

# Characterization of the role of CD8<sup>+</sup>T cells in breast cancer immunity following mammaglobin-A DNA vaccination using HLA-class-I tetramers

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## Abstract

**Introduction** Mammaglobin-A(mam-A) is expressed in over 80% of human breast tumors. We recently reported that mam-A DNA vaccination resulted in breast cancer immunity in a preclinical model. Here we investigated whether mam-A HLA-class-I tetramers could be used to monitor and define the role of CD8<sup>+</sup>cytotoxic T-lymphocytes(CTL) in mediating breast cancer immunity following mam-A DNA vaccination.

**Study Design** Mam-A DNA vaccination was performed in HLA-A2<sup>+</sup>huCD8<sup>+</sup> transgenic mice. HLA-A2 tetramers carrying the immunodominant mamA2.1 peptide were used to monitor CD8<sup>+</sup>CTL. Human breast cancer colonies were developed in immunodeficient SCID-beige mice. ELISPOT was used to correlate frequency of mamA2.1 tetramer<sup>+</sup>CD8<sup>+</sup>T cells and IFN- $\gamma$  production [spots per million cells (spm)] in human subjects.

**Results** Vaccination of HLA-A2<sup>+</sup>huCD8<sup>+</sup> mice with mam-A DNA vaccine, but not empty vector, led to the expansion of mamA2.1 tetramer<sup>+</sup>CD8<sup>+</sup>T-cells in peripheral blood (<0.5% pre-vaccination compared to >2.0% post-vaccination). CD8<sup>+</sup>T cells from vaccinated mice specifically lysed UACC-812(HLA-A2<sup>+</sup>/mam-A<sup>+</sup>, 25% lysis) but

not MDA-MB-415(HLA-A2<sup>-</sup>/mam-A<sup>+</sup>) or MCF-7(HLA-A2<sup>+</sup>/mam-A<sup>-</sup>) breast cancer cells. Adoptive transfer of purified CD8<sup>+</sup>T cells from vaccinated mice into immunodeficient SCID-beige mice with established human breast cancer colonies led to tetramer<sup>+</sup>CD8<sup>+</sup> T-cell infiltration with regression of UACC-812 but not MCF-7 tumors. HLA-A2<sup>+</sup> breast cancer patients revealed increased frequency of mamA2.1 tetramer<sup>+</sup>CD8<sup>+</sup> T-cells compared to normal controls (2.86  $\pm$  0.8% vs. 0.71  $\pm$  0.1%,  $P = 0.01$ ) that correlated with the IFN- $\gamma$  response to mamA2.1 peptide (48.1  $\pm$  20.9 vs. 2.9  $\pm$  0.8 spm,  $P = 0.03$ ).

**Conclusions** CD8<sup>+</sup> T-cells are crucial in mediating breast cancer immunity following mam-A DNA vaccination. Mam-A HLA-class-I tetramers can be effectively used to monitor development of CD8<sup>+</sup> T-cells following mam-A vaccination.

**Keywords** Breast cancer · Mammaglobin A · DNA vaccination · HLA class I tetramers · CD8<sup>+</sup> T cells

## Abbreviations

Mam-A	Mammaglobin A
HLA	Human leukocyte antigen
CTL	Cytotoxic T lymphocytes
TAA	Tumor associated antigens
SPM	Spots per million cells
PBMC	Peripheral blood mononuclear cells

## Introduction

Mammaglobin-A (mam-A), a novel breast cancer specific protein, was recently identified using differential screening approach [1–4]. Mam-A is a 10 kDA glycoprotein related

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to the family of epithelial secretory proteins that includes rat estramustine-binding protein and human clara cell protein (CC10/uteroglobulin). Mam-A is expressed in over 80% of human breast cancers [5–10]. Its expression is consistent both in non-invasive and invasive breast cancer, and in all nuclear grades of breast cancer. In addition, it is frequently produced by metastatic breast cancer cells [2]. Due to its exclusive expression by breast cancer cells, mam-A has been shown to be a superior marker for detection of malignant cells in peripheral blood, bone marrow, and lymph nodes [5–7]. These properties also make mam-A a unique candidate protein for breast cancer immunotherapy.

Plasmid DNA vaccination can induce expression of exogenous proteins in a host [11–15] and is a useful strategy to induce tumor immunity. However, the use of DNA vaccination in cancer settings is limited by lack of tumor specific antigens (TAA). The definition of immune responses against a broadly expressed tumor antigen such as mam-A should be of benefit in developing immunotherapy in human subjects. We recently demonstrated that mam-A DNA vaccination could lead to immunity against breast cancer in HLA-A2<sup>+</sup>huCD8<sup>+</sup> double transgenic mice [16]. Human mam-A gene was cloned into the PCI-neo vector and administered intramuscularly into the host. Vaccinated mice demonstrated specific lysis of mam-A producing human breast cancer colonies both in vitro and in vivo. However, the effector mechanisms underlying the development of breast cancer immunity following mam-A vaccination remain undefined.

In this study, we used mam-A HLA class I tetramers to monitor and define the role of CD8<sup>+</sup> CTL in the development of breast cancer immunity following mam-A DNA vaccination. We demonstrated that CD8<sup>+</sup> CTL are important mediators of immunity against human breast cancer following DNA vaccination and can be detected in the peripheral blood using mam-A HLA class-I tetramers both in murine models and breast cancer patients.

## Methods

### Mice

C57BL/6 mice (H-2<sup>b</sup>) carrying the HLA-A\*0201 (HLA-A2<sup>+</sup>) or human CD8 (huCD8<sup>+</sup>) genes were kindly provided by Dr. Victor H. Engelhard (University of Virginia, Charlottesville, VA) and Dr. Linda A. Sherman (The Scripps Research Institute, La Jolla, CA), respectively [17, 18]. First generation (F1) mice from a cross between these two mouse transgenic lines (HLA-A2<sup>+</sup>/huCD8<sup>+</sup>) were used in this study. Severe combined immunodeficient (SCID)-beige mice were obtained from Taconic (Germantown, NY). All

animal use protocols followed federal and institutional guidelines and were approved by the Animal Studies Committee at Washington University School of Medicine.

