T-helper cells, which are essential for coordinated memory immune responses to antigen. Thus, due to its degeneracy, this pool could be effective at generating persistent and protective immunity in most ovarian or breast cancer patients, regardless of HLA genetic background.

On the basis of our prior demonstration of spontaneous anti-FR α immunity in a subset of breast cancer and ovarian cancer patients (13), we conducted a phase I clinical trial study to assess the immunogenicity and safety profile of a multipeptide FR α peptide vaccine comprised of FR30, FR56, FR76, FR113, and FR238 (Table 1) admixed with GM-CSF. This 22-patient trial enrolled women with ovarian, primary, peritoneal, or breast cancers that had completed treatment (surgery, chemotherapy, radiation, or a combination of the above) at least 90 days previously and showed no evidence of disease following their treatment. Perhaps the largest barrier to the generation of effective antitumor immunity is local and systemic tumor-induced immunosuppression, often mediated through regulatory T cells (Tregs; ref. 14). Several strategies to inhibit immunosuppressive enzymes (IDO), surface markers (PD-L1, CTLA-4), and Tregs (Denileukin Diftitox, cyclophosphamide; refs.15–22) have or are currently

undergoing testing to determine whether they improve responses to vaccines. For this trial, we selected cyclophosphamide based on its long safety history, cost effectiveness, oral administration, and its status as an FDA-approved agent for ovarian cancer and breast cancer patients (23–27). The dose and schedule were selected on the basis of a small previous study that showed decreased levels of circulating Tregs in 9 of 9 advanced cancer patients (28). Directly targeting immunosuppressive Tregs, plus careful timing of vaccination for when disease burden is lowest, provides an opportunity to generate meaningful immune responses with the goal of preventing or delaying disease recurrence. Thus, the primary objectives were to determine the safety of the combination therapy protocol (cyclophosphamide followed by vaccine) and the ability of this regimen to generate anti-FR α immune responses.

Patients and Methods

Eligibility and enrollment

This single arm phase I study enrolled women of 18 years or older with histologically confirmed stage II–III breast cancer or stage II–IV ovarian (including primary peritoneal and fallopian tube) cancer that had completed systematic therapy (except for hormonal treatment or bisphosphonates) at least 90 days prior to registration and were without evidence of disease. Patients could enroll after initial diagnosis or after a complete response to therapy for recurrent disease. Additional eligibility criteria included Eastern Cooperative Oncology Group performance status of 0–1 and adequate hematologic, renal, and hepatic functions. Exclusion criteria included active infection, immunocompromising conditions, autoimmunity, systemic steroid use within 30 days of registration, concurrent thyroid replacement therapy, NYHA class III/IV congestive heart failure, myocardial infarction or stroke within 6 months of registration, another invasive malignancy within 5 years of enrollment, pregnancy, or breast feeding. A decision was made not to require FR α expression in the primary tumor because: (i) this was a phase I safety trial; (ii) lack of a validated antibody IHC test for expression, and (iii) lack of data that FR α would or would not be expressed on recurrent tumors, regardless of primary tumor expression. With respect to the latter, we have previously shown that it is possible for recurrent ovarian lesions to express FR α even though the primary lesion may not have shown positivity (5). All patients were at Mayo Clinic (Rochester, MN). Protocol chairs obtained study approval from the institutional review board and filed assurances with the Department of Health and Human Services. The study was conducted in accordance with the U.S. Common Rule. Written, informed consent was required for enrollment. The study was registered on Clinicaltrials.gov accessed at NCT01606241.

Preparation of the vaccine

Peptides were produced by Polypeptide Systems, Inc. After reconstituting, the five peptides were pooled into single vials containing 550 μ g of each peptide (representing a 10% overfill to allow complete retrieval of the appropriate dose), lyophilized, and stored at -80°C until use. On the day of vaccination, vials were brought to room temperature over 2 hours. A total of 1.1 mL of 1% DMSO in sterile water was added and the vial swirled until the peptides solubilized. GM-CSF was added and mixed gently. Three syringes were prepared with approximately 0.4 mL of vaccine.

Table 1. Vaccine FR α peptides

Sequence	Position	Designation	Length (aa)
RTLLNVCMNAKHHKEK	30–46	FR30	17
QCRPWRKNACCSTNT	56–70	FR56	15
KDVSYLRYFNWNHCGEMA	76–93	FR76	18
LGPWIIQQVDQSWRKERV	113–129	FR113	17
PWAAWPFLLSLALMLLWL	238–255	FR238	18

For stability testing of the vaccine, reconstituted peptides were separated chromatographically using an Agilent Zorbax 300SB C18 RP column with a water:acetonitrile:formic acid gradient to assess stability quarterly during the first year after mixing, then every 6 months to the present time, with no significant degradation observed during the vaccination period. Concentrations (mg/mL) at manufacture and 2 years following reconstitution were 0.58 and 0.74 for FR30; 0.6 and 0.63 for FR56; 0.68 and 0.61 for FR76; 0.75 and 0.73 for FR112; and 0.76 and 0.78 for FR238.

Vaccinations

Prior to registration, patients underwent baseline screening evaluation. Within 14 days of study registration, prior to each treatment cycle, 4 weeks after completion of treatment, and 3, 6 and 12 months thereafter until disease recurrence or a maximum of 18 months postregistration, patients underwent a complete physical exam, assessment of performance status, blood chemistries, and metabolic panel, urinalysis for proteinuria, TSH, ANA, and RF testing, toxicity assessments (CTCAE v. 4.0), and research blood draws for immune monitoring. Disease status was radiographically evaluated at registration and as per standard of care by the treating physician.

Patients received a standard tetanus booster (DT, Sanofi) just prior to FR α vaccination unless they indicated that they had received a booster within 1 year of registration. This was done as a positive control to verify that the patients demonstrated a measurable immune response and prior human observations that concurrent administration of different vaccines did not appear to influence immunogenicity (29). All patients received one cycle of priming with 50 mg of cyclophosphamide administered orally twice daily on days 1–7 and 15–21 of a 28-day cycle (cycle 1). Patients were vaccinated intradermally at 3 sites in forearm (on a side where no axillary lymph node dissection [ALND] was performed) or the outer thigh (if bilateral ALNDs were performed) with a mixture of 500 μ g each of FR30, FR56, FR76, FR113, and FR238 admixed with 125 μ g of GM-CSF (Sargramostim, trade name Leukine from Genzyme) on day 1 of a 28-day cycle for a maximum of 6 vaccination cycles (cycles 2–7).

