Published in final edited form as:
Hum Immunol. 2012 January ; 73(1): 11–16. doi:10.1016/j.humimm.2011.10.017.

Identification of HLA-A24 restricted CD8+ Cytotoxic T-Cell Epitopes Derived from Mammaglobin-A, A Human Breast Cancer Associated Antigen

Venkataswarup Tiriveedhi[1], **Nayan J Sarma**[1], **Vijay Subramanian**[1], **Timothy P. Fleming**[2], **William E. Gillanders**[1], and **T. Mohanakumar**[1,2]

[1]Department of Surgery, Washington University School of Medicine, Saint Louis, MO

[2]Department of Pathology & Immunology, Washington University School of Medicine, Saint Louis, MO

Abstract

Human breast cancer associated antigen, Mammaglobin-A (Mam-A), potentially offers a novel therapeutic target as breast cancer vaccines. In this study, we define the $CD8^+$ CTL response to Mam-A derived candidate epitopes presented in the context of HLA-A24 (A*2402). HLA-A24 has a frequency of 72% in Japanese, 27% in Asian-Indian and 18% in Caucasian populations. Using HLA-binding prediction algorithm we identified seven HLA-A24 restricted Mam-A-derived candidate epitopes (MAA24.1–7). Membrane stabilization studies with TAP-deficient T2 cells transfected with HLA-A2402 (T2.A24) indicated that MAA24.2 (CYAGSGCPL) and MAA24.4 (ETLSNVEVF) have the highest HLA-A24 binding affinity. Further, two $CD8^+$ CTL cell lines generated *in vitro* against T2.A24 cells individually loaded with Mam-A-derived candidate epitopes showed significant cytotoxic activity against MAA24.2 and MAA24.4. In addition, the same $CD8^+$ CTL lines lysed the HLA-$A24^+$/Mam-A^+ stable transfected human breast cancer cell line AU565 and MDA-MB-361. However, these CTLs had no cytotoxicity against HLA-$A24^-$/Mam-A^+ and HLA-$A24^+$/Mam-A^- breast cancer cell lines. In summary, our results define HLA-A24-restriced, Mam-A-derived, $CD8^+$ CTL epitopes which can potentially be employed for Mam-A-based breast cancer vaccine therapy to breast cancer patients with HLA-A24 phenotype.

Keywords

Vaccine; HLA-A24; CD8 T cell; Mammaglobin-A; Breast Cancer

1. Introduction

American cancer society facts and figures 2010 confirm that breast cancer remains the most common cancer, representing an estimated 27% of all new cancer cases in women. Conventional treatment approaches for advanced breast cancers are highly invasive and

Address all correspondence to: T. Mohanakumar, Ph.D., Washington University School of Medicine, Box 8109, 3328 CSRB, 660 South Euclid Avenue, Saint Louis, MO 63110, Telephone: (314) 362-8463, Fax: (314) 747-1560, kumart@wustl.edu.

mostly offer only a palliative effect [1]. Plasmid DNA vaccine by its immunomodulatory effect offers novel anti-tumor therapeutic approach [2]. Tumor associated antigens (TAA) offer specific immunological targets by inducing antigen-specific cytotoxic cellular responses [3]. Early studies demonstrating the generation of a cellular immune response against malaria and HIV peptides in human beings by DNA vaccination have offered promising clinical application of this immunization technique [4, 5].

Mammaglobin-A (Mam-A), is a 10kDa breast cancer associated protein, related to the family of epithelial secretory proteins.[6] Mam-A is expressed in up to 80% of human breast cancers including non-invasive, invasive, and metastatic breast cancer [7]. Because of its almost universal expression by all kinds of breast cancers Mam-A offers a superior antitumor breast cancer target over other TAA like MUC1 and Her-2/neu which are expressed only in 20–30% of breast cancers [8, 9]. Previous studies have demonstrated that vaccination with dendritic cells pulsed with Her-2/neu-derived peptides induced specific CD8+ cytotoxic lymophocyte (CTL) response in breast cancer patients [9]. However, the limited frequency of expression of Her2/neu on breast tumors makes it suitable for only a very limited group of patients to be considered for clinical immune therapies against breast cancer [10, 11]. We have previously demonstrated that Mam-A DNA vaccination could lead to immunity against breast cancer in HLA-A2+huCD8+ double transgenic mice [12]. Human Mam-A gene when cloned into the PCI-neo vector and administered intramuscularly into these transgenic mice demonstrated specific lysis of Mam-A producing human breast cancer colonies both *in vitro* and *in vivo* [12].