### Study subjects

Ten HLA-A2<sup>+</sup> breast cancer patients and HLA-A2<sup>+</sup> healthy female volunteers were enrolled after obtaining informed consent. HLA typing was performed using sequence-specific oligonucleotide probes that provided low-medium resolution for HLA-A genes (Dynal Biotech, Lafayette Hill, PA, USA). Written informed consent was obtained from all subjects, and the protocol was reviewed and approved by the Human Studies Committee at Washington University School of Medicine.

### Preparation of tetramers

Using the HLA class I-peptide binding prediction program from the Bio informatics & Molecular Analysis Section of the National Institutes of Health at [http://bimas.dcr.t.nih.gov/molbio/hla\\_bind/](http://bimas.dcr.t.nih.gov/molbio/hla_bind/), we have previously described seven mam-A-derived peptides that bind to the HLA-A\*0201 molecule [19]. The sequences of these peptides are described in Table 1. Of these seven peptides, MamA2.1 (LIYDSSLCDL) was found to be an immunodominant peptide [19]. Therefore, mam-A2.1 tetramers were developed to monitor mam-A specific T cells following mam-A DNA vaccination. The tetramers were developed by Beckman Coulter Immunomics (San Diego, CA) as previously described [20, 21]. In addition, a “negative” tetramer carrying an unrelated peptide was prepared as control. Tetramers were used to stain target cells at a concentration of 10 μl per 200 μl of whole blood or 1 × 10<sup>6</sup> peripheral blood mononuclear cells (PBMC). RBC lysis buffer from the manufacturer was used to remove the RBCs prior to flow cytometry.

**Table 1** Sequences of HLA-A2 restricted Mam-A derived peptides

Peptide	Amino acid position	Sequence	HLA-binding score
Mam-A2.1	83–92	LIYDSSLCDL	150.7
Mam-A2.2	2–10	KLLMVMLLA	148.0
Mam-A2.3	4–12	LMVMLLAAL	60.3
Mam-A2.4	66–74	FLNQTDETL	48.2
Mam-A2.5	73–81	TLSNVEVFM	27.3
Mam-A2.6	32–40	MQLIYDSSL	13.6
Mam-A2.7	32–40	TINPQVSKT	2.4

## Antibodies and flow cytometry

Unconjugated and fluorochrome-conjugated mouse anti-human CD8 (clone SFCI21Thy2D3) and isotype control antibodies were purchased from Beckman Coulter, Inc (Fullerton, CA). CD8<sup>+</sup> T cells were purified from mouse spleens using purification kit purchased from Miltenyi Biotec (Auburn, CA).

## Breast cancer cell lines

All breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). HLA typing of the cell lines was performed as described above, and mam-A expression was determined by reverse transcriptase-polymerase chain reaction [4]. Breast cancer cell lines were cultured in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 10% defined fetal bovine serum (HyClone, Logan, UT, USA), 100 mM non-essential amino acids, 2 mM L-glutamine, 25 mM HEPES, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 µg/ml streptomycin (Gibco) at 37°C in a 5% CO<sub>2</sub> incubator. Human breast cancer colonies were established in immunodeficient SCID-beige mice by injecting 8-week-old SCID beige mice (Taconic) with 40 × 10<sup>7</sup> breast cancer cells resuspended in 300 µl of BD Matrigel basement membrane matrix (BD Biosciences, San Diego CA) into the scruff region of the neck. Tumor measurements were obtained using calipers by two different investigators who were blinded to experimental treatments.

## Peptides

Mam-A HLA-A2 peptides (Table 1) were synthesized by Research Genetics (Huntsville, AL, USA). The purity of peptides was determined by high-performance liquid chromatography and mass spectrometry. The peptides were dissolved in DMSO (Sigma, St. Louis, MO, USA) at a concentration of 10 mg/ml and stored at -70°C until use.

## Loading of TAP-deficient T2 cells with mam-A peptides

Briefly, T2 cells (1 × 10<sup>6</sup>/ml) were incubated in flat-bottom 96-well plates at 25°C in the presence of each peptide (40 µg/ml) in 200 µl of RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 10% defined fetal bovine serum (HyClone, Logan, UT), 100 µM non-essential amino acids, 2 mM L-glutamine, 25 mM HEPES, 1 mM sodium pyruvate, 100 units/ml penicillin, and

100 µg/ml streptomycin. Three micrograms per millilitre of human β<sub>2</sub>m (Sigma) was also added to the cultures. The loaded T2 cells were washed thoroughly after 24 h and used to develop the human CD8<sup>+</sup> CTL line.

## Generation of human CD8<sup>+</sup> CTL

After monocyte and dendritic cell depletion by adherence to plastic for 90 min at 37°C, peripheral blood lymphocytes (PBMC, 2 × 10<sup>6</sup>) were cultured in 2 ml of RPMI-1640 medium supplemented as described above in 24-well plates in the presence of a pool of irradiated (10,000 rads) T2 cells (1 × 10<sup>6</sup>) individually loaded with the mam-A-derived peptides or control FLU peptide (GILGFVFTL) as described above. β<sub>2</sub>m (3 µg/ml) and CD28.2 anti-CD28 mAb (500 ng/ml, BD Biosciences) were also added to the cultures. In addition, recombinant human IL-2 (20 U/ml, Chiron, Emeryville, CA) and human T-STIM<sup>®</sup> without phytohemagglutinin (10%, BD Biosciences) were added to the cultures after 24 h. The T cells (2 × 10<sup>5</sup>) were restimulated every 8–10 days with irradiated, peptide-loaded T2 cells (1 × 10<sup>6</sup>) in the presence of irradiated (3,000 rads) autologous peripheral blood lymphocytes (2 × 10<sup>6</sup>) in 24-well plates in 2 ml of culture medium supplemented with IL-2, β<sub>2</sub>m, and anti-CD28. After stimulation, the CD8<sup>+</sup> CTL were purified by negative selection in a MiniMacs separation column using anti-CD4-coated microbeads (Miltenyi Biotec, Auburn, CA). The cytotoxic activities of the resulting CD8<sup>+</sup> CTL lines were analyzed after six stimulations.