Treatment was discontinued if a patient developed grade ≥ 3 toxicity or grade ≥ 2 neurologic problems during cyclophosphamide that did not resolve within 3 weeks. Vaccinations were discontinued if the patient developed a grade ≥ 2 allergic reaction, autoimmune reaction, neurologic difficulties, any grade 3+ adverse events; antinuclear antibody value >3 ; or urine protein ≥ 2.0 gm/24 hours for more than 4 weeks.

IHC

Expression of FR α was ascertained as previously described (5). Briefly, tissue sections from the primary tumors of patients on the study were incubated with the monoclonal antibody mab343 (kindly provided by Dr. Phillip Low, Purdue University, West Lafayette, IN) or an isotype-matched control antibody after antigen retrieval. The mMACH3 system (Biocare Medical) with horseradish peroxidase detection was used. Tissue samples were scored by a pathologist specializing in breast and ovarian cancers (D.W. Visscher). Samples were considered positive for FR α expression if at least 10% of the epithelial cells expressed FR α . Expression of FR α was not used as eligibility criteria. Staining was scored as negative (0), weak

(1+), moderate (2+), and strong (3+) membrane staining. The percent of cells within each section stained at intensity was recorded to calculate an H-score for each sample. The H-score is a weighted score that captures both the proportion of positive staining and its intensity, and thus more representative of the staining of the entire tumor section. The H-score for staining each sample was defined as: H-score = 3 \times (% at 3+) + 2 \times (% at 2+) + 1 \times (% at 1+) + 0 \times (% at 0). H-score values can range from zero (no membrane staining) to a maximum of 300 (100% membrane staining at 3+).

PBMC preparation and cryopreservation

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation, as described previously (30). PBMCs were cryopreserved in liquid nitrogen at 10 million PBMCs/mL in RPMI containing 10% (v/v) DMSO, 110 mg/mL human serum albumin, and 2.7 mg/mL HEPES. For serum preparation, whole blood was allowed to clot and then the serum was immediately frozen at -80°C . Peripheral blood samples were also obtained from 12 healthy volunteer women for comparison of Treg levels.

Flow cytometry

Treg frequencies were assessed using flow cytometry. PBMCs obtained at baseline (i.e., before cyclophosphamide treatment) and at the end of cycle 1 were surface-labeled with fluorochrome-conjugated antibodies against CD4, CD25, and CD127 (31–33). Cells were then permeabilized and labeled with anti-FoxP3. Samples were analyzed by flow cytometry (BD LSRII), and data were analyzed using FlowJo software (v.10.0.8). Within the lymphocyte gate, CD4 $^{+}$ 25 $^{+}$ cells were assessed for CD127 and FoxP3, and Tregs (defined as CD4 $^{+}$ 25 $^{+}$ 127 $^{\text{lo}}$ FoxP3 $^{+}$) were calculated as a percentage of total cells in the lymphocyte gate.

IFN γ enzyme-linked ELISpot assay

A 2-day, direct *ex vivo*, IFN γ enzyme-linked immunosorbent spot (ELISpot) assay was used to determine frequencies of peptide-specific T lymphocytes, a modification of which we have described previously (34). Initially an FBS-based assay was used for assessing immunity in the first 15 patients but then we switched to a human serum (HS)-based assay because concerns that low signal-to-noise ratio (SNR) may interfere with reliable detection of antigen-specific immune responses. The SNR was 0.92 ± 0.22 for the FBS-based assay and 1.74 ± 0.19 for the HS-based assay ($P = 0.043$). On day 1, 2.5×10^5 PBMCs/well were plated in triplicate into 96-well round bottom plates in 200 μ L of RPMI1640 containing l-glutamine, penicillin, streptomycin, and 10% AB (of FBS) serum (T-cell medium) along with cyclin D1 (control) peptide (MELLLVNKLKWNLAA; ref. 34), FR30, FR56, FR76, FR113, FR238, FR α protein (Sino Biological Inc.), tetanus toxoid (TT; List Biological Laboratories Inc.), or Phytohemagglutinin (PHA; Sigma-Aldrich). Peptides were plated at 10 μ g/mL, PHA and tetanus were used at 1 μ g/mL, and FR α protein at 100 ng/mL. The cells were incubated at 37°C at 5% CO_2 . On the same day as plating the cells, nitrocellulose-backed (NC plates) plates were coated with 10 μ g/mL anti-IFN γ antibody (MabTech) in PBS at 50 μ L/well for 24 hours. After 24 hours, the cells were transferred to the washed and blocked NC plates and further incubated at 37°C for further 24 hours, followed by washing three times using PBS containing 0.05% Tween-20. The plate was then incubated for 2.5 hours at room temperature

in 50 μ L/well PBS containing 5 μ g/mL biotinylated anti-IFN γ Ab, washed three times with PBS, and further incubated with 100 μ L/well streptavidin-alkaline phosphatase at a dilution of 1:1,000 in PBS for 2 hours at room temperature. After washing three times in PBS, the plate was incubated with 100 μ L/well AP-colorimetric substrate for 20–30 minutes, rinsed with cool tap water, and allowed to dry completely. After drying overnight, the plates were read on an AID ELISpot reader, which provides quantitative spot information based on the number of stimulated cells that secrete IFN γ . Antigen-specific T-cell frequencies were defined as the average number of spots elicited by a given antigen minus the average number of spots with no added antigen.

ELISA

Antibody (IgG) responses were assessed using a typical ELISA. Briefly, 96-well microplates (Thermo Fisher Scientific) were coated with 20 ng/mL of FR30, FR56, FR76, FR113, FR238, and cyclin D1 peptides, or with 100 ng/mL TT or FR α protein. Human IgG was used as a protein standard that was added at a concentration range of 250 to 1.95 μ g/mL. After blocking the plates with 1% BSA, patient serum samples were added at a 1:100 dilution incubated for 2 hours at room temperature. Anti-human IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology) and ELISA substrate (BD Biosciences) was then added in standard fashion, and absorbance was read at 450 nm on a plate reader.