The definition of immune responses against broadly expressed breast cancer-specific antigens such as Mam-A should be of great help in the development of new therapeutic strategies including single chain trimer based techniques for the treatment and prevention of breast cancer. In this regard, we have previously identified four HLA-A2, A3 and B7 restricted Mam-A-derived epitopes recognized by breast cancer patients *in vivo* and showed that a CD8+ CTL line developed *in vitro* against these specifc epitopes [13–15]. In our current study, we have identified 2 immunogenic HLA-A24 restricted Mam-A peptides, which is a common allele in Japanese and Asian-Indian population [16]. Using these epitopes we demonstrate that an *in vitro* generated CD8+ CTL-cell can specifically lyse human breast cancer cells in a Mam-A specific and HLA-A24 restricted manner.

2. Materials and Methods

2.1. Study subjects

Two HLA-A24 (*A2402) positive healthy female volunteers were enrolled in this study after obtaining informed consents. HLA typing was performed using sequence-specific oligonucleotide probes that provided low-medium resolution for HLA-A alleles (Dynal Biotech, Lafayette Hill, PA).

2.2. Breast cancer cell lines

Breast cancer cell lines in this study were obtained from the American Type Culture Collection (Manassas, VA). The 3 breast cancer cell lines used were AU-565, MDA-MB-361, and MCF-7. AU-565 and MDA-MB-361 cell lines express Mam-A [13]. Breast Cancer cell lines were cultured until they reached optimal confluency. The presence of Mam-A in the cell lines were determined by reverse transcriptase-polymerase chain reaction and western blot analysis.

2.3. Cloning of HLA-A*2402 cDNA

Total RNA from PBLs of HLA-A24 healthy donor was extracted and polyA RNA isolated using Ambion polyA kit. cDNA mixture was synthesized from the RNA by reverse transcription reaction using Superscript II and oligo(dT) primer as described previously. PCR was performed using 2 μL of the reverse transcription mixture, Pfu DNA polymerase (Stratagene, La Jolla, CA), and 50 pmol of forward and reverse primers in a total reaction volume of 50 μL. Nucleotide sequences of the forward and reverse primers are 5-GATCGACTCAGATGATATCCAGACGCCGAGGATGGCCGTCATG-3 and 5 CGCGGATCCGCGGCCGCAGGGAGCACAGGTCAGCGTGGGAAGATC-3, which contain HLA-A24 gene-specific sequence flanked by *Eco*RV and *Bam*HI restriction sequence, respectively. The amplification protocol consisted of denaturation for 45s at 98°C, annealing for 45s at 58°C, and extension for 4 min at 72°C for a total of 30 cycles, using a BioRad Mycycler™ (Hercules, CA). The PCR product was purified and cloned into pIRESpuro mammalian expression vector (Clontech, Palo Alto, CA). Cell with stable transfection were selected by growing them in whole media containing 2μg/mL puromycin.

2.4. Candidate Peptides

Mam-A-peptides that bind the HLA-A24 molecule were identified using the HLA class I-peptide binding computer algorithm from the Bioinformatics & Molecular Analysis Section of the National Institutes of Health at http://www.syfpeithi.de/Scripts/MHCServer.dll/EpitopePrediction.htm. Seven Mam-A-derived peptides with varying levels of binding affinity for the HLA-A24 molecule were synthesized for this study (Table 1). EBV peptide sequence TYGPVFMSL which has been shown to bind HLA-A24 allele was used as positive control. Peptides were synthesized by peptide2.0 Inc (Chantilly, VA). The purity of peptides was determined by high-performance liquid chromatography and mass spectrometry.

