Safety and Preliminary Evidence of Biologic Efficacy of a Mammaglobin-A DNA Vaccine in Patients with Stable Metastatic Breast Cancer

Venkataswarup Tiriveedhi1,2, Natalia Tucker1, John Herndon1, Lijin Li1, Mark Sturmoski1, Matthew Ellis3,4, Cynthia Ma3,4, Michael Naughton3,4, A. Craig Lockhart3,4, Feng Gao3,4, Timothy Fleming1,4, Peter Goedegebuure1,4, Thalachallour Mohanakumar1,4,6, and William E. Gillanders1,4

Abstract

Purpose: Mammaglobin-A (MAM-A) is overexpressed in 40% to 80% of primary breast cancers. We initiated a phase I clinical trial of a MAM-A DNA vaccine to evaluate its safety and biologic efficacy.

Experimental Design: Patients with breast cancer with stable metastatic disease were eligible for enrollment. Safety was monitored with clinical and laboratory assessments. The CD8 T-cell response was measured by ELISPOT, flow cytometry, and cytotoxicity assays. Progression-free survival (PFS) was described using the Kaplan–Meier product limit estimator.

Results: Fourteen subjects have been treated with the MAM-A DNA vaccine and no significant adverse events have been observed. Eight of 14 subjects were HLA-A2+, and the CD8 T-cell response to vaccination was studied in detail. Flow cytometry demonstrated a significant increase in the frequency of MAM-A–specific CD8 T cells after vaccination (0.9% ± 0.5% vs. 3.8% ± 1.2%; P < 0.001), and ELISPOT analysis demonstrated an increase in the number of MAM-A–specific IFNγ-secreting T cells (41 ± 32 vs. 215 ± 67 spm; P < 0.001). Although this study was not powered to evaluate progression-free survival (PFS), preliminary evidence suggests that subjects treated with the MAM-A DNA vaccine had improved PFS compared with subjects who met all eligibility criteria, were enrolled in the trial, but were not vaccinated because of HLA phenotype.

Conclusion: The MAM-A DNA vaccine is safe, capable of eliciting MAM-A–specific CD8 T-cell responses, and preliminary evidence suggests improved PFS. Additional studies are required to define the potential of the MAM-A DNA vaccine for breast cancer prevention and/or therapy. Clin Cancer Res; 20(23); 5964–75. ©2014 AACR.

Introduction

Progress in basic and translational immunology has confirmed the importance of the immune system in cancer prevention and has renewed interest in vaccine therapy for cancer (1). DNA vaccines are safe, well tolerated, and can typically be given in an ambulatory facility (2). Although breast cancer is commonly thought to be less immunogenic than melanoma or renal cell cancer, there is increasing evidence of a crosstalk between the immune system and breast cancer, and this crosstalk strongly suggests that successful development of a breast cancer vaccine could have a clinical impact. Evidence of this crosstalk includes the clinical significance of immune infiltrates in breast cancer (3), clear evidence of preexisting immune responses to several breast cancer antigens including HER2/neu (4–6), MUC1 (7), and MAM-A (8–10), the increased prevalence of regulatory T cells in breast cancer patients (11), and upregulation of inhibitory molecules of the CD28 receptor family on breast cancer–specific T cells (12). Most patients with breast cancer are diagnosed with local–regional disease and typically have no evidence of disease after standard treatment modalities, providing a window-of-opportunity to generate effective antitumor immune responses to prevent recurrent disease (13). Peoples and colleagues recently confirmed the potential of breast cancer vaccine therapy in patients with stable metastatic breast cancer (1).
this clinical context, demonstrating that administration of a HER2/neu peptide vaccine was associated with a survival advantage in a prospective study of patients with node-negative breast cancer with no evidence of disease (14). Taken together, the dynamic interaction between breast cancer and the immune system and preliminary evidence of the efficacy of first-generation breast cancer vaccines provide strong rationale for the clinical evaluation of breast cancer vaccine strategies.

MAM-A was first identified using a differential screening approach directed at the isolation of novel human breast cancer–associated genes (15). MGBA encodes MAM-A, a 10-kDa glycoprotein that is related to a family of epithelial secretory proteins. Of note, MAM-A has several unique properties that make it an exceptional target for breast cancer vaccine therapy. First, MAM-A is expressed almost exclusively in breast cancer (16–19). Second, MAM-A is overexpressed in 40% to 80% of primary breast cancers (20–24). Overexpression of MAM-A in a significant percentage of breast cancer suggests that many patients with breast cancer are likely to be candidates for vaccine therapy and is particularly relevant for the development of vaccine strategies for the prevention of breast cancer. Third, MAM-A overexpression is evident in noninvasive, invasive, and metastatic breast cancer (21). This consistency of expression in breast cancers confirms that MAM-A is an attractive target for vaccine therapy. Finally, we have demonstrated that MAM-A is capable of eliciting an immune response in patients with breast cancer (8, 9, 25, 26), and that a DNA vaccine targeting MAM-A is capable of successfully generating breast cancer immunity in preclinical models (10).