## Mammaglobin-A cDNA construct and vaccination

Mam-A cDNA was derived from the human breast cancer cell line MDA-MB-415 [4]. The mam-A cDNA was modified by PCR to yield *EcoRI* ends and cloned into the *EcoRI* site at the multiple cloning site of the PCI-neo vector (Promega, Madison, WI, USA). HLA-A2<sup>+</sup>/huCD8<sup>+</sup> mice were injected intramuscularly in the quadriceps with 100 µg of the mammaglobin-A cDNA or the vector alone (control) along with 0.5% bupivacaine. The mice were vaccinated four times at 2-week intervals.

## Human ELISPOT assay

MultiScreen<sup>®</sup> 96-well filtration plates (Millipore, Bedford, MA) were coated overnight at 4°C with 5.0 µg/ml of a capture human IFN-γ-specific monoclonal antibody (BD Biosciences, Franklin Lakes, NJ) in 0.05 M carbonate-bicarbonate buffer (pH: 9.6). The plates were blocked with 1% BSA for 1 h and washed (3×) with PBS. Subsequently,

$3 \times 10^5$  CD8<sup>+</sup> T cells were cultured in triplicate or quadruplicate wells in the antibody-coated plates in 200  $\mu$ l of RPMI-1640 medium supplemented as described above in the presence of mamA2.1 peptide (40  $\mu$ g/ml) and irradiated autologous PBMC. Cells cultured in the presence of the GILGFVFTL influenza-derived epitope (FLU) were used as a positive control. Cells cultured alone were used as a negative control. After 24 h, the plates were washed with PBS (3 $\times$ ) and PBS supplemented with 0.05% Tween-20 (3 $\times$ ). Biotinylated human IFN- $\gamma$ -specific monoclonal antibody (BD Biosciences) in PBS/BSA/Tween-20 (2.0  $\mu$ g/ml) was added to the wells. After an overnight incubation at 4°C, the plates were washed (3 $\times$ ) and horseradish peroxidase-labeled streptavidin (BD Biosciences), diluted 1:2000 in PBS/BSA/Tween-20, was added to the wells. After 2 h, 3-amino-9-ethylcarbazole substrate reagent (BD Biosciences) was added to the wells for 5–10 min. The plates were washed with tap water to stop the reaction and air-dried. Spots were analyzed in an ImmunoSpot Series I analyzer (Cellular Technology, Cleveland, OH) that was designed to detect spots with pre-determined criteria for spot size, shape, and colorimetric density. The number of spots in the negative control cultures was subtracted from the number of spots in the experimental cultures. Results are expressed as spots per million cells (spm).

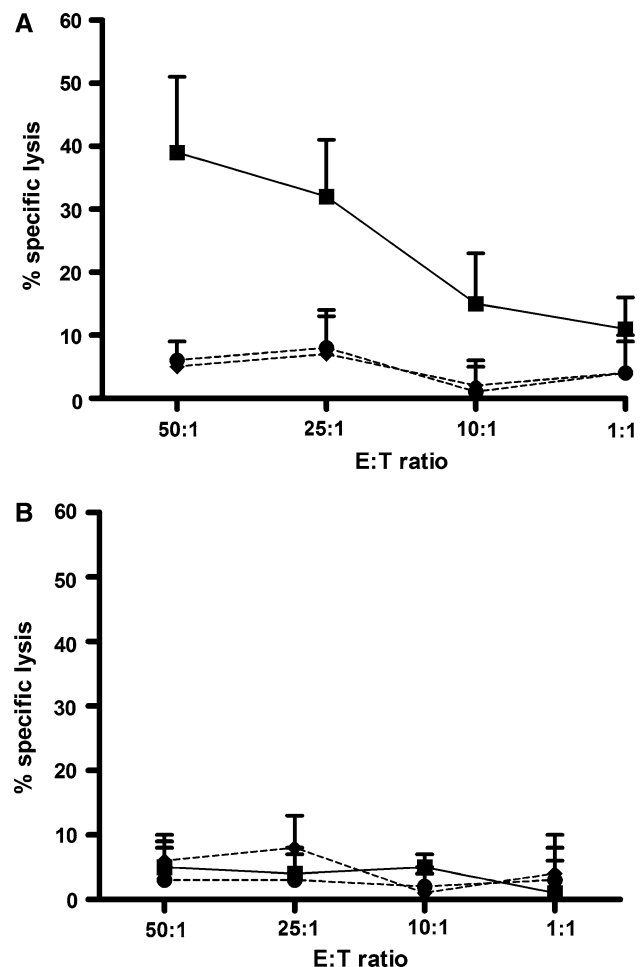
#### Cytotoxicity assay

The CD8<sup>+</sup> T cells were co-cultured overnight with target breast cancer cells at a ratio of 50:1 and cytotoxicity measured using Cytotox 96<sup>®</sup> LDH release assay according to the manufacturer's protocol (Promega). The percent specific lysis was calculated as follows: [(experimental LDH release) – (spontaneous LDH release)/(maximum LDH release) – (spontaneous LDH release)]  $\times$  100.

## Results

#### Development of mam-A specific cytotoxic CD8<sup>+</sup> T cell lines

Mam-A specific CD8<sup>+</sup> CTL lines were developed from two HLA-A2 positive healthy female subjects by stimulating PBMC with T2 cells individually pulsed with the seven peptides in equal concentrations. The cytotoxicity of the resulting CD8<sup>+</sup> CTL lines (purity >95%, data not shown) was assessed in vitro against UACC-812 (HLA-A2<sup>+</sup> MamA<sup>+</sup>), MCF-7 (HLA-A2<sup>+</sup> Mam<sup>-</sup>), and MDA-MB-415 (HLA-A2<sup>-</sup> MamA<sup>+</sup>) human breast cancer cell lines at varying effector:target ratios (Fig. 1). CD8<sup>+</sup> CTL lines demonstrated cytotoxicity against the UACC-812 (>35%