2.5. Membrane stabilization assay

The HLA-A24 binding ability of the Mam-A derived peptides were analyzed by cell membrane stabilization of the HLA-A24 molecule in the TAP-deficient T2 cells transfected with HLA-A*2402(T2.A24). T2.A24 (1×10^6/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 g/ml streptomycin along with 3μg/ml of human β2m (Sigma). After 16 hours of incubation, the T2.B7 cells were washed (3×) and the levels of HLA-A24 expression were determined by flow cytometric analysis. Briefly, the T2.A24 cells were incubated for 30 min at 4°C with the anti-HLA-A24-FITC (MBL International, Woburn, MA) and resuspended in phosphate-buffered saline (PBS) supplemented with 2% bovine serum albumin (BSA), 25 mM HEPES, and 0.02% sodium azide. The cells were then washed (3×) and incubated for 30 min at 4°C with FITC-conjugated goat anti-mouse IgG (10 μg/ml, Becton Dickinson, Franklin Lakes, NJ). The cells were then washed (3×), fixed in 1% paraformaldehyde, and used in a single-color flow cytometric analysis in a FACScan flow cytometer (Becton Dickinson). T2.B7 cells incubated in the presence of the EBV peptide sequence TYGPVFMSL that binds to HLA-A24 was used as positive control. Results expressed as the mean fluorescence shift corresponded to the difference between the mean fluorescence obtained with T2.A24 cells incubated in the presence of the peptide (experimental) and the mean fluorescence obtained with ‘unloaded’ T2.A24 cells cultured in the absence of peptides (negative control).

2.6. Generation of Mam-A reactive HLA-A24 restricted CD8+ CTL lines

We generated two CD8+ CTL cell lines against HLA-A24-restricted Mam-A epitopes *in vitro* as described previously. Briefly peripheral blood lymphocytes (2×10^6) 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 (5,000 rads) T2.A24 cells (1×10^6) loaded with Mam-A-A24.1–7 or control EBV peptide TYGPVFMSL. β2m (3 μg/ml), CD3 and CD28 mAb (500 ng/ml, BD Biosciences) were added to the cultures. In addition, recombinant human IL-2 (20 U/ml, Chiron, Emeryville, CA) and 5% T cell growth factor prepared from pooled human splenocyte supernatant were added to the cultures after 24 hrs. PBLs (2×10^5) were restimulated every 8–10 days with irradiated, peptide-loaded T2.A24 cells (1×10^6) in the presence of irradiated (3,000 rads) autologous peripheral blood lymphocytes (2×10^6) as antigen presenting cells in 24-well plates in 2 ml of culture medium supplemented with IL-2, β2m, anti-CD3 and anti-CD28. After stimulation, the CD8+ CTL were purified by negative selection (Human CD8+ enrichment kit, Stem cell, Canada). The purity of resulting CD8+ T cells obtained by this protocol were verified to be >95% (data not shown).The Mam-A and peptide specific cytotoxic activities of the resulting CD8+ CTL lines were analyzed 7 days after 4 stimulations. At the end of 4 stimulations, CD8+ CTLSs were isolated by immunomagnetic separation (Stem Cell, Canada) and the resulting purity was verified to be >95%.

2.7. Cytotoxicity assay

The CD8+CTL efficiency to lyse the target cells was investigated by non-irradiative LDH cytotoxicity assay (Promega, Madison, WI). Peptide-loaded T2.A24 cells or breast cancer cells (5×10^3 cells) in 100μl of complete medium at were plated in triplicate cultures in round bottom 96-well plates in the presence of varying numbers of CD8+T cells (6.25:1 to 50;1) and incubated at 37°C in a humidified 5% CO_2 incubator for 4 hrs. Non-transfected cell lines was used to test non-specific lysis by NK cells. Maximal 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 protocol and measured using UV/Visible spectrophotometer at 450 nm. The percentage specific lysis was calculated as follows: [(experimental LDH release − spontaneous LDH release)/(maximum LDH release − spontaneous LDH release)] × 100. Epitope-specificity of breast cancer cell lysis by CD8+CTLs was further determined in a cold target inhibition assay by analyzing the capacity of peptide-loaded unlabeled T2.A24 cells to block the lysis of breast cancer cells at an inhibitor to target ratio of 50:1.