The observation that direct administration of recombinant DNA can generate potent immune responses in rodents established the field of DNA vaccines in the early 1990s (27). Since that time, DNA vaccines have remained an area of intense research interest, and vaccines targeting infectious disease and cancer have progressed into clinical trials. DNA vaccination offers several potential advantages. First, the presence of the full-length cDNA provides multiple potential epitopes, thus avoiding the need for patient selection based on MHC restriction. Second, bacterial plasmid DNA contains immunostimulatory unmethylated CpG motifs that may act as potent immune adjuvants (28, 29). Finally, DNA vaccines are relatively easy to prepare with high purity and high stability relative to proteins and other biologic agents, facilitating clinical translation of this platform, particularly in early-phase clinical trials. In clinical trials of infectious disease and cancer, DNA vaccine strategies have been shown to be safe and effective in developing immune responses to malaria (30), HIV (31), and prostate cancer (32). Several reviews have recently been published summarizing progress in the field (2, 33, 34).

To explore the potential of a novel MAM-A DNA vaccine, we initiated an open-label phase I clinical trial in patients with metastatic breast cancer. Patients with stable metastatic disease were eligible for enrollment. Subjects were treated with three intramuscular injections of 4 mg plasmid at one-month intervals using an FDA-approved carbon dioxide–powered jet delivery device. Safety was closely monitored with eight or more clinical and laboratory assessments in the first 24 weeks of the trial. The immune response to the vaccine was measured by ELISPOT analysis, multiparameter flow cytometry, and cytotoxicity assays. Evidence of biologic efficacy was assessed by measuring progression-free survival (PFS).

Materials and Methods

Study rationale and objectives

We recently initiated a single-institution, open-label, phase I clinical trial of a novel MAM-A DNA vaccine in patients with breast cancer with stable metastatic disease (ClinicalTrials.gov identifier: NCT00807781). The primary objective of the trial was to evaluate the safety of the MAM-A DNA vaccine. Secondary and exploratory objectives included measuring the CD8 T-cell response to the vaccine and the impact on PFS. The MAM-A DNA vaccine is composed of a closed circular DNA plasmid based on the pING parental vector and is designed to express the human MAM-A breast cancer–associated antigen under a strong viral promoter (Fig. 1).

Study participants

The phase I clinical trial was approved by the Siteman Cancer Center Protocol Review and Monitoring Committee and the Washington University School of Medicine Human Studies Committee. A written informed consent was obtained from all subjects before enrollment/participation in the study. Men or women 18 years or older with metastatic breast cancer were eligible for enrollment. Eligible subjects had metastatic breast cancer that had been stable for at least 30 days after the last dose of chemotherapy, or that had been stable for at least 30 days on endocrine therapy, documented adequate organ and marrow function, ECOG status of less than or equal to 2, and negative urine or serum b-HCG pregnancy test for women of reproductive age. Subjects were considered ineligible if they had received an investigational drug within 30 days of

Translational Relevance

MAM-A is an important breast cancer–associated antigen with exquisite tissue specificity. We initiated a phase I clinical trial to evaluate the safety and efficacy of a MAM-A DNA vaccine. Patients with breast cancer with stable metastatic disease were eligible. We demonstrate that (i) the MAM-A DNA vaccine is safe, with no significant adverse events observed, (ii) there is a significant increase in the frequency and number of MAM-A–specific CD8 T cells after vaccination, and (iii) preliminary evidence suggests improved progression-free survival. Additional studies are required to define the clinical potential of a MAM-A DNA vaccine for breast cancer treatment or prevention.
enrollment, had known brain metastases, known allergy, or history of serious adverse reaction to vaccines, autoimmune disease requiring management with immunosuppression, or history of failing greater than two chemotherapy regimens for metastatic disease. After enrollment, the primary breast cancers of the subjects were evaluated by immunohistochemistry (IHC) for MAM-A expression (only subjects with MAM-A+ cancers were eligible for vaccination), and their HLA phenotype was determined (initially, only subjects with HLA-A2 and/or HLA-A3 were eligible for vaccination to facilitate immune monitoring as immunodominant epitopes have been identified for these alleles; refs. 8–10). Screen failures were not vaccinated, but were followed for progression of disease. Subjects who had screen failures based on HLA phenotype, withdrawal of consent, and inability to perform MAM-A immunohistochemical staining were considered to be appropriate for inclusion in a comparator group.

Figure 1. The MAM-A DNA vaccine is a plasmid DNA vaccine designed to drive MAM-A expression with a strong viral promoter. The pING parental vector contains the following elements: (i) a eukaryotic promoter and enhancer from the Towne strain of CMV; (ii) a polylinker region to facilitate cloning; (iii) donor and acceptor splice sites and a poly adenylation signal sequence derived from the bovine growth hormone gene; (iv) the ColE1 origin of replication and (v) a gene conferring kanamycin resistance.
Vaccination schedule

Study subjects were vaccinated with intramuscular injections of 4 mg plasmid DNA vaccine at day 1 (week 1), day 29 ± 7 (week 4), and day 57 ± 7 (week 8) with at least 21 days between vaccinations. All vaccinations were given intramuscularly using an FDA-approved carbon dioxide–powered jet delivery device (Needle Free Biojector 2000).