Statistical considerations

The study was designed to assess the immunogenicity and safety profile of a multi-epitope FR α peptide vaccine comprised of FR30, FR56, FR76, FR113, and FR238 admixed with GM-CSF. The sample size was powered for the immune monitoring endpoint. Assuming that half of the participants would develop immune responses, 22 patients were required to achieve a lower 95% confidence interval of 30% true responses, which was deemed the lowest proportion of participant responses that would support continued development of the vaccine. The primary clinical endpoint was the percentage of patients who developed a severe toxicity (defined as a grade 3–5 AE by NCI-CTC version 4.0 criteria that was possibly, probably or definitely related to treatment). The sample size of 22 patients resulted in a maximum width of the 90% binomial confidence interval for the percentage of patients who develop severe toxicity of $\pm 17.5\%$.

Mean levels of antigen-specific T cells and antibodies were compared using the Paired Student's *t* test (Microsoft Excel 2010; Microsoft Inc.). A patient was considered to have responded to vaccination if they had developed a 3-fold or greater increase in FR α -specific T cells or antibodies at any point during the vaccine or observation periods. For comparing the relative immunogenicity of the peptides, if patients had undetectable T-cell immunity, a positive response was defined as was ≥ 50 antigen-specific T cells/million PBMC or at any point during the trial period. None of the patients had undetectable levels of antibodies prior to vaccination. Mean levels of Tregs in patients at baseline were compared with Tregs in normal healthy women using an unpaired Student *t* test. Correlations between the baseline levels and the magnitude of change of T-cell frequency were examined using linear regression analysis (Prism, v.6, GraphPad Software, Inc.).

Rigid protocol criteria for moving the vaccine forward into future clinical testing (e.g., phase II or phase I combinations) were established. For this, mean values derived from triplicates with coefficients of variations of greater than 20% were excluded from

further analysis. Postimmunization values were thereafter considered elevated relative to preimmunization values if they were at least double and with nonoverlapping standard deviations. If preimmunization values were zero or below after subtracting the background, the postimmunization mean values must have been above zero with a SD that was nonoverlapping with the preimmunization values. If at least 30% of patients developed an immune response (T cell or antibody) following vaccination, as measured in the vaccine period, then the vaccine was considered a candidate for future investigation.

Results

Patient characteristics

Twenty-two female patients were treated in this phase I trial at Mayo Clinic. Table 2 summarizes the patient characteristics including the disease type, grade, and histology. Fourteen patients had been previously diagnosed with ovarian cancer and eight with breast cancer. Four of the ovarian cancer patients had recurrent disease at the time of study enrollment. Patients were enrolled regardless of FR α (Materials and Methods). All 8 breast cancer patients were diagnosed with ER⁺PR⁺HER2/neu⁻ cancers consistent with the high frequency of this phenotype in the patient population diagnosed with breast cancer.

Vaccination against the FR α is safe

Twenty-two patients were evaluable for toxicity assessments. Overall, vaccination was well tolerated and was not associated with significant grade ≥ 3 toxicity (Table 3). One patient developed a treatment-related grade 3 injection site reaction. Grade 2 reactions observed during the active vaccination phase included lymphopenia (3 patients), fatigue (3 patients), neutropenia (2 patients), leukopenia (2 patients), and injection site reactions (2 patients). Two patients developed grade 3–4 infections during priming (cyclophosphamide) phase that were not treatment-related. Tests for autoimmunity (rheumatoid factor, anti-nuclear antibodies, and thyroid stimulating hormone) did not show elevation for any participants throughout the vaccination schedule (data not shown because all were negative and below limits of detection of an abnormal response).

Circulating Tregs are not decreased following cyclophosphamide treatment

Frequencies of Tregs from pretreatment and post-cyclophosphamide blood samples were compared in 17 evaluable patients (Fig. 1A and B). Prior to priming with cyclophosphamide, the mean frequency of Tregs was $0.6 \pm 0.1\%$ (\pm SEM) of the total lymphocyte population. After cyclophosphamide, the mean frequency was $0.7 \pm 0.1\%$, which was not significantly different than mean pretreatment levels ($P = 0.16$). In contrast to prior studies, in this patient population with no evidence of disease, Tregs were not significantly elevated ($127 \pm 13\%$ (\pm SEM) of normal, $P = 0.2$) relative to levels seen in 12 normal healthy women.

Vaccination generates T-cell but not antibody immunity to FR α after immunization

The frequency of IFN γ -producing T cells specific for FR α peptides, FR α protein, TT, and control peptide was compared between pretreatment samples and multiple posttreatment samples, during vaccine treatment (up to 30 days following the last vaccination). PBMCs from the vaccine period were

Table 2. Patient and tumor characteristics

Breast cancer cohort <i>N</i> = 8		Ovarian cancer cohort <i>N</i> = 14	
Median age (range)	48 (39–61)	Median age (range)	57 (35–68)
T stage		Site	
T1	3 (37.5%)	Ovary	13 (92.9%)
T2	4 (50.0%)	Fallopian tubes	1 (7.1%)
T4	1 (12.5%)		
N stage		FIGO stage	
N1	5 (62.5%)	II	2 (14.3%)
N2	2 (25.0%)	III	12 (85.7%)
N3	1 (12.5%)		
Histology		Histology	
Invasive ductal carcinoma	5 (62.5%)	Serous	10 (71.4%)
Invasive lobular carcinoma	1 (12.5%)	Endometrioid	1 (7.1%)
Mix of IDC and ILC	2 (25.0%)	Clear cell	1 (7.1%)
		Mixed	2 (14.3%)
Prior systemic therapy		Prior systemic therapy	
Neoadjuvant chemotherapy	4 (50.0%)	Neoadjuvant carboplatin/paclitaxel	3 (21.4%)
Adjuvant chemotherapy	4 (50.0%)	Adjuvant carboplatin-based regimen	9 (64.3%)
Adjuvant endocrine therapy	8 (100.0%)	Adjuvant IP cisplatin/paclitaxel → carboplatin/paclitaxel	3 (21.4%)
		Adjuvant IP cisplatin/paclitaxel → BEV	1 (7.1%)
Grade 1 fatigue	3 (37.5%)	Grade 1 fatigue	6 (42.9%)
Race		Race	
White	7 (88%)	White	13 (93%)
Not reported	1 (13%)	Not reported	0
Unknown	0	Unknown	1 (7%)
FR H score		FR H score	
0	8 (100%)	0	0
1–100	0	1–100	7 (50.0%)
101–200	0	101–200	3 (21.4%)
201–300	0	201–300	2 (14.3%)
No tumor available	0	No tumor available	2 (14.3%)