3. Results

3.1. Identification of candidate HLA-A24 restricted CD8+CTL epitopes derived from Mam-A

Using a HLA class I-peptide binding prediction software algorithm we determined seven 9-mer candidates Mam-A derived epitopes for the HLA-A24 molecule. Peptides with varying levels of binding affinity for the HLA-A24 molecule were identified (Table 1) and synthesized by Peptide2.0 Inc (Chantilly, VA). EBV peptide sequence TYGPVFMSL was used as positive control.

3.2. Membrane stabilization assay

Based on the computer-assisted analysis, we screened Mam-A 9-mer peptides with highest binding probability for HLA-A24. Seven Mam-A peptides were analyzed (Table 1). The actual binding affinity of the epitopes to HLA-A24 molecule was determined by membrane stabilization assay using TAP deficient T2 cells transfected with HLA-A*2402(T2.A24). As shown in figure 1, the peptides MAA24.2 (CYAGSGCPL) and MAA24.4 (ETLSNVEVF)

displayed a high affinity for HLA-A24 molecule (176±41 A.U and 145±39 A.U, respectively) almost comparable to the binding affinity of EBV derived peptide (TYGPVFMSL) (257±61 A.U). In contrast, the peptides MAA24.3 (IYDSSLCDL) MAA24.6 (VLMLAALSQ) and MAA24.7 (ELKECFLNQ) demonstrated significantly low binding compared to EBV derived peptide. These results clearly demonstrate a discrepancy between the computer based prediction and actual binding capacity of Mam-A derived epitopes. This is expected since the binding affinity for a given epitope in a MHC class I groove is determined by both its amino acid sequence as well as the 3 dimensional structure of the binding motif. Several studies have documented this phenomenon between the predicted and the experimental binding affinity for epitopes in the MHC class I groove [17, 18]. Based on the HLA-A24 membrane stabilization assays as shown in figure 1, we identified 2 Mam-A epitopes, MAA24.2 and MAA24.4 which demonstrate high binding affinities to HLA-A24 molecule.

3.3. Specific lysis of HLA-A24$^+$/Mam-A$^+$ human breast cancer cells by HLA-A24 restricted CD8+ CTLs from HLA-A24 subjects

In order to determine the cytotoxic reactivity of HLA-A24 restricted CD8+CTLs against human breast cancer cell lines that endogenously express HLA-A24 and Mam-A, we determined the specific lysis of 4 different breast cancer cell lines by CD8+CTL lines generated *in vitro*. The 3 breast cancer cell lines used were AU-565, MDA-MB-361, and MCF-7. AU-565 and MDA-MB-361 cell lines express Mam-A, while MCF-7 did not express Mam-A. The 3 human breast cancer cell lines used for this assay were transfected with HLA-A24. The specific expression of mRNA of HLA-A24 and Mam-A is given in figure 2. The CD8+ CTLs generated from two HLA-A24 positive healthy donor were stimulated by pooled T2.A24 cells loaded with all seven peptides (MAA24.1–7) derived from Mam-A. As indicated in figure 3, both CD8+ CTLs demonstrated significant cytotoxicity against the transfected breast cancer cell line AU-565, which expresses both HLA-A24 and Mam-A (8±2% at 6.25:1; 19±4% at 12.5:1; 31±6% at 25:1; and 59±9% at 50:1) but not against non-transfected AU-565 (HLA-A24$^-$/Mam-A$^+$). Similarly, transfected MDA-MB 361 (HLA-A24$^+$/Mam-A$^+$), demonstrated significant cytotoxicity over the wild type MDA-MB 361 (HLA-A24$^-$/Mam-A$^+$) at all E:T ratios (12.5, 25 and 50). More importantly, no cytotoxicity was observed against both transfected and non-transfected MCF-7 which lacks Mam-A expression. These results demonstrate that the generated HLA-A24 restricted CD8+CTLs were specific against breast cancer cells expressing Mam-A epitopes presented in the context of HLA-A24.