Safety monitoring and study procedures

Safety was closely monitored after vaccination with eight or more clinical and laboratory assessments in the first 24 weeks of the trial. After each vaccination, study subjects were carefully observed for 60 minutes. Vital signs were monitored and adverse events were recorded. The injection site was inspected for evidence of a local reaction. Subjects were given a “Diary Card” where they recorded temperature and symptoms daily for 5 days. Laboratory tests performed on the day of vaccination included complete blood count, comprehensive metabolic panel, and serum or urine pregnancy test (in women of childbearing potential). Patients were followed actively for 52 weeks after vaccination, and then until disease progression or death, whichever occurred first. Toxicity was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0. Subjects had standard-of-care clinical assessments and imaging studies (CT scans) during follow-up to assess tumor stability.

Peripheral blood specimens were obtained for immune monitoring and other correlative studies before vaccination and at serial time points after vaccination. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll–Hypaque density gradient centrifugation (Pharmacia) and stored at −135°C until evaluation. The CD8 T cells were isolated from PBMC by positive selection using a MACS Bead Isolation Kit (Miltenyi Biotec Inc.; ref. 35).

Breast cancer cell lines and cell culture

The breast cancer cell lines, UIACC-812 (MAM-A+/HLA-A2+), MCF-7 (MAM-A+/HLA-A2+), MDA-MB-415 (MAM-A+/HLA-A2+), and MDA-MB-134 (MAM-A+/HLA-A2+), were obtained from the ATCC. The cell lines were ordered in 2004 by Dr. Mohanakumar’s laboratory. No specific authentication of the cell lines was performed in our laboratory. All experiments were performed on the cell lines below 30th passage. Breast cancer cell lines were cultured in RPMI1640 culture medium at 37°C in 5% CO2 incubator until they were 80% confluent. The presence of MAM-A in the cell lines was confirmed by reverse transcriptase-PCR (Supplementary Fig. S1) and Western blot analysis (data not shown). siRNA oligonucleotides and monoclonal antibodies used in the cell culture inhibition studies were obtained from Santa Cruz Biotechnology.

ELISPOT assay

PBMCs were cultured overnight in complete RPMI1640 and viability was determined by Trypan blue exclusion. PBMC with viability of at least 90% were used for ELISPOT analysis (10, 36). CD8 T cells were enriched by MACS bead negative selection using immunomagnetic separation cocktails (Stem Cell Technologies). Purified CD8 T cells (2 × 10^5 cells, >90% purity) were cultured in triplicate with MAM-A2.1 (LIYDSSLCDL, 20 μg/mL) in 96-well ELISPOT plates (Multiscreen IP plate) precoated with IFNy monoclonal antibody (mAb: 4 μg/mL) in the presence of autologous-irradiated T-cell–depleted PBMCs (3 × 10^4) for 48 to 72 hours in humidified 5% CO2 at 37°C. Subsequently, the plates were washed and developed to detect the number of spots in individual wells using an ImmunoSpot analyzer (Cellular Technology). CD8 T cells plus autologous irradiated T-cell–depleted PBMCs cultured in medium without MAM-A2.1 peptide were used as a negative control, whereas CD8 T cells plus autologous irradiated T-cell–depleted PBMCs cultured with phytohemagglutinin (5 μg/mL) were used as a positive control. The number of spots in control wells was subtracted from the number of spots in experimental wells and reported in the final results as spots per million cells (spm ± SEM).

Flow cytometry

We previously demonstrated that LIYDSSLCDL, designated MAM-A2.1, is an immunodominant HLA-A2–restricted epitope (10, 36). MAM-A2.1 tetramers were developed by Beckman Coulter Immunomics to monitor the MAM-A–specific CD8 T-cell response after MAM-A DNA vaccination. An HLA-A2 tetramer incorporating an unrelated peptide from influenza (Flu), GILGFVFTL, was also prepared and used as a control. Tetramers were used to stain target cells at a concentration of 10 μL per 200 μL final volume of CD8 T cells (1 × 10^6 CD8 T cells/mL). Antibodies used for flow cytometry included CD8-FITC (BD Biosciences), MAM-A2.1/Tetramer-PE, and Flu-peptide/Tetramer-PE. Samples were analyzed using a FACSCalibur/LSRII flow cytometer (Becton Dickinson), and cell sorting was performed using a Vantage cell sorter (Becton Dickinson). Data were analyzed using BD FACSDiva software. Gates were set according to isotype controls.