available from 14 of 21 patients. In Fig. 1C, the preimmunization and highest postimmunization (as observed during the vaccination period only) T-cell frequencies are plotted as previously described (35, 36), excluding values from the observation period at 3, 6, and 12 months following the final vaccination. The median number of postvaccination (not including observation) period samples was 5.5 (range 1–6). The preimmunization IFN γ T-cell frequency to FR30 was 47 ± 14 (\pm SEM, $n = 14$) T cells/million PBMCs which increased to 315 ± 14

to T cells/million PBMCs ($P = 0.00003$). To FR56, T cells increased from 52 ± 13 to 322 ± 52 ($P = 0.0001$), FR76 from 43 ± 14 to 307 ± 55 ($P = 0.00005$), to FR113 from 27 ± 11 to 274 ± 68 ($P = 0.001$), and FR238 from 33 ± 8 to 176 ± 37 ($P = 0.002$). Analysis of data from the FBS-based assay yielded similar information (Supplementary Fig. S1).

There was a minor, albeit statistically significant, increase in the reactivity to the pan-DR binding cyclin D1 peptide from 15 ± 10 to 45 ± 15 ($P = 0.02$). Although preexistent immunity appeared to be high for FR α , which has many potential epitopes, the mean frequency increased significantly from 88 ± 21 to 239 ± 44 T cells ($P = 0.0008$), which indicates that the vaccine is generating T cells that are recognizing naturally processed antigens. Consistent with tetanus boosters in several of the patients prior to FR α vaccination, the mean TT T-cell frequency increased from 541 ± 67 to 1007 ± 68 TT-specific T cells per million PBMC ($P = 0.00005$; Fig. 1D).

In contrast to increased T-cell immunity, increased mean antibody responses to the peptides encompassing the vaccine were not observed following immunization (Fig. 1E). Serum for antibody assessments was available from 21 patients. The median number of postvaccination (not including observation) period serum samples was 6.0 (range 3–6) and the maximum was 6. The preimmunization mean antibody concentration to FR30 was 7.0 ± 2.3 μ g/mL (\pm SEM, $n = 21$), which increased to 9.1 ± 2.4 μ g/mL ($P = 0.53$). To FR56, mean antibodies increased from 5.1 ± 1 to 6.7 ± 1.0 ($P = 0.26$), to FR76 from 3.8 ± 1.0 to 4.9 ± 1.3 ($P = 0.5$), to FR113 from 2.3 ± 0.6 to 2.8 ± 0.8 ($P = 0.28$), and to FR238 from 3.1 ± 1.6 to 5.9 ± 1.8 ($P = 0.88$). These slight nonsignificant increases did

Table 3. AEs at least possibly related to study treatment

Grade	2	3	4
During priming with cyclophosphamide (<i>N</i> = 21)			
Decreased lymphocyte counts	3	0	0
Decreased white blood cell counts	3	0	0
Decreased neutrophil counts	1	0	0
Fatigue	2	0	0
Nausea	1	0	0
Sinus pain	1	0	0
Sepsis	0	0	1
During vaccination and observation period (<i>N</i> = 21)			
Eye disorders	1	0	0
Arthralgia	1	0	0
Fatigue	1	0	0
Myalgia	1	0	0
Injection site reaction	2	1	0
Neutrophil count decreased	2	0	0
Dyspnea	1	0	0
White blood cell decreased	1	0	0
Lymphocyte count decreased	3	0	0
General disorder, admin site conditions	1	0	0