3.4. Inhibition of cytotoxicity by HLA-A24 Abs

To confirm the presentation of the Mam-A24 peptide by the HLA-A24 molecule, the cytotoxic activity of MAA24.2 and MAA24.4 the anti-Mam-A CD8+ CTL line was determined in the presence of either anti-HLA-A24 or anti-HLA-A2 mAbs. As shown in Figure 4, addition of the non-specific isotype control MOPC-11 and anti-HLA-A2 mAb did not inhibit the lysis of the transfected AU-565 breast cancer cell line whereas addition of the anti-HLA-A24 Abs demonstrated significant decrease in the cytotoxic activity (from 42 ± 9% to 5 ± 3%) against the transfected AU-565 breast cancer cell line. These results confirm the HLA-A24-restriction of the Mam-A peptide recognition by the anti-Mam-A CD8+ CTL line.

3.5. Specific dominance of generation of MAA24.2 and MAA24.4 restricted CD8+T-cells

In order to determine whether HLA-A24 restricted Mam-A derived epitopes identified above could induce CD8+CTLs, PBLs from two HLA-A24+ healthy female individuals were stimulated *in vitro* in presence of a pool of T2.A24 cells individually loaded with all HLA-A24 binding Mam-A-derived peptides. Since the affinity of a given peptide to a MHC

class I molecule does not necessarily correlate with its ability to generate CD8+ CTL response, we used all the peptides to examine the profile of CD8+ CTL reactivity. After 4 stimulations, the cytotoxic activities of the resulting CD8+ CTL lines and their epitope specificity was evaluated using a standard LDH release assay over transfected AU-565 breast cancer cell line. T2 cells loaded with EBV derived epitope (TYGPVFMSL) and empty T2.A24 (no peptide) were used as negative controls. The assay was performed at effector to target (E:T) ratio 50, 25, 12.5 and 6.25 in triplicates. The results shown in figure 5 demonstrate that both CD8+CTL lines showed significant cytotoxic activity against Mam-A epitopes MAA24.2 and MAA24.4 (39±7% and 33±5%, respectively). However, MAA24.5, MAA24.6 and MAA24.7 showed very minimal cytotoxic activity (all less than 10% individually).

4. Discussion

Successful cancer-specific immunotherapy is based on the identification of tumor-specific antigens and potent CTL epitope. Proteins that are selectively expressed in cancer cells become good targets for such an approach, including MAGE from melanoma, and 5T4 of renal cell carcinoma [19, 20]. Advances in the understanding of the structural basis for MHC class I-bound peptides, a reverse immunological approach has developed, and numerous CTL epitopes for various tumor specific antigens have been successfully identified [21, 22]. The CD8+ CTLs play a critical role in host immunity to tumors due to their ability to recognize tumor-associated antigens in an MHC-restricted manner on several tumor cells and induce apoptotic cell death [23].

DNA vaccines due to their ability to induce both cytotoxic T-cell and humoral responses raised interest for their potential use in cancer immunotherapy [2]. Mam-A gene is shown to express in about 80% of primary breast carcinomas [6]. We investigated whether Mam-A-reactive T cells were expanded in breast cancer patients. Furthermore, we identified HLA-A24 restricted Mam-A derived CD8+ CTL epitopes and demonstrated that it is possible to develop Mam-A-reactive CD8+ CTL lines *in vitro* that recognize breast cancer cells naturally expressing Mam-A derived peptides. We have previously identified Mam-A epitopes presented in the context of HLA-A2, A3 and B7, the common HLA class I alleles in general population [13–15]. In this communication, we describe the identification of HLA-A24 restricted Mam-A epitopes which is also present in the population with high frequency. The HLA-A24 occurs in population at a frequency of 72% in Asian, 27% in Indian and and 18% in Caucasian populations [16]. Therefore, identification of HLA-A24 restricted Mam-A derived CD8+ cytotoxic T cell epitopes in conjunction to previously identified HLA class I locus specific HLA A2, A3 and B7 derived epitopes will provide novel strategies in designing validation for Mam-A vaccination. The efficiency of cDNA vaccination could be tested with the tetramer staining approach using the identified Mam-A immunodominant HLA-class I restricted epitopes. MHC class I-tetramer peptide technology offers a novel approach to quantify and monitor the Mam-A specific cytotoxic T-cell responses following cDNA vaccination [24, 25]. Furthermore, to increase the efficiency of cytotoxic T-cell responses to Mam-A tumor associated antigens by single chain trimers consisting of MHC class I molecule covalently linked with the identified immunodominant peptides can potentially result in a better cytotoxic T-cell responses over the cDNA vaccination [26, 27]. To further enhance the efficiency this single chain trimer approach can be combined with cDNA vaccination.