Cytotoxicity assay

The ability of MAM-A–specific CD8 T cells to lyse breast cancer cell lines was determined using an LDH cytotoxicity assay (Promega). Breast cancer cells (5 × 10^5 cells) in 100 μL of complete medium were plated in triplicate cultures in round bottom 96-well plates in the presence of varying numbers of CD8 T cells (E:T ratios of 6.25:1 to 50:1) and incubated at 37°C in a humidified 5% CO2 incubator for 4 hours. Controls were breast cancer target cells alone or CD8 T cells isolated from normal subjects. Maximum release was determined by adding Triton X-100 (1%) to the target cells. A colorimetric measurement of the released LDH was developed as per manufacturer’s instructions and measured by spectrophotometer at 450 nm. The percent-specific lysis was calculated using the formula [(experimental LDH release − spontaneous LDH release)/(maximum LDH release − spontaneous LDH release)] × 100.
Western blot analysis

Total proteins were extracted from cells with lysis buffer [50 mmol/L HEPES (pH 7.6), 150 mmol/L NaCl, 1% Triton X-100, 30 mmol/L Na₂PO₄, 10% glycerol, 1 mmol/L benzamidine, 1 mmol/L diethiothreitol, 10 μg of leupeptin/mL, 1 mmol/L phenylmethylsulfonfonyl fluoride, 50 mmol/L NaF, 1 mmol/L sodium orthovanadate, 10 mmol/L sodium pyrophosphate decahydrate, and 10 mmol/L β-glycerophosphate (Sigma Aldrich)]. After cell lysis, the supernatant was collected after centrifugation at 15,000 × g for 15 minutes at 4°C (37). Protein concentration was determined with a Bradford Assay Kit from Bio-Rad. Total proteins were separated on a 4%–12% SDS-polyacrylamide gradient gel and transferred onto a nitrocellulose membrane. The membranes were blocked overnight at 4°C in Tris-buffered saline with 0.05% Tween 20 (5% nonfat milk in 10 mmol/L Tris-HCl, 100 mmol/L NaCl, 0.1% Tween 20, pH 7.6). The membranes were incubated first with primary antibodies diluted at 1:1,000 in blocking buffer at room temperature for 2 hours and then with a horseradish peroxide–conjugated secondary IgG mAb diluted at 1:5,000 for 1 hour. All primary and secondary antibodies were obtained from Santa Cruz Biotechnology. The membrane was developed using a chemiluminescence kit (Millipore) and analyzed using Bio-Rad Universal Hood II. Densitometric analysis was done using the software provided by Bio-Rad.

Gene expression analysis

Relative expression of HLA-A2, MAM-A, NKG2D, DAP-10, perforin, and actin were analyzed using 6-carboxyfluorescein–labeled quantitative real-time reverse transcription PCR primers (Applied Biosystems) as per the manufacturer’s recommendations (25). Briefly, total RNA was extracted from 1 × 10⁶ cells using TRIzol reagent (Sigma Aldrich). RNA samples were quantified by absorbance at 260 nm. The RNA was reverse-transcribed and RT-PCR was performed in a final reaction volume of 20 μL using iCycler 480 Probes Master (Roche Diagnostics). Each sample was analyzed in triplicate. Cycling conditions were set as an initial denaturation step at 95°C for 15 minutes, followed by 40 cycles at 95°C for 30 seconds, followed by 61°C for 1 minute.

Statistical analysis

As a phase I study, the data analysis was primarily descriptive in nature. The differences in CD8 T-cell frequencies over time (pre-versus postvaccination) were compared using two-way ANOVA for repeated measurement data or Friedman rank-sum test as appropriate, and results were expressed in mean ± SEM. Correlation analysis was performed using Spearman rank test. PFS of the vaccinated patients was described using Kaplan–Meier product limit estimator and compared with a subset of screen failures who were considered to be appropriate for comparative analysis (HLA phenotype) by log-rank test. PFS was calculated on the basis of the date of study registration. The distributions of demographic and clinical characteristics between two groups were also compared by two-sample t test or Fisher exact test as appropriate. All analyses were two-sided and significance was set at a P value of 0.05. Statistical analyses were performed using statistical packages SAS 9.2 (SAS Institute).

Results

Study subjects and vaccine safety

A total of 53 subjects were consented and enrolled in phase 1 clinical trial between December 2009 and May 2013. Following enrollment, the HLA phenotypes of the subjects were determined and breast cancer specimens were evaluated for MAM-A protein expression. Fourteen subjects met all eligibility criteria and were vaccinated. Thirty-nine subjects were screen failures. Ten of the 14 vaccinated subjects were followed for at least 52 weeks and detailed immune monitoring was completed in 8 of the 14 vaccinated subjects. The remaining 6 vaccinated subjects were not HLA A2+, and detailed immune monitoring is ongoing in these patients, pending validation of epitopes and reagents for the specific HLA alleles expressed by these subjects. Data from all 14 vaccinated subjects are included in the safety analyses and in the analyses of PFS.