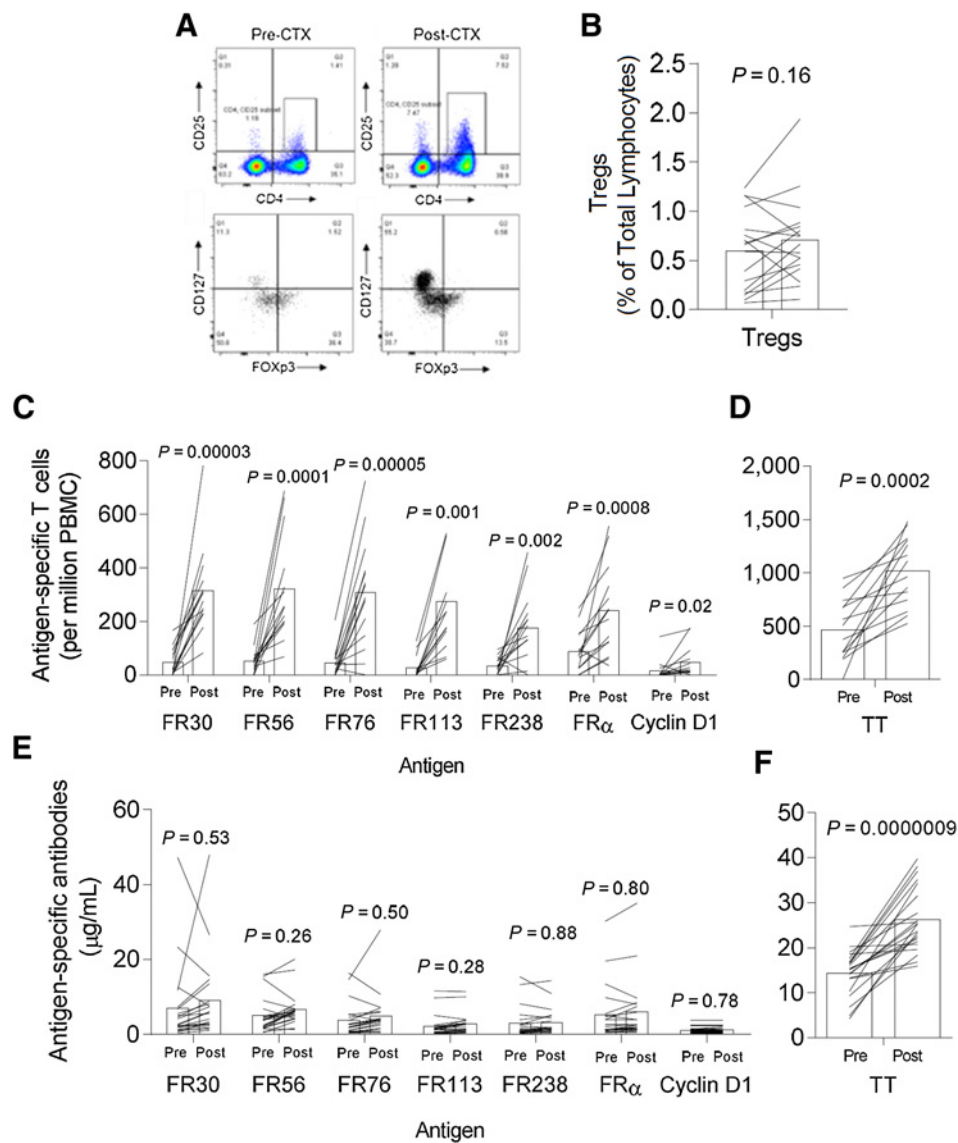


Figure 1. Vaccination generates T-cell but not antibody immunity to FR α following immunization. **A**, Representative set of flow cytometry dot plots demonstrating detection of Tregs using CD4, CD25, FoxP3, and CD127. **B**, Bars show the mean levels of Tregs in 17 evaluable patients measured on time points 0 and 1 month before and after treatment with cyclophosphamide just before vaccination. Each line traces the pre- and post-cyclophosphamide Treg levels for a single unique patient. **C**, Mean ($n = 14$ patients) preimmunization (Pre) and highest postvaccination (Post) frequency of antigen-specific T-cell frequencies (per million PBMC plated) that recognize vaccine antigens, FR30, FR56, FR76, FR113, and FR238. Also shown, frequencies to control cyclin D1 and the FR α protein. Shown are the high postvaccination values for the vaccine period which includes measurements up to 30 days following final injection. **D**, Mean preimmunization (Pre) and highest postvaccination frequency of tetanus toxoid-specific T cells for the same patients in **C**. **E**, Mean preimmunization (Pre) and highest postvaccination (Post) frequency of antigen-specific antibodies ($\mu\text{g/mL}$) that recognize vaccine antigens, FR30, FR56, FR76, FR113, FR238, control cyclin D1, and the FR α protein. **F** shows the mean preimmunization (Pre) and highest postvaccination (Post) frequency of tetanus toxoid-specific antibodies ($\mu\text{g/mL}$) for same patients in **C**. P values shown were calculated using the paired Student t test. For **C-F**, each line traces the pre- and post-antigen-specific T-cell levels for a single unique patient measured during the vaccine period.

not lead to an overall increase in antibodies which could bind the whole FR α protein (5.3 ± 1.6 to 5.9 ± 1.8 ng/mL, $P = 0.8$). Despite the lack of immune responses to the FR α , the mean TT serum antibody concentration increased from 14.4 ± 1.1 to 26.2 ± 1.6 ($P = 0.0000009$), also consistent with tetanus boosters in several of the patients prior to FR α vaccination series (Fig. 1F).

T-cell immunity slowly increases over the vaccine course

The T-cell data was also examined at each individual time point over the course of vaccination, enabling an analysis of the duration to maximal immunity over the vaccine period (Fig. 2A-H). For this analysis, there were samples from 14, 12, 9, 11, 10, 11, and 10 patients for time points 0, 2, 3, 4, 5, 6, and 7, respectively. Time for maximal immunity was estimated

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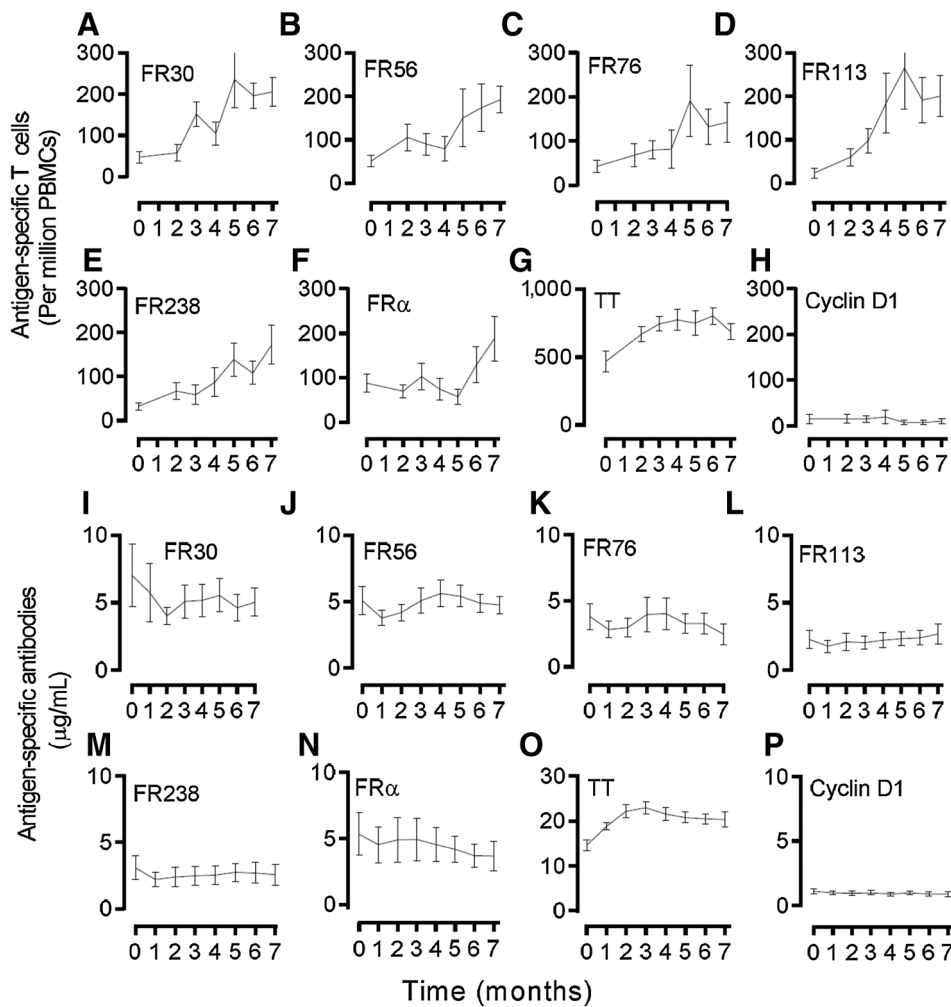