Using a HLA class I-peptide binding prediction computer program, seven Mam-A-derived peptides (MAA24.1–7) with varying levels of binding affinity for the HLA-A24 molecule were determined (Table 1). The actual binding affinity of the identified epitopes to HLA-A24 molecule was determined by membrane stabilization assay using TAP deficient T2

cells transfected with HLA-A*2402(T2.A24). The peptides MAA24.2 and MAA24. displayed a high affinity for HLA-A24 molecule comparable to the binding affinity of EBV derived peptide (RVRARLRPL). This discrepancy between the computer predicted and experimental binding affinity for a given epitope in the HLA-class I groove is most likely explained by the findings that binding affinity for an individual epitope to each HLA class I molecule is determined by both its amino acid sequence and the 3 dimensional structure of the binding motif within the HLA class I groove [13, 28, 29]. To determine whether CD8+ CTLs generated against Mam-A-derived epitopes presented by HLA-A24 were able to recognize human breast cancer cells. We generated two CD8+ CTL lines from two HLA-$A24^+$ healthy individuals using pooled T2.A24 cells individually pulsed with pooled Mam-A-derived peptides MAA24.1–7. Upon addition of these CD8+CTLs to the HLA-A24 transfected breast cancer cell lines AU-565 and MDA-MB-361 which are HLA-A24+/Mam-A+ showed specific lysis. However, only minimal lytic activity against the wild-type AU-565 and MDA-MB-361 which are HLA-A24−/Mam-A+. Similarly no lytic efficiency was noted upon addition of these CTLs to breast cancer cell line MCF-7, which is Mam-A−. The lack of reactivity of against wild type AU-565 (HLA-$A24^-$/Mam-A^+), MDA-MB 361 (HLA-$A24^-$/Mam-A^+), MCF-7 (HLA-$A24^-$/Mam-A^-) and transfected MCF-7 (HLA-$A24^+$/Mam-A^-) clearly demonstrate that the generated CD8+CTLs exhibited the cytotoxic reactivity against human breast cancer cells in a Mam-A specific and HLA-A24 restricted manner. Furthermore, of the CD8+CTLs generated with T2.A24 cell lines pulsed individually with peptides MAA24.1–7, only those CTLs generated against MAA24.2 and MAA24.4 showed highest specific lytic activity against HLA-24 transfect AU-565 breast cancer cell line. This data demonstrates that MAA24.2 and MAA24.4 are the immunodominant HLA-A24 restricted Mam-A epitope sequences. Other sequences did not induce significant lysis. This might be due to the significantly lower binding affinity of peptides, MAA24.1, MAA24.3, MAA24.5–7 for the HLA-A24 molecule as compared to the other two peptides. Breast cancers often display a heterogenous nature of antigenicity due to the differential proteosomal cleavage of the Mam-A protein [30]. In this regard, a potential limitation of our study is that we were able to analyze only a single breast cancer cell line which cannot fully evaluate the heterogenic nature of breast cancer associated tumor antigens *in vivo*. At the same time, our results demonstrate the feasibility of four Mam-A derived epitopes, MAA24.2 and MAA24.4 presented in the context of HLA-A24 molecule to induce CD8+CTL responses specifically against HLA-$A24^+$ Mam-A expressing breast cancer cells tested *in vitro*. These results will offer further insights into determining the ability of single chain construct Mam-A DNA vaccines in inducing potent CD8+ CTL and Ab mediated responses in populations with high frequency of HLA-A24 allele.

In conclusion, we identified two Mam-A derived epitopes, MAA24.2 and MAA24.4 presented in the context of HLA-A24 molecule which can be used to generate CD8+CTL *in vitro*. The generated CD8+CTLs *in vitro* demonstrated the ability to effectively lyse breast cancer cells which presented endogenously processed natural Mam-A peptide in a HLA-A24 restricted fashion. Results presented in this study with Mam-A epitopes for HLA-A24 along with already identified Mam-A epitopes for HLA A2, A3 and B7 provides opportunity for efficient breast vaccine development. Further, the identified peptide sequences would be used in future vaccine studies involving the single trimer technique and for developing tetramer staining of the HLA-A24/Mam-A responsive cytotoxic T-cells following Mam-A vaccination.