Of the 39 screen failures, 9 were excluded based on HLA phenotype, 2 were excluded because of withdrawal of consent, and one was excluded because of inability to perform immunohistochemical staining for MAM-A expression. These 12 subjects met all other inclusion/exclusion criteria and were considered suitable for comparative analysis. The 27 screen failures that were not considered suitable for comparative analysis were excluded because their breast cancers were MAM-A–negative (8/27), insurance denial (6/27), disease progression before completion of screening studies (4/27), lost to follow up (4/27), or ineligibility secondary to failing more than two prestudy chemotherapy regimens, dialysis, locally advanced disease, noncompliance or concurrent trastuzumab therapy (1 each). Of the vaccinated subjects, the median age was 51 years. Ninety-three percent were female, 86% were white, and 100% were ER+.

All subjects had received endocrine therapy and the majority had bone-only metastatic disease (57%; Table 1). Similar demographics were noted in the comparative analysis screen-failure group. None of the baseline demographic or clinical characteristics were significantly different between the vaccinated and comparative analysis screen failure groups. Of note, screen failure and vaccinated patients received similar therapies after enrollment (Supplementary Fig. S6).

In the current study, positive MAM-A expression was an inclusion criteria. MAM-A expression was quantified using the Allred scoring system (38), which is a composite score based on the proportion of cells staining positive, and the staining intensity. MAM-A IHC was performed on 36 subjects, as some subjects were identified as screen failures. The average Allred score for the 36 subjects was 3.9 (n = 36, includes screen failures). Twenty-four of 36 subjects (66%) had positive MAM-A expression (defined as Allred score ≥3). The average proportion score for the 36 subjects was 2.4 (n = 36, includes screen failures).
Vaccine safety was closely monitored following vaccination with eight or more clinical and laboratory assessments in the first 24 weeks of the trial. The most common grade 1 toxicity was malaise/flu-like symptoms (4/14). Other grade 1 toxicities potentially attributable to vaccination included vaccine site tenderness (1/14), rash (1/14), and precipitation of a shingles episode (designated as “infection”; 1/14). A shingles episode treated with Valtrex (also designated as “infection”) was the only grade 2 toxicity (1/14). There were no grade 3 or 4 toxicities reported (Table 2).

Mammaglobin-A DNA vaccination elicits MAM-A2.1–specific CD8 T cells

We previously demonstrated that LIYDSSLCDL, designated MAM-A2.1, is an immunodominant HLA-A2–restricted epitope derived from MAM-A (10, 36). In preclinical studies, we observed that MAM-A2.1–specific CD8 T cells expanded following MAM-A DNA vaccination of HLA-A2 transgenic mice (36). In the current study, we performed tetramer and ELISPOT analyses to measure the MAM-A2.1–specific CD8 T-cell response to vaccination in HLA-A2+ subjects. The frequency of CD8+ MAM-A2.1+ T cells significantly increased following vaccination (0.9% ± 0.5% vs. 3.8% ± 1.2%; P < 0.001; Fig. 2A, top), with longitudinal analysis demonstrating significant responses in 6 of 8 subjects. Two subjects (023 and 030) did not show a significant increase in the frequency of CD8+ MAM-A2.1+ T cells. Of note, subject 023 had a significant frequency of CD8+ MAM-A2.1+ T cells before vaccination that increased marginally after vaccination (2.8% prevaccination vs. 3.4% at 12 months), whereas subject 030 had a low frequency of CD8+ MAM-A2.1+ T cells before vaccination that marginally increased after vaccination

<table>
<thead>
<tr>
<th>Table 1. Patient demographics and baseline characteristics</th>
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<tr>
<td><strong>Baseline characteristics</strong></td>
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<td>Gender, n (%)</td>
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<tr>
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<td>Female</td>
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<td>Race, n (%)</td>
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<td>Biomarker profile, n (%)</td>
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<td>Her-2+</td>
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<td>Prior therapy, n (%)</td>
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<td>Surgery</td>
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Table 2. Adverse events

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<td>Rash</td>
<td>1 (7.1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Infection</td>
<td>1 (7.1)</td>
<td>1 (7.1)</td>
<td>0</td>
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vaccination (0.7% vs. 1.6% at 12 months). ELISPOT analyses demonstrated that the number of MAM-A2.1–specific CD8 T cells capable of IFNγ secretion increased significantly after vaccination in 6 of 8 subjects (41 ± 32 vs. 215 ± 67 spm CD8 T cells; P < 0.001; Fig. 2B, bottom). Consistent with the tetramer analyses, no significant change in the frequency of IFNγ+ CD8 T cells was noted in subjects 023 (241 vs. 277 spm) or 030 (18 vs. 41 spm). Taken together, these data suggest that MAM-A DNA vaccination is capable of inducing potent MAM-A–specific CD8 T-cell responses in patients with breast cancer.