Figure 2. T-cell immunity increases slowly over the vaccine course. Shown in **A-H** are times courses of antigen-specific T-cell frequencies (T cells per million PBMC, mean \pm SEM) for FR30, FR56, FR76, FR113, FR238, FR α protein, TT, and control cyclin D1 peptide, respectively, in 14 evaluable patients. Shown in **I-P** are times courses of antigen-specific antibody frequencies ($\mu\text{g/mL}$, mean \pm SEM) for 21 evaluable patients. For all panels, time 0 months represents the baseline levels before cyclophosphamide treatment. T-cell frequencies were not measured at time point 1 month.

to be 5 months for FR30, 7 months for FR56, 5 months for FR76, 5 months for FR113, and 7 months for FR238 with a median time to maximal immunity at 5 months. Excluding a patient who received only four of the six vaccines did not change the curves or conclusions (data not shown). In contrast, the levels of FR α -specific antibodies tended to remain even over the vaccine period with a slight downward trend observed at month 1 at the time of first vaccine (Fig. 2I-P). TT-specific antibodies increased to a maximum at the time of the second FR α immunization, a rapid response consistent with immunologic memory.

T-cell immunity to FR α is durable

One of the key hallmarks of an efficacious vaccine is the ability to generate durable immunity to prevent disease. To determine whether immunologic memory was achieved, we collected and analyzed blood specimens at 10, 13 and 18 months following start of vaccination (observation period). Similar to that depicted in Fig. 1, we determined the frequency of IFN γ -producing T cells specific for FR α peptides, FR α protein, TT, and control peptide that were compared between pre-treatment samples and multiple post-treatment observation period samples. Assays were done separately from the vaccine period. PBMCs from the observation period were available

from 16 of 21 patients, 11 of which overlapped with samples from the vaccine period.

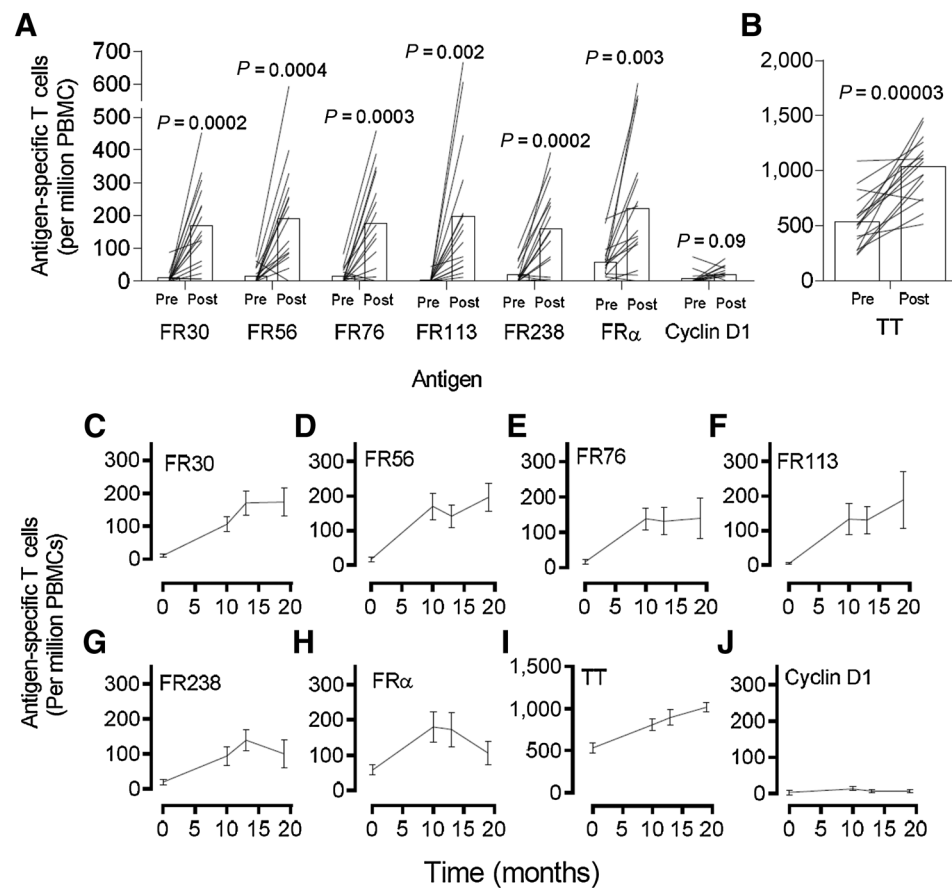
In Fig. 3A, the preimmunization and highest observation period (i.e., not including vaccination period samples) T-cell frequencies are plotted. The median number of observation period samples was 2 (range 2–3) and the maximum was 3. The pre-immunization IFN- γ T-cell frequency to FR30 was 10 ± 5 (\pm SEM, $n = 16$) T cells/million PBMCs compared with the highest observation frequency of 169 ± 32 to T cells/million PBMCs ($P = 0.0002$). To FR56, T cells increased from 16 ± 6 to 191 ± 40 ($P = 0.0004$), to FR76 from 16 ± 6 to 178 ± 38 ($P = 0.0004$), to FR113 from 5 ± 2 to 197 ± 52 ($P = 0.001$), to FR238 from 20 ± 8 to 160 ± 32 ($P = 0.0002$). There was a minor but insignificant increase in the reactivity to the pan-DR binding cyclin D1 peptide from 8 ± 5 to 21 ± 5 ($P = 0.09$). The mean frequency of FR α -specific T cells increased 58 ± 14 to 222 ± 51 T cells ($P = 0.0008$), which indicates that the vaccine is generating persistent T cells that recognize naturally processed antigens. The mean TT T cell frequency increased from 535 ± 62 to 1034 ± 71 TT-specific T cells per million PBMC ($P = 0.00005$; Fig. 3B).

The T cell data was also examined at each individual time point over the course of observation, enabling an analysis of the duration of immunity over the observation period

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Figure 3.