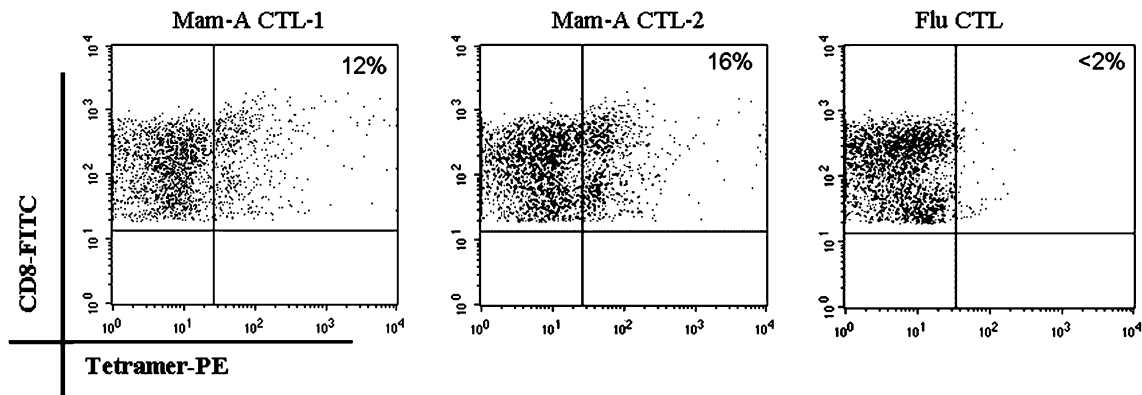


**Fig. 1** Development of mam-A specific HLA-A2 restricted CD8<sup>+</sup> CTL lines. PBMCs isolated from two HLA-A2<sup>+</sup> healthy individuals (CTL-1, CTL-2) were stimulated six times in vitro with pooled T2 cells individually loaded either with the HLA-A2 binding mam-A derived (Mam-A2.1–2.7) or control Flu (GILGFVFTL) peptide. The mam-A specificity and HLA-A2 restriction of the CD8<sup>+</sup> CTL lines, (A) Mam-A specific CTLs, (B) FLU CTLs, was evaluated after six stimulations by testing them against UACC-812 (■, HLA-A2<sup>+</sup> Mam-A<sup>+</sup>), MCF-7 (◆, HLA-A2<sup>+</sup> Mam-A<sup>-</sup>), and MDA-MB-415 (●, HLA-A2<sup>-</sup> Mam-A<sup>+</sup>) human breast cancer cell lines using LDH release assay at varying effector: target ratios. All experiments were done in triplicate cultures and results are presented as mean  $\pm$  standard error

specific lysis at 50:1 E:T ratio) but not against MCF-7 and MDA-MB-415 breast cancer cell lines (<10% specific lysis) demonstrating that the CTL lines were mam-A specific and HLA-A2 restricted (Fig. 1A). A control cell line developed against the influenza peptide did not kill any of the breast cancer cell lines (<5% specific lysis, Fig. 1B).

#### Specificity of mam-A2.1 tetramers

The specificity of the mamA2.1 tetramers was next analyzed. Towards this, the two mam-A CTL lines (CTL-1, CTL-2) and the control FLU line were stained with the



**Fig. 2** Specificity of mamA2.1 tetramers. CD8<sup>+</sup> CTL lines were generated from two HLA-A2<sup>+</sup> healthy individuals by in vitro stimulations with pooled T2 cells individually loaded with the HLA-A2 binding mam-A derived peptides (CTL-1 and CTL-2) or

control flu-peptide (Flu CTL). The mam-A specificity and HLA-A2 restriction of the CD8<sup>+</sup> CTL lines was subsequently confirmed. Then, the CTL lines were stained with human anti-CD8 and mamA2.1 tetramers and analyzed using flow cytometry

mamA2.1 tetramers and human anti-CD8 mAbs and analyzed using flow cytometry (Fig. 2). Both CTL-1 and CTL-2 lines revealed high frequency of mamA2.1 tetramer<sup>+</sup> CD8<sup>+</sup> T cells (12% and 16%, respectively). In contrast, the frequency of mamA2.1 tetramer positive cells in the control FLU cell line was less than 2%. These data highly suggested that the mamA2.1 tetramers could specifically bind to mam-A specific and HLA-A2 restricted CD8<sup>+</sup> CTL. It is noteworthy that the CD8<sup>+</sup> CTL lines were developed by stimulating with all seven HLA-A2 binding peptides. Therefore, we expected the CD8<sup>+</sup> CTL lines to be heterogeneous containing CD8<sup>+</sup> T lymphocytes specific for the other HLA-A2 binding mam-A peptides shown in Table 1. The percentage of tetramer staining (12% and 16%) in these two cell lines further indicate that CD8<sup>+</sup> T cells specific for peptides other than mamA2.1 do not bind to mamA2.1 tetramers.