Acknowledgments

Funding Source: This project was funded by DOD/CDMRP-BCRP W81XWH-06-1-0677 (WG). TM is funded the BJC Foundation. The authors would like to thank Ms. Billie Glasscock for her assistance in submitting this manuscript.

Abbreviations Used

MamA	Mammaglobin-A
TAA	Tumor associated antigens
CTL	cytotoxic lymophocyte

References

1. Olin JJ, Muss HB. New strategies for managing metastatic breast cancer. Oncology (Williston Park). 2000; 14(5):629. [PubMed: 10853456]
2. Ada GL. The immunological principles of vaccination. Lancet. 1990; 335(8688):523. [PubMed: 1689787]
3. Bocchia M, Bronte V, Colombo MP, De Vincentiis A, Di Nicola M, Forni G, Lanata L, Lemoli RM, Massaia M, Rondelli D, Zanon P, Tura S. Antitumor vaccination: where we stand. Haematologica. 2000; 85(11):1172. [PubMed: 11074658]
4. Wang R, Doolan DL, Le TP, Hedstrom RC, Coonan KM, Charoenvit Y, Jones TR, Hobart P, Margalith M, Ng J, Weiss WR, Sedegah M, de Taisne C, Norman JA, Hoffman SL. Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. Science. 1998; 282(5388):476. [PubMed: 9774275]
5. Calarota S, Bratt G, Nordlund S, Hinkula J, Leandersson AC, Sandstrom E, Wahren B. Cellular cytotoxic response induced by DNA vaccination in HIV-1-infected patients. Lancet. 1998; 351(9112):1320. [PubMed: 9643795]
6. Watson MA, Fleming TP. Mammaglobin, a mammary-specific member of the uteroglobin gene family, is overexpressed in human breast cancer. Cancer Res. 1996; 56(4):860. [PubMed: 8631025]
7. Watson MA, Dintzis S, Darrow CM, Voss LE, DiPersio J, Jensen R, Fleming TP. Mammaglobin expression in primary, metastatic, and occult breast cancer. Cancer Res. 1999; 59(13):3028. [PubMed: 10397237]
8. Finn OJ, Jerome KR, Henderson RA, Pecher G, Domenech N, Magarian-Blander J, Barratt-Boyes SM. MUC-1 epithelial tumor mucin-based immunity and cancer vaccines. Immunol Rev. 1995; 145:61. [PubMed: 7590831]
9. Peoples GE, Goedegebuure PS, Smith R, Linehan DC, Yoshino I, Eberlein TJ. Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. Proc Natl Acad Sci U S A. 1995; 92(2):432. [PubMed: 7831305]
10. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science. 1989; 244(4905):707. [PubMed: 2470152]
11. Lohrisch C, Piccart M. HER2/neu as a predictive factor in breast cancer. Clin Breast Cancer. 2001; 2(2):129. [PubMed: 11899784]
12. Narayanan K, Jaramillo A, Benshoff ND, Campbell LG, Fleming TP, Dietz JR, Mohanakumar T. Response of established human breast tumors to vaccination with mammaglobin-A cDNA. J Natl Cancer Inst. 2004; 96(18):1388. [PubMed: 15367572]
13. Jaramillo A, Majumder K, Manna PP, Fleming TP, Doherty G, Dipersio JF, Mohanakumar T. Identification of HLA-A3-restricted CD8+ T cell epitopes derived from mammaglobin-A, a tumor-associated antigen of human breast cancer. Int J Cancer. 2002; 102(5):499. [PubMed: 12432553]
14. Jaramillo A, Narayanan K, Campbell LG, Benshoff ND, Lybarger L, Hansen TH, Fleming TP, Dietz JR, Mohanakumar T. Recognition of HLA-A2-restricted mammaglobin-A-derived epitopes by CD8+ cytotoxic T lymphocytes from breast cancer patients. Breast Cancer Res Treat. 2004; 88(1):29. [PubMed: 15538043]
15. Ilias Basha H, Tiriveedhi V, Fleming TP, Gillanders WE, Mohanakumar T. Identification of immunodominant HLA-B7-restricted CD8+ cytotoxic T cell epitopes derived from mammaglobin-A expressed on human breast cancers. Breast Cancer Res Treat. 2011; 127(1):81. [PubMed: 20544273]