T-cell immunity to the FR α is durable after immunization. **A**, Mean ($n = 16$ patients) preimmunization (Pre) and highest observation (Post) frequency of antigen-specific T-cell frequencies (per million PBMC plated) that recognize vaccine antigens, FR30, FR56, FR76, FR113, and FR238. Also shown are T-cell frequencies to control cyclin D1 and the FR α protein. Shown are the high postvaccination values for the observation period which includes measurements at months 10, 13, and 18. **B**, Mean preimmunization (Pre) and highest postvaccination frequency of tetanus toxoid-specific T cells for same patients in **A**. **C–J** are time courses of antigen-specific T-cell frequencies (T cells per million PBMC, mean \pm SEM) for FR30, FR56, FR76, FR113, FR238, FR α protein, TT, and control cyclin D1 peptide, respectively, in 16 evaluable patients. P values shown were calculated using the paired Student t test. For panels **A** and **B**, each line traces the pre- and highest observation period (post) antigen-specific T-cell levels for a single unique patient measured during the vaccine period.



(Fig. 3C–J). For this analysis, there were samples from 16 patients at each of the time point. As shown, FR α -peptide specific T-cell immunity appeared consistently elevated during the observation period, demonstrating immunologic memory.

All the peptides in the vaccine appear to be immunogenic in most patients

To determine the relative immunogenicity of the vaccine component we analyzed the percentage of patients that responded (either in the vaccine or observation period, $n = 19$ patients) to each of the individual peptides based on having a 3 fold increased T cell frequency over baseline. In case of zero baselines, a minimum frequency of 50 antigen-specific T cells was the cutoff for response. T-cell immunity to all of the peptides was frequently observed with 79% to FR30, 74% to each of FR56, FR76, and FR113, and 58% to FR238 (Fig. 4A). Antibody response rates were minimal at 14% for FR30, 14% for FR56, 5% for each of FR76 and FR113, and 10% for FR238 (Fig. 4B). All patients in this analysis responded to vaccine with a T-cell response with the majority (17/19) of patients responding to multiple epitopes (Fig. 4C). The median number of epitopes that patients demonstrated T-cell immunity was 3. Six of 19 patients responded with antibody responses for a median of 0 epitopes to which there was antibody responses. On the basis of the more rigid protocol criteria for advancing the vaccine (Materials and Methods) 19 of 21 (~91%) patients demonstrated an antigen-specific response (either antibody or T-cell response) in the vaccine period.

Tumor expression of FR α

FR α protein expression was observed on pre-enrollment archived tumor specimens from 12 of 12 assessable ovarian cancer patients and 0 of 8 breast cancer patients (Table 2). Two ovarian cancer tissue samples did not contain evaluable tumor. Finally, the T-cell response to vaccine peptides did not appear to correlate with FR α positivity or negativity in the primary tumor (FR30, $P = 0.36$; FR56, $P = 0.31$; FR76, $P = 0.73$; FR113, $P = 0.69$; FR238, 0.82).

Clinical outcomes

Although the trial was not designed to evaluate clinical outcomes, all patients remained alive at last follow-up, at least two years following initiation of immunizations. Ovarian cancer patients in first remission demonstrated a median of relapse-free survival of 528 days, whereas median survival was not reached for the ovarian cancer patients in second remission and the breast cancer patients.

Discussion

There were two main goals of this phase I clinical trial using a multi-epitope FR α peptide vaccine. The first was to assess the safety, since this approach was the first-in-human trial of the FR α vaccine and second was to assess the ability of this vaccine to generate T-cell and antibody immunity against FR α . With respect to the first goal, the vaccine immunizations was found to be uniformly safe overall and associated only with mild to

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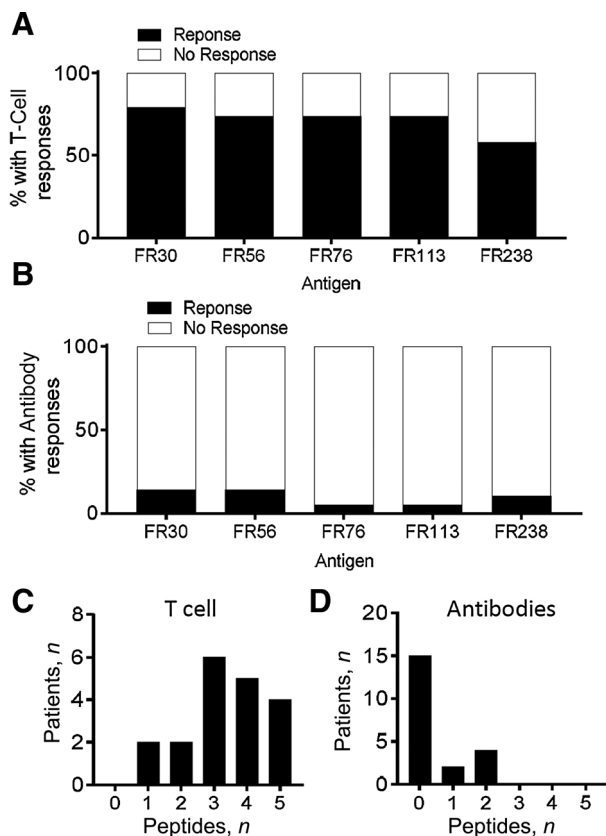


Figure 4.

All the vaccine epitopes are immunogenic in most patients. **A** and **B** show the percent of patients who responded to vaccine epitopes with either T-cell responses (**A**) or antibody responses (**B**). **C** and **D**, The distribution of T-cell and antibody responses to the individual epitopes.

moderate toxicities, despite the fact that some normal healthy tissues, notably the kidney and the choroid plexus, express FR α (10, 37). One potential reason for the reasonable safety profile is that the vaccine targets CD4 T cells whose activity is regulated by HLA class II which is typically only expressed on hematopoietic cells. As normal tissues apparently shed very little FR α relative to cancer tissues, it is likely that there is very little presentation of FR α in normal healthy tissue for the vaccine-generated T cells to respond to (38). The trial enrolled adults who had previously been diagnosed with either breast or ovarian cancer and who had completed curative intent treatment (surgery, chemotherapy). Participants were allowed to continue concurrent hormonal therapy, if indicated. This might explain the musculoskeletal-related side effect profile (myalgias, arthralgias) seen in some of the breast cancer patients, since all breast cancer patients enrolled in the study were noted to be hormone positive (ER/PR positive, HER2/neu negative; ref. 39). This biased enrollment was secondary to competing studies at our institution for patients with hormone negative and HER2/neu-positive breast cancers, respectively.