#### Induction of mamA2.1 tetramer<sup>+</sup> CD8<sup>+</sup> CTL following mam-A DNA vaccination

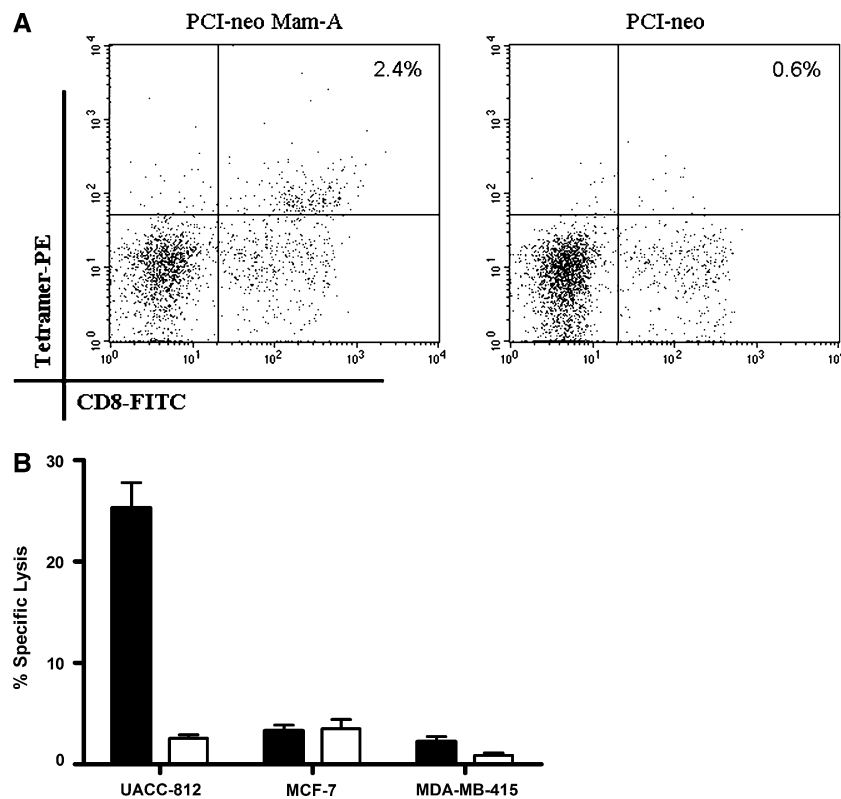
Using HLA-A2<sup>+</sup> huCD8<sup>+</sup> double transgenic mice, we have previously demonstrated that mam-A DNA vaccination can induce breast cancer specific immunity. We further hypothesized that CD8<sup>+</sup> CTL would be important mediators of breast cancer specific immunity following mam-A DNA vaccination. The HLA-A2<sup>+</sup> huCD8<sup>+</sup> double transgenic mice were vaccinated using the PCI-neo vector cloned with full length human mam-A gene. The vaccination schedule consisted of four doses of two-weekly intramuscular injections. Following vaccination, the frequency of mamA2.1 tetramer<sup>+</sup> CD8<sup>+</sup> T cells was analyzed in the peripheral blood. As control, mice were injected with the PCI-neo vector alone without the mam-A gene. As demonstrated in Fig. 3A, mamA2.1 tetramer<sup>+</sup> CD8<sup>+</sup> T cells

were found to be significantly expanded in the peripheral blood of vaccinated (2.4% of PBMC) but not control mice (0.6%,  $P = 0.01$ ).

We next investigated whether the increase in mamA2.1 tetramer<sup>+</sup> CD8<sup>+</sup> T cells correlated with the development of breast cancer immunity in the vaccinated mice. First, CD8<sup>+</sup> T cells from the spleen of control and vaccinated mice were isolated. Following this, cytotoxicity against breast cancer cell lines was analyzed using LDH release assay at varying effector: target ratio (Fig. 3B). The vaccinated mice demonstrated increased cytotoxicity towards the UACC-822 cell line (>25% lysis, E:T ratio 50:1) but not the MCF-7, or MDA-MB-415 cell lines (<5% lysis). In contrast, CD8<sup>+</sup> T cells from mice that received the “empty” vector did not reveal cytotoxicity against any cell line. Taken together, these results indicated that the induction of mamA2.1 tetramer<sup>+</sup> CD8<sup>+</sup> T cells correlates with development of HLA class-I restricted breast cancer specific immunity.

#### Tumor infiltration of mamA2.1 tetramer<sup>+</sup> CD8<sup>+</sup> CTL in vivo

Human breast cancer colonies were established in immunodeficient SCID-beige mice. Following this, whole splenocytes or fractionated CD8<sup>+</sup> T cells isolated from vaccinated (mam-A DNA) or control (PCI-neo vector alone) HLA-A2<sup>+</sup> huCD8<sup>+</sup> mice were adoptively transferred into the SCID-beige mice. SCID-beige mice with UACC-812 human breast tumors that received either whole splenocytes or fractionated CD8<sup>+</sup> T cells from vaccinated transgenic mice revealed tumor regression starting at 2-weeks with 50% reduction in tumor volume at 4-weeks (Fig. 4A). The rate of tumor regression observed with adoptive transfer of CD8<sup>+</sup> T cells was similar to that with



**Fig. 3** Induction of mamA2.1 tetramer<sup>+</sup> CD8<sup>+</sup> cytotoxic T cells in the peripheral blood of vaccinated hosts. **(A)** HLA-A2<sup>+</sup>huCD8<sup>+</sup> transgenic mice ( $n = 4$ ) were vaccinated with PCI-neo vector cloned with full length human mam-A gene. As control, mice ( $n = 4$ ) received “empty” PCI-neo vector injections. Following the last vaccination dose, the frequency of mamA2.1 tetramer positive CD8<sup>+</sup> T cells in the peripheral blood was analyzed using flow cytometry. Results of a representative vaccinated and control animal are demonstrated. **(B)** HLA-A2<sup>+</sup>huCD8<sup>+</sup> transgenic mice were vaccinated

with either mam-A DNA ( $n = 4$ , black bars) or empty vector ( $n = 4$ , white bars). Following the last vaccination dose, CD8<sup>+</sup> T cells from the spleen were isolated from each animal. Then, mam-A specificity and HLA-A2 restriction of the CD8<sup>+</sup> T cells were evaluated by testing them against the UACC-812 (HLA-A2<sup>+</sup> Mam-A<sup>+</sup>), MCF-7 (HLA-A2<sup>+</sup> Mam-A<sup>-</sup>), and MDA-MB-415 (HLA-A2<sup>-</sup> Mam-A<sup>+</sup>) at effector: target ratios of 50:1, 25:1, 10:1, and 1:1. Results of E:T ratio 50: 1 are presented here as the mean  $\pm$  standard error of triplicate cultures of all mice in respective groups