**MAM-A–specific CD8 T cells induced after vaccination are cytotoxic**

To assess whether vaccine-induced MAM-A–specific CD8 T cells are capable of lysing MAM-A+ breast cancers, we performed *in vitro* cytotoxicity assays against breast cancer cell lines. Purified CD8 T cells from responders were tested against a panel of breast cancer cell lines including UACC-812 (MAM-A+/HLA-A2+), MCF-7 (MAM-A+/HLA-A2+), MDA-415 (MAM-A+/HLA-A2+), and MDA-134 (MAM-A+/HLA-A2+). MAM-A and HLA-A2 expression was confirmed in breast cancer cell lines by RT-PCR (Supplementary Fig. S1). CD8 T cells purified from PBMCs of responders at 12 months after vaccination were able to lyse MAM-A+/HLA-A2+ UACC-812 breast cancer cells (44.3 ± 13.9% at 50:1 E/T ratio), but not control breast cancer lines (MCF-7, 2.2 ± 0.8%; MDA-415, 1.8 ± 0.6% or MDA-134, 1.5 ± 0.4%; Fig. 3A). These studies confirm the MAM-A–specific cytotoxicity, and MHC class I restriction to HLA-A2, of the MAM-A DNA vaccine-induced CD8 T cells.

**IFNγ and TNFα contribute to the cytolytic activity of MAM-A–specific CD8 T cells**

IFNγ and TNFα have been shown to contribute to the cytolytic activity of antigen-specific CD8 T cells (39). To determine the impact of MAM-A DNA vaccination on TNFα expression in MAM-A–specific CD8 T cells, purified CD8 T cells from vaccinated subjects were cocultured with UACC-812 breast cancer cells, and TNFα expression was determined by RT-PCR and immunoblot analysis. Within the sensitivity of our detection system, we were not able to perform ELISA-based approach to TNFα detection. TNFα expression increased 7.8-fold after DNA vaccination (relative expression compared with actin 2.2 ± 0.6 vs. 17.1 ± 3.7; Fig. 3B). Similar results were observed when TNFα expression was assessed by immunoblot (Supplementary Fig. S2). Both IFNγ and TNFα contribute to the cytolytic activity of MAM-A–specific CD8 T cells as the ability to lyse UACC-812 cells was significantly reduced following incubation with mAb specific for IFNγ (16.6% ± 7.1% vs. 43% ± 15% for isotype control), TNFα (14.3% ± 8.5%), or both (2.4% ± 1.1%; Fig. 3C). Taken together, these results demonstrate that MAM-A DNA vaccination is capable of inducing MAM-A–specific CD8 T cells that are cytolytic and IFNγ and TNFα contribute to this cytolytic activity.

**NKG2D signaling contributes to the cytolytic activity of MAM-A–specific CD8 T cells**

NKG2D has been shown to be upregulated upon activation of CD8 T cells (40). After vaccination, NKG2D mRNA expression was upregulated in purified CD8 T cells cultured with UACC-812 breast cancer cells (0.3 ± 0.2 relative
Figure 3. MAM-A DNA vaccination induces MAM-A–specific CD8 T cells capable of specifically lysing MAM-A+ breast cancer cell lines in a NKG2D/DAP10/perforin-dependent manner. A, CD8 T cells isolated from HLA-A2+ subjects 12 months after vaccination were tested for cytolytic activity on a panel of breast cancer cell lines at various E:T ratios. Only the HLA-A2+/MAM-A+ breast cancer cell line, UACC-812, was effectively lysed. B, CD8 T cells were purified from PBMCs at the indicated time points after vaccination, and TNFα production in response to stimulation with UACC-812 breast cancer cells was determined by qRT-PCR. Similar results were observed for IFNγ production (data not shown). C, UACC-812–specific cytolytic activity was determined in the presence of antibodies to isotype control, IFNγ, or TNFα. Error bars represent the mean ± SEM from the 6 HLA-A2+ responders. D, UACC-812–specific cytolytic activity was determined in the presence of antibodies to isotype control, NKG2D, IFNγ, or TNFα, or control or NKG2D siRNA. Error bars represent the mean ± SEM from the 6 HLA-A2+ responders. E, purified CD8 T cells from subjects vaccinated with the MAM-A DNA vaccine were stimulated with UACC-812 breast cancer cells at a 50:1 T-cell to breast cancer cell ratio as indicated. Representative immunoblots for NKG2D (left); DAP10 (middle), and perforin (right). Actin expression represents a protein loading control. F, purified CD8 T cells were tested for UACC-812–specific cytolytic activity in the presence of isotype control, NKG2D, DAP-10, or perforin antibody, or control, NKG2D, DAP-10, or perforin siRNA. Error bars represent the mean ± SEM from the 6 HLA-A2+ responders.
expression compared with actin before vaccination vs. 13.7 ± 2.4 at 12 months; \( P < 0.05 \); Fig. 3D). Similar increases in NKG2D protein expression were also observed (Fig. 3E). Of note, NKG2D mRNA and protein expression were downregulated in the presence of anti-IFN\(\gamma\) mAb (3.7 ± 0.9 relative expression compared with actin), anti-TNF\(\alpha\) mAb (5.8 ± 1.4), or both (1.3 ± 0.5) suggesting important roles for IFN\(\gamma\) and TNF\(\alpha\) in the induction of NKG2D expression (Fig. 3D and Supplementary Fig. S3). Specific ablation or antibody neutralization of NKG2D significantly reduced the cytolytic activity of MAM-A–specific CD8 T cells (Fig. 3F).