Contradictory to what was expected and what was seen in other studies using metronomic cyclophosphamide (28), Tregs did not decrease consistently with exposure to cyclophosphamide. In some patients Tregs actually increased for

unclear reasons despite a cumulative cyclophosphamide dose of 1,400 mg. Comparisons with previously published values are complicated by the fact that Tregs have been quantitated and reported in literature in numerous ways. The method reported here (CD127^{lo}/FoxP3⁺ cells within the CD4⁺/CD25⁺ gate) is more rigorous than studies using fewer markers (40). Assessment was done one week after the last cyclophosphamide dose; other studies have reported on continuing cyclophosphamide treatment until the administration of vaccine. It is likely that the variation in Tregs levels is quite high and that the relatively minor increases or decreases observed reflect normal variation. In addition, many studies in the literature assess cyclophosphamide effects on Tregs in patients with advanced metastatic disease, whereas this clinical trial enrolled patients who did not have any evidence of disease at the time of treatment with cyclophosphamide. Levels and function of peripheral Tregs may differ with disease stage, noting our finding that the presently described patient population did not have elevated Tregs relative to control.

ELISpots were utilized to estimate peptide antigen-specific T-cell responses to the 5 FR α peptides. Most patients responded to at least 3 of the 5 peptides resulting in the generation of immunity able to recognize naturally processed antigen. However, despite having established a cutoff, it is notable that it was an arbitrary cutoff because at present there is no way to know what levels of antigen-specific T cells are required for disease protection. The high frequency of responses observed is consistent with the degeneracy of the epitopes with respect to predicted HLA-DR binding (13). Antigen-specific T-cell responses during the observation period were relatively similar to those observed during active priming with the FR α vaccine. Despite the high prevalence of immunity to the FR α , it is important to note that the assay used was optimized to detect immunity but was not validated to the extent to provide an assured accurate assessment of the frequency of T cells, including rigorous control of all of the constituent assay components. Future implementation of the vaccine in clinical trials, thus, needs to be accompanied by validation programs to better depict the longitudinal increase in antigen-specific immune effectors (41, 42). Additional analyses could also include cell separations to specifically determine whether CD4 T cells or the primary responding population or whether CD8 T cells are also activated as a result of exposure to class I binding peptides embedded within the longer vaccine peptides as we have previously suggested in earlier studies (13).

Assessment of antibody responses revealed that some, albeit a small number, of patients demonstrated a vaccine-induced antibody immune response to one or more of the peptides. However, there were no responses to intact FR α protein, which is the only response that would be expected to have clinical relevance. It is not currently known whether directing both cellular and humoral immune responses against FR α are necessary for efficacy. In some situations (e.g., HER2/neu-positive breast cancer), antibody responses appear to be helpful (43). However, anti-FR α -based antibody treatment strategies did not show great responses in clinical trials (44).

Given the enrollment of several patient populations and a small total number of subjects, this trial cannot assess the clinical efficacy of the vaccine, nor was it designed for that purpose. While it is noteworthy that the ovarian cancer patients that were in first remission demonstrated a median progression-free survival of

528 days following the initiation of the immunization and that all patients are still alive, a carefully controlled clinical trial in which patients are enrolled within a defined period following conventional treatment should enable a better understanding of the role of vaccine in delaying or preventing disease recurrence. Alternatively, there are other possible uses of vaccines in the course of a patient's treatment. One potential example is combination with checkpoint blockade, a setting in which we and others have demonstrated synergistic activity in murine models with respect to tumor regression and lasting protection from disease relapse (45, 46).

Identified over three decades ago, GM-CSF has been widely used as a vaccine adjuvant with notable immune promoting properties including increasing cytotoxic T cell activity, upregulation of co-stimulatory molecules (e.g., CD80 and CD86), and increasing the migratory and antigen-presenting properties of antigen-presenting cells (reviewed in refs. 47, 48). In our study, we chose to use GM-CSF as the vaccine adjuvant primarily due to (i) our past successful experience with the cytokine in human trials, (ii) its outstanding safety profile, and (iii) its association with clinically effective peptide vaccination strategies (49–54). Thorough examination of GM-CSF as a vaccine adjuvant has demonstrated that its use is not straightforward and under certain conditions, such as concurrent delivery with montanide, the cytokine may actually be immune suppressive and detrimental to the generation of immunity (55). Other adjuvants have recently emerged as alternatives to GM-CSF and other commonly used adjuvants (e.g., montanide), including poly-ICLC, STING agonists, CpG ODN, and even checkpoint modulators such as anti-PD-1 (45, 56–58). Future clinical testing should reveal whether these newer vaccine adjuvants demonstrate superior activity relative to GM-CSF.

Tumor expression of FR α was not an eligibility requirement for this trial. This decision was made based on the high proportion of patients with serous ovarian cancer whose tumors express FR α , the observation that breast cancers noted to be initially negative for FR α expression may develop FR α expression at the time of recurrence. In the current cohort, 12 of 12 (100%) evaluable ovarian cancer patients and 0 out of 8 breast cancer patients were noted to have FR α expression in their tumor samples. These results are comparable with previous studies of the expression of FR α in these tumors (5). FR α expression in hormone-positive breast cancer is estimated to be around 20%, with higher reported rates in hormone negative (triple negative) breast cancers (59). Whether tumor expression of FR α should be an inclusion criterion in future studies is debatable, as there is rationale to vaccinating against antigens that are not expressed by the tumor at disease onset but are expressed at the time of recurrence.

In summary, this first-in-human phase I study targeting FR α with a multi-peptide vaccine after priming with metronomic cyclophosphamide showed minimal untoward and no severe

unanticipated toxicities. Projected clinical efficacy of vaccine-induced antitumor immunity cannot easily be derived from the data but supports continued development of the vaccine perhaps as a means to prevent recurrence or to be used in combination with immune checkpoint blockade (45).

Disclosure of Potential Conflicts of Interest

K.R. Kalli is listed as a co-inventor on a patent on immunity to folate receptors, which is owned by the Mayo Clinic and licensed to TapImmune, Inc. M.S. Block reports receiving commercial research support from TapImmune, Inc. G. Wilson is an employee of and has ownership interests (including patents) in TapImmune, Inc. K.L. Knutson has ownership interests (including patents) in and is a consultant/advisory board member for TapImmune, Inc. No potential conflicts of interest were disclosed by the other authors.

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