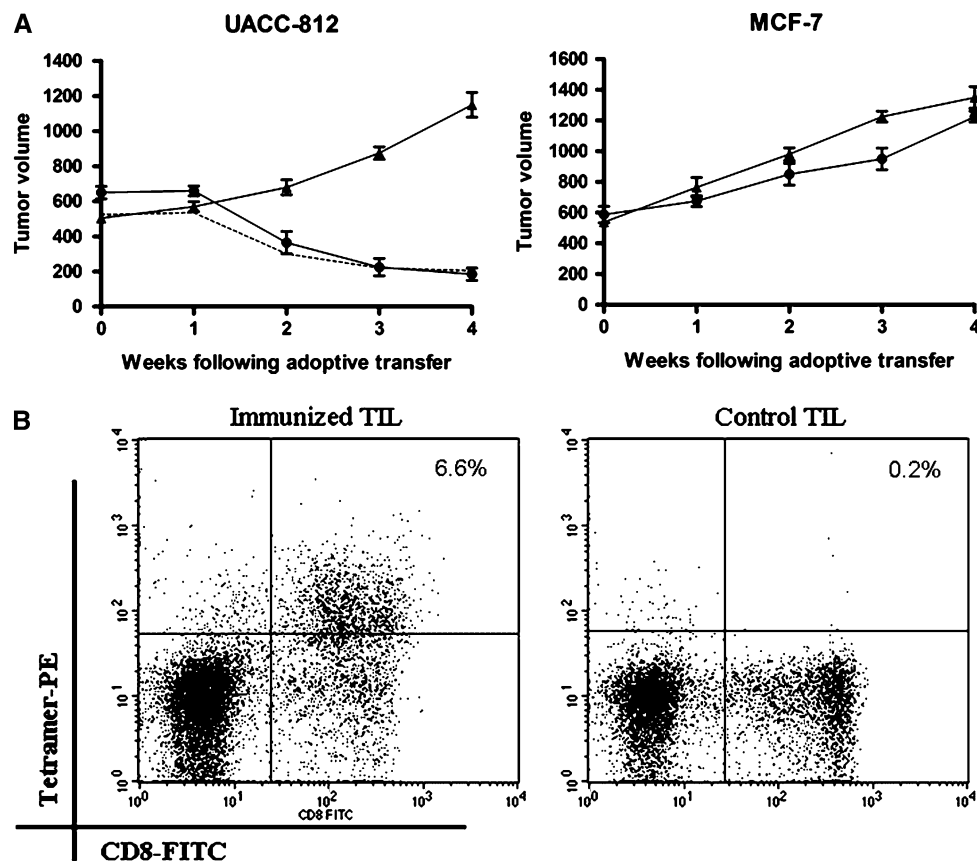
whole splenocytes. No tumor regression was observed in the case of MCF-7 (HLA-A2<sup>+</sup>Mam-A<sup>-</sup>) cell lines. In contrast, splenocytes from control mice did not induce regression of any tumor colonies. Furthermore, depletion of CD8<sup>+</sup> T cells from splenocytes of the vaccinated mice before reconstitution into SCID-beige mice prevented regression of UACC-812 breast cancer colonies (data not shown).

UACC-812 tumors from SCID-beige mice that received splenocytes from vaccinated or control HLA-A2<sup>+</sup>huCD8<sup>+</sup> transgenic mice were excised at 2-weeks following the adoptive transfer and the tumor infiltrating lymphocytes isolated with mechanical dissociation. The tumor infiltration of mamA2.1 tetramer<sup>+</sup> CD8<sup>+</sup> T cells was analyzed using flow cytometry. As shown in Fig. 4B, the regressing UACC-812 tumors from SCID-beige that had received splenocytes from vaccinated HLA-A2<sup>+</sup>huCD8<sup>+</sup> transgenic mice revealed infiltration with mamA2.1 tetramer<sup>+</sup>CD8<sup>+</sup> T cells (6.6%). In contrast, no mamA2.1 tetramer<sup>+</sup> CD8<sup>+</sup> T cells (<0.2%) were detected in UACC-812 tumors from

SCID-beige mice that received splenocytes from control transgenic mice.

#### Expansion of mamA2.1 tetramer<sup>+</sup> T cells in breast cancer patients

The above data demonstrated that mamA2.1 tetramers could detect mam-A specific cytotoxic CD8<sup>+</sup> T cells in the murine model. We next investigated whether the mamA2.1 tetramers could be utilized to monitor CD8<sup>+</sup> CTL in human subjects with breast cancer. Towards this, PBMC isolated from ten HLA-A2 positive breast cancer patients and normal subjects were analyzed using the mam-A2.1 tetramers. The clinical and demographic profile of the study subjects is given in Table 2. In this preliminary analysis, only Caucasians were included. In addition, none of the patients were currently lactating. There was no significant difference in age (patients:  $54.2 \pm 2.7$  vs. controls  $45.7 \pm 3.5$ ,  $P = 0.08$ ), gravida (patients:  $1.4 \pm 0.3$  vs. controls  $1.8 \pm 0.4$ ,  $P = 0.46$ ), BMI (patients  $28.5 \pm 1.3$  vs.



**Fig. 4** Mam-A specific CD8<sup>+</sup> CTLs induce regression of established tumor colonies. (A) HLA-A2<sup>+</sup>huCD8<sup>+</sup> transgenic mice were vaccinated with mam-A DNA or empty PCI-neo vector. Subsequently, whole splenocytes from vaccinated (circles) and control (triangles) mice or fractionated CD8<sup>+</sup> T cells from vaccinated mice (broken line) were adoptively transferred into SCID-beige mice ( $n = 3$  in each group) with previously established UACC-812 (HLA-A2<sup>+</sup> Mam-A<sup>+</sup>) or MCF-7 (HLA-A2<sup>+</sup>Mam-A<sup>-</sup>) human breast cancer cell lines. Tumor volume was analyzed weekly for 4 weeks and the results are presented as the mean  $\pm$  standard error of the three SCID-beige mice in each group. (B) Infiltration of mamA2.1 tetramer<sup>+</sup> CD8<sup>+</sup> T cells in

regressing human breast cancer colonies. HLA-A2<sup>+</sup>huCD8<sup>+</sup> transgenic mice were vaccinated with mam-A cDNA or empty PCI-neo vector. Subsequently, splenocytes from vaccinated and control mice were adoptively transferred into SCID-beige mice ( $n = 3$  in each group) with previously established UACC-812 (HLA-A2<sup>+</sup>Mam-A<sup>+</sup>) human breast cancer cell line. At 2 weeks, the tumors from both groups were excised and the tumor infiltrating lymphocytes (TIL) were stained using mamA2.1 tetramers and human anti-CD8. A representative flow cytometry dot plot from both groups is demonstrated

controls  $26.2 \pm 1.2$ ,  $P = 0.20$ ). Tumor stage distribution of breast cancer patients was: (I = 4, II = 4, III = 1, IV = 1).