The NKG2D adapter protein DAP-10 has been shown to be an important downstream mediator of NKG2D-induced cytolytic activity (41), and perforin has been identified as a key cytotoxic effector molecule released by activated CD8 T cells (42). To determine whether DAP-10 and/or perforin contribute to NKG2D-mediated cytolytic activity after vaccination, we assessed DAP-10 and perforin expression in MAM-A–specific CD8 T cells after coculture with UACC-812 breast cancer cells. Expression of DAP-10 and perforin were increased in purified CD8 T cells following vaccination (DAP-10: 0.5 ± 0.3 relative expression compared with actin before vaccination vs. 9.3 ± 2.7 at 12 months; \( P < 0.05 \); perforin: 1.7 ± 0.6 relative expression compared with actin before vaccination vs. 16.5 ± 4.1 at 12 months, \( P = 0.05 \); Fig. 3E, middle and right panels, and Supplementary Figs. S4 and S3). Specific ablation or antibody neutralization of NKG2D was associated with downregulation of DAP-10 expression (Supplementary Fig. S4), and reduced perforin expression (Supplementary Fig. S5), suggesting a critical role for NKG2D in the regulation of DAP-10 and perforin expression in MAM-A–specific CD8 T cells after vaccination. Similarly, antibodies to IFN\(\gamma\), TNF\(\alpha\), or both strongly inhibited the expression of both DAP-10 (Supplementary Fig. S4) and perforin (Supplementary Fig. S5) in CD8 T cells cultured with UACC-812 cells.

Specific ablation of NKG2D or DAP10 expression was associated with a significant reduction in MAM-A–specific CD8 T-cell cytolytic activity (DAP-10: 44.3% ± 13.9% vs. 3.8% ± 1.6%; \( P < 0.05 \); NKG2D: 44.3% ± 13.9% vs. 4.7% ± 1.3%; \( P < 0.05 \); Fig. 3F). Cytotoxicity was mediated almost exclusively through perforin, as specific ablation or antibody neutralization of perforin reduced cytolytic activity to almost zero. On the basis of these results, we conclude that MAM-A DNA vaccination is capable of inducing MAM-A–specific CD8 T cells with significant cytolytic activity. This cytolytic activity is dependent on NKG2D, DAP10, and perforin expression.

**Preliminary evidence suggests that MAM-A DNA vaccination is associated with improved PFS**

Although this study was not powered to evaluate the impact of MAM-A vaccination on PFS, clinical outcome was one of the exploratory objectives. We compared progression-free survival between subjects who received the vaccine and subjects who met all eligibility criteria and were enrolled in the trial but were screen failures because of HLA phenotype. PFS was determined by blinded review of case report forms, medical records, and diagnostic imaging results. PFS of the vaccinated patients was described using Kaplan–Meier product limit estimator and compared with a subset of screen failures by log-rank test. MAM-A vaccination was associated with a prolonged PFS (6-month PFS, 53% vs. 33%; \( P = 0.011 \)).

**Discussion**

Preclinical studies by our group (10) and others (43–45) have demonstrated that MAM-A is an exceptional target for breast cancer vaccine therapy based on its exquisite tissue specificity (16–19), overexpression in 40% to 80% of breast cancers (20–24), and evidence of preexisting immunity in patients with breast cancer (8, 9, 25, 26). On the basis of these preclinical studies, we initiated a phase I clinical trial to evaluate the safety and biologic efficacy of a novel plasmid MAM-A DNA vaccine. The MAM-A DNA vaccine is based on the plNG vector, driving MAM-A expression by a strong viral promoter. Patients with breast cancer with stable metastatic disease were eligible for participation. To date, 14 subjects have been successfully treated with the vaccine, including 8 HLA-A2+ subjects that were studied with in-depth immune monitoring analyses using PBMC collected before, during, and after vaccination.
The results of the phase I clinical trial demonstrate the safety of the MAM-A DNA vaccine and compelling preliminary evidence of biologic efficacy. Specifically, we have made three important observations: (i) the MAM-A DNA vaccine is safe, with no significant adverse events observed in the trial. The most common grade 1 toxicity was malaise/flu-like symptoms; (ii) there was a significant increase in the frequency and number of MAM-A–specific CD8 T cells following vaccination, as measured by tetramer analysis and ELISPOT; and (iii) preliminary evidence suggests that subjects who were vaccinated had improved PFS compared with subjects who were enrolled in the trial but were not vaccinated because of HLA type.

Induction of effective antitumor immunity requires that a cancer vaccine be capable of eliciting robust type I immunity. In the last two decades, considerable progress has been made in understanding the complex regulatory and signaling networks that control immune responses. It is increasingly clear that CD8 T-cell responses are tightly regulated by the immune system, presumably to avoid deleterious autoimmune responses. In the context of breast cancer, regulatory networks such as the increased prevalence of T regulatory cells (Treg; ref. 11) and upregulation of inhibitory molecules on breast cancer–specific T cells (12) are present that restrain breast cancer immune responses. These regulatory networks may also limit the T-cell response to therapeutic cancer vaccines. In a previous study evaluating the CD4 T-cell response to vaccination in 7 of the first 9 patients of this cohort, we demonstrated that the frequency of Treg was significantly decreased following MAM-A DNA vaccination, whereas the frequency of CD4+ICOShi MAM-A–specific T cells was increased (35). These CD4+ICOShi T cells produced higher levels of IFNγ and lower levels of IL10 after vaccination.