PBMC were isolated and kept frozen at  $-135^{\circ}\text{F}$  until analysis. After thawing at the time of analysis, the cells were suspended in complete medium (described in the methodology) and incubated overnight at  $37^{\circ}\text{C}$  at 5%  $\text{CO}_2$ . All samples were tested in quadruplicate and analyzed at the same time. As shown in Table 2, breast cancer patients revealed an increased frequency of mamA2.1 tetramer<sup>+</sup> T cells compared to normal subjects ( $2.86 \pm 0.8\%$  vs.  $0.71 \pm 0.1\%$ ,  $P = 0.01$ ). There was no staining of peripheral T cells in either group with a control tetramer (Cancer patients  $0.55 \pm 0.2\%$  vs. Normal control  $0.39 \pm 0.3\%$ ,  $P = 0.8$ ).

Based on murine studies, we speculated that the frequency of mam-A2.1 tetramer<sup>+</sup> T CD8<sup>+</sup> T cells would

correlate with IFN- $\gamma$  production in response to mamA2.1. Towards this, fractionated CD8<sup>+</sup> T cells from PBMC of breast cancer patients or normal subjects were cultured with irradiated autologous PBL and mamA2.1 peptide. IFN- $\gamma$  production was analyzed using ELISPOT assays. As shown in Table 2, breast cancer patients had significantly increased IFN- $\gamma$  production in response to mamA2.1 ( $48.1 \pm 20.9$  vs.  $2.9 \pm 0.8$ ,  $P = 0.03$ ). There was no difference in the immune response against the control FLU peptide (Cancer patients  $66 \pm 23$  spm vs. Normal control  $79 \pm 43$ ,  $P = 0.6$ ).

## Discussion

Induction of immunity against the plasmid encoded proteins involves several mechanisms. Following transfection,

**Table 2** Demographic profile with mamA2.1 tetramer<sup>+</sup> and mamA2.1 specific IFN- $\gamma$  secreting CD8<sup>+</sup> T cells in the study subjects

Breast cancer patients									
Patient	Age	Race	Gravida	Menopause	BMI	Stage	Tetramer frequency (%)	IFN- $\gamma$ ELISPOT (spm)	
01	54	C	0	N	29.3	I	5.5	98	
02	41	C	1	N	31.3	IV	0	3	
03	55	C	3	N	34.8	IIA	1.2	8	
04	55	C	2	N	27.1	III	2.6	11	
05	69	C	1	Y	29.9	IIA	8.0	212	
06	48	C	2	N	25.2	IIA	2.5	14	
07	61	C	3	Y	24.4	I	3.4	55	
08	63	C	0	Y	33.1	I	3.1	65	
09	51	C	1	N	21.2	I	0.1	2	
10	45	C	1	N	29.1	IIA	2.2	13	
Mean	54.2 $\pm$ 2.7 <sup>a</sup>		1.4 $\pm$ 0.3 <sup>b</sup>		28.5 $\pm$ 1.3 <sup>c</sup>		2.86 $\pm$ 0.8 <sup>d</sup>	48.1 $\pm$ 20.9 <sup>e</sup>	
Normal subjects									
01	56	C	4	Y	27.1	NA	1.1	2	
02	45	C	0	N	28.2		0.2	3	
03	34	C	0	N	21.7		0.1	5	
04	61	C	1	Y	28.8		0.9	0	
05	27	C	2	N	30.3		0.5	1	
06	60	C	3	Y	31.7		0.9	5	
07	48	C	2	N	26.5		1.1	1	
08	37	C	2	N	23.0		0.8	0	
09	44	C	1	N	21.2		0.2	4	
10	45	C	3	N	23.5		1.3	8	
Mean	45.7 $\pm$ 3.5 <sup>a</sup>		1.8 $\pm$ 0.4 <sup>b</sup>		26.2 $\pm$ 1.2 <sup>c</sup>		0.71 $\pm$ 0.1 <sup>d</sup>	2.9 $\pm$ 0.8 <sup>e</sup>	

*P* values: <sup>a</sup> 0.08, <sup>b</sup> 0.46, <sup>c</sup> 0.20, <sup>d</sup> 0.01, <sup>e</sup> 0.03

somatic cells express the plasmid genes. The encoded proteins are transcribed intracellularly and then processed and presented on the cell surface in the context of MHC class I. CD8<sup>+</sup> T cells can recognize the MHC class I/peptide complexes and induce effector functions [22]. In addition, the transfected somatic cells can cross-prime antigen presentation cells (APC) following cell death. The primed APC can activate CD4<sup>+</sup> or CD8<sup>+</sup> T cells by presenting the plasmid proteins in the context of MHC class II or I, respectively. Alternatively, the plasmid DNA can directly transfect the APC and activate CD4<sup>+</sup> and CD8<sup>+</sup> T cells. It has been postulated that CD8<sup>+</sup> CTL are important effector T cells in tumor specific immune response against melanomas, renal, and ovarian cell carcinoma [23–25]. In addition, metastatic effusions from breast cancer patients have been shown to contain HLA class I-restricted autologous tumor-specific CD8<sup>+</sup> CTL [26]. However, it is unclear whether CD8<sup>+</sup> T cells can induce breast cancer immunity.

Several breast cancer associated antigens have been identified including MUC-1, HER-2, CEA, and p53. Even though HER-2 and MUC-1 have been tried in cancer

immunotherapy, the proportion of breast tumors expressing any one of these antigens is low. Hence, despite the demonstration that in vitro generated CD8<sup>+</sup> CTL can recognize these antigens, attempts to induce active breast cancer-specific immunity with these antigens have been limited to very selected patients [27]. Mam-A is a novel breast-tumor associated antigen that is a unique target for inducing CD8<sup>+</sup> CTL due to its widespread and specific expression in primary as well as metastatic breast cancer [1–4]. Therefore, definition of immune responses against mam-A in breast cancer patients should be of value not only for monitoring tumor immunity but also identifying candidates for possible vaccination.

DNA vaccination can induce CD8<sup>+</sup> CTL that have been shown to be important mediators of immunity against a variety of tumors including melanomas, renal, and ovarian cell carcinoma [23–25]. With specific tetramers, it is possible to enumerate antigen-specific T cells using flow cytometry. Tetramer assays are highly sensitive and less labor intensive for immune monitoring compared to other techniques like ELISPOT, limiting dilution, and trans vivo delayed type hypersensitivity assays [28]. HLA class-I



tetramers carrying TAA epitopes have been used to monitor melanoma-specific immune responses following DNA vaccination [29, 30]. The use of tetramers for mam-A requires knowledge of immunodominant peptides. Therefore, we characterized the immunodominant peptides of the mam-A protein (Table 1). The HLA class-I peptide binding prediction program from the Bio informatics & Molecular Analysis Section of the National Institutes of Health predicted seven mam-A peptides that could be presented in the context of HLA-A2 [19]. Of these seven peptides, mamA2.1 revealed the highest HLA-A2 binding affinity (Table 1). To determine the immunogenicity of these peptides, CD8<sup>+</sup> CTL lines were developed by repeated stimulation of PBMC isolated from HLA-A2 female donors with peptides pulsed TAP-2 deficient T cells. The CD8<sup>+</sup> CTL were confirmed to be HLA-A2 restricted and mam-A specific. Both cell lines revealed significant cytotoxicity as well as IFN- $\gamma$  production in response to mamA2.1 peptide [19]. Therefore, mamA2.1 peptide was used in the tetramer assays.

Vaccination of HLA-A2<sup>+</sup>huCD8<sup>+</sup> transgenic mice led to expansion of mamA2.1 tetramer<sup>+</sup> CD8<sup>+</sup> T cells in the peripheral blood. The expansion of mamA2.1 tetramer<sup>+</sup> CD8<sup>+</sup> T cells was a direct result of mam-A DNA vaccination since empty vectors did not increase tetramer<sup>+</sup> cells. To correlate development of mamA2.1 tetramer<sup>+</sup> T cells with breast cancer specific immunity, we isolated CD8<sup>+</sup> T cells from the vaccinated and control mice and tested their ability to induce tumor lysis in vitro. The CD8<sup>+</sup> T cells from the vaccinated mice, but not control, induced lysis of human breast cancer cells in vitro. Furthermore, the lysis of cancer cells was mam-A specific and HLA-A2 restricted since breast cancer cells not expressing mam-A or HLA-A2 were not affected. We also demonstrated the ability of these tetramer<sup>+</sup> CD8<sup>+</sup> T cells to infiltrate into the tumor resulting in tumor regression, in vivo. Whole splenocytes as well as fractionated CD8<sup>+</sup> T cells from vaccinated mice both induced regression of human breast cancer colonies in SCID-beige mice. The tumor regression with fractionated CD8<sup>+</sup> T cells paralleled that observed with whole splenocytes. Again, only UACC-812 cancer cells that express both mam-A and HLA-A2 demonstrated tumor regression while MCF-7 cells that are mam-A negative continued to grow. Taken together, these data indicate that mam-A DNA vaccination leads to the development of HLA-restricted CD8<sup>+</sup> CTL that can effectively kill mam-A expressing human breast cancer colonies both in vivo and in vitro. Significantly, after completion of the vaccination, CD8<sup>+</sup> T cells can alone mediate tumor lysis. Furthermore, the CD8<sup>+</sup> CTL can be effectively monitored using mam-A HLA class I tetramers. Nevertheless, we do not exclude the role of other immune cells like CD4<sup>+</sup> T cells and natural killer cells in breast cancer immunity.

There was an expansion of mamA2.1 positive CD8<sup>+</sup> CTL in breast cancer subjects compared to normal controls. However, the levels of mamA2.1 tetramer<sup>+</sup> CD8<sup>+</sup> T cells varied in the patient population. It is known that patients with similar tumor stage have variable disease recurrence. One of the factors that can influence the rate of disease recurrence is immunity against TAA. We hypothesize that patients with low frequency of mam-A specific T cells would have sub-optimal breast cancer immunity and, therefore, a poorer long-term outcome. This is supported by the fact that patient 02 with stage IV tumor had no detectable mamA2.1 reactive CD8<sup>+</sup> T cells and no IFN- $\gamma$  production upon stimulation with mamA2.1 peptide. Such patients may be good candidates for mam-A DNA vaccination. However, we cannot exclude the possibility that these patients are tolerant to mam-A peptides accounting for the unresponsiveness. Therefore, further studies are required to address these questions and correlate mam-A tetramer<sup>+</sup> T cell frequency in breast cancer patients with disease outcome. The use of DNA vaccination, unlike peptide vaccination, is not restricted by HLA specificities. Therefore, mam-A DNA vaccination can be used in subjects with specificities other than HLA-A2. In this report we included patients with HLA-A2 since this is the most prevalent HLA class I allele in the Caucasian population (estimated gene frequency 28.6%, [http://www.ashi-hla.org/publicationfiles/archives/prepr/mori\\_gf.htm](http://www.ashi-hla.org/publicationfiles/archives/prepr/mori_gf.htm)). We have also recently identified the HLA-A3 (gene frequency in Caucasian population 13.4%) binding immunogenic mam-A peptides [31] that would enable surveillance of breast cancer immunity in the majority of patients.

It is noteworthy that our murine model, although unique for characterizing the role of CD8<sup>+</sup> T cells, is a xenograft model. Mam-A is not a self-antigen in the double transgenic mice used in our experiments. Although the properties of mam-A described earlier make it a viable candidate for breast cancer vaccination, it can be argued that there may be tolerogenic mechanisms in human subjects that may prevent mam-A DNA vaccination. We were able to easily develop human CD8<sup>+</sup> CTL against mam-A from healthy females (Fig. 1). In fact, even a single stimulation of peripheral blood lymphocytes with mam-A peptides induced lysis of human breast cancer cells in vitro (data not shown). Furthermore, there was expansion of mam-A specific T cells in breast cancer patients compared to normal subjects (Table 2). These data suggest that mam-A immunity can be achieved in human subjects. However, this question can definitively be addressed only by a human trial.

In conclusion, here we demonstrated that mam-A DNA vaccination leads to the development of CD8<sup>+</sup> CTL that can induce breast cancer immunity. These CD8<sup>+</sup> CTL can be detected in the peripheral blood of vaccinated hosts using mam-A peptide HLA class-I tetramers. Studies are in

progress to use mam-A DNA to vaccinate against human breast cancer. The results presented here strongly indicate that the effect of vaccination can be monitored using mam-A tetramers.

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