We present evidence here that the MAM-A DNA vaccine is able to induce type I immune responses despite the presence of these regulatory networks. MAM-A DNA vaccination results in induction of MAM-A–specific CD8 T cells that are capable of producing both IFNγ and TNFα, and lysing MAM-A+ breast cancer cells. MAM-A–specific CD8 T cells also have increased NKG2D, DAP-10, and perforin expression. Of note, in CD8 T cells, NKG2D signaling has been shown to augment proliferation and cytotoxicity upon antigen encounter, suggesting that NKG2D functions as a costimulatory molecule (46). Our studies demonstrate that after MAM-A DNA vaccination, there is specific upregulation of NKG2D and DAP-10 expression (Fig. 3). Although this response is not unique to MAM-A vaccination, it does provide strong supporting evidence for the efficacy of the MAM-A DNA vaccine. Taken together, the results of these two studies strongly suggest that the MAM-A DNA vaccine is capable of inducing strong type I immune responses. The humoral response to vaccination was not measured.

Current cancer vaccine clinical development paradigms emphasize the early assessment of cancer vaccine efficacy in an appropriate clinical context (47). These paradigms have been endorsed in the literature and in FDA guidance documents. Although treatment of patients with metastatic disease is appropriate for first-in-human studies of a novel biologic therapeutic cancer vaccine treatment of patients with metastatic disease does have unique limitations. First, it is very difficult to assess the biologic efficacy of cancer vaccines in patients with metastatic disease, as the time interval from vaccine administration to subsequent disease progression in patients with metastatic disease may be too short to develop a productive antitumor immune response. Second, patients with metastatic disease have typically received multiple prior cancer treatments, which may be detrimental to the immune system. The fact that preliminary evidence suggests that the MAM-A DNA vaccine was associated with improved PFS in patients with metastatic breast cancer is notable, supporting ongoing clinical development of the MAM-A DNA vaccine. Studies to define the clinical potential of a MAM-A DNA vaccine are particularly important given the safety of the vaccine, evidence of biologic efficacy, exquisite tissue specificity, and near-universal expression of MAM-A in breast cancer, and the potential of MAM-A as a target for a breast cancer prevention vaccine.

Disclosure of Potential Conflicts of Interest
T. Fleming holds a patent on mammaglobin that is owned by Washington University. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: V. Tiriveedhi, M. Ellis, C. Ma, A.C. Lockhart, P. Goedegebuure, T. Mohanakumar, W.E. Gillanders
Development of methodology: V. Tiriveedhi, J. Herndon, T. Fleming, P. Goedegebuure, T. Mohanakumar, W.E. Gillanders, Lijin Li
 Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): V. Tiriveedhi, N. Tucker, M. Sturmoski, M. Ellis, C. Ma, M. Naughton, A.C. Lockhart, W.E. Gillanders, Lijin Li
 Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V. Tiriveedhi, N. Tucker, C. Ma, A.C. Lockhart, F. Gao, W.E. Gillanders
 Writing, review, and/or revision of the manuscript: V. Tiriveedhi, N. Tucker, M. Ellis, C. Ma, A.C. Lockhart, F. Gao, P. Goedegebuure, W.E. Gillanders
 Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Tucker, J. Herndon, C. Ma, W.E. Gillanders
 Study supervision: A.C. Lockhart, W.E. Gillanders
 Other (involved in the GMP manufacture and quality control of the mammaglobin DNA vaccine): T. Fleming

Acknowledgments
The authors thank the Alvin J. Siteman Cancer Center at Washington University School of Medicine and Barnes-Jewish Hospital (St. Louis, MO) for the use of the Biologic Therapy Core Facility, the Clinical Trials Core and Biostatistics Core, which provided critical support for the vaccine manufacture and validation, design and performance of the clinical trial, and data analysis, respectively. The authors are also extremely grateful to George and Diana Holway, whose generous gift provided the resources necessary to help realize the promise of new discovery.

Grant Support
This project was funded by research grants DOE/CDMRP (CRIP W81XWH-06-1-0677) (to W.E. Gillanders) and Gateway for Cancer Research P-06-016 (to W.E. Gillanders). V. Tiriveedhi, P. Goedegebuure, and T. Mohanakumar are funded by the Foundation for Barnes-Jewish Hospital. N. Tucker was supported by a NCI grant T32 CA009261. The Siteman Cancer Center is supported in part by NCI Cancer Center Support Grant #P30 CA91842.

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Received January 23, 2014; revised August 18, 2014; accepted August 19, 2014; published online December 1, 2014.


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*Clin Cancer Res* 2014;20:5964-5975.

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