16. Date Y, Kimura A, Kato H, Sasazuki T. DNA typing of the HLA-A gene: population study and identification of four new alleles in Japanese. Tissue Antigens. 1996; 47(2):93. [PubMed: 8851721]

17. Kuhns JJ, Batalia MA, Yan S, Collins EJ. Poor binding of a HER-2/neu epitope (GP2) to HLA-A2.1 is due to a lack of interactions with the center of the peptide. J Biol Chem. 1999; 274(51): 36422. [PubMed: 10593938]

18. Tanaka Y, Amos KD, Joo HG, Eberlein TJ, Goedegebuure PS. Modification of the HER2/NEU-derived tumor antigen GP2 improves induction of GP2-reactive cytotoxic T lymphocytes. Int J Cancer. 2001; 94(4):540. [PubMed: 11745441]

19. Oaks MK, Hanson JP Jr, O'Malley DP. Molecular cytogenetic mapping of the human melanoma antigen (MAGE) gene family to chromosome region Xq27-qter: implications for MAGE immunotherapy. Cancer Res. 1994; 54(7):1627. [PubMed: 8137270]

20. Tykodi SS, Thompson JA. Development of modified vaccinia Ankara-5T4 as specific immunotherapy for advanced human cancer. Expert Opin Biol Ther. 2008; 8(12):1947. [PubMed: 18990081]

21. Sato Y, Roman M, Tighe H, Lee D, Corr M, Nguyen MD, Silverman GJ, Lotz M, Carson DA, Raz E. Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. Science. 1996; 273(5273):352. [PubMed: 8662521]

22. Ulmer JB, Deck RR, Dewitt CM, Donnhly JI, Liu MA. Generation of MHC class I-restricted cytotoxic T lymphocytes by expression of a viral protein in muscle cells: antigen presentation by non-muscle cells. Immunology. 1996; 89(1):59. [PubMed: 8911141]

23. Zoller M, Christ O. Prophylactic tumor vaccination: comparison of effector mechanisms initiated by protein versus DNA vaccination. J Immunol. 2001; 166(5):3440. [PubMed: 11207302]

24. Bousso P. Generation of MHC-peptide tetramers: a new opportunity for dissecting T-cell immune responses. Microbes Infect. 2000; 2(4):425. [PubMed: 10817645]

25. Bharat A, Benshoff N, Fleming TP, Dietz JR, Gillanders WE, Mohanakumar T. Characterization of the role of CD8+T cells in breast cancer immunity following mammaglobin-A DNA vaccination using HLA-class-I tetramers. Breast Cancer Res Treat. 2008; 110(3):453. [PubMed: 17874294]

26. Huang CH, Peng S, He L, Tsai YC, Boyd DA, Hansen TH, Wu TC, Hung CF. Cancer immunotherapy using a DNA vaccine encoding a single-chain trimer of MHC class I linked to an HPV-16 E6 immunodominant CTL epitope. Gene Ther. 2005; 12(15):1180. [PubMed: 15800656]

27. Wang B, Primeau TM, Myers N, Rohrs HW, Gross ML, Lybarger L, Hansen TH, Connolly JM. A single peptide-MHC complex positively selects a diverse and specific CD8 T cell repertoire. Science. 2009; 326(5954):871. [PubMed: 19892989]

28. Flower DR. Towards in silico prediction of immunogenic epitopes. Trends Immunol. 2003; 24(12): 667. [PubMed: 14644141]

29. Liu W, Meng X, Xu Q, Flower DR, Li T. Quantitative prediction of mouse class I MHC peptide binding affinity using support vector machine regression (SVR) models. BMC Bioinformatics. 2006; 7:182. [PubMed: 16579851]

30. Tammen H, Kreipe H, Hess R, Kellmann M, Lehmann U, Pich A, Lamping N, Schulz-Knappe P, Zucht HD, Lilischkis R. Expression profiling of breast cancer cells by differential peptide display. Breast Cancer Res Treat. 2003; 79(1):83. [PubMed: 12779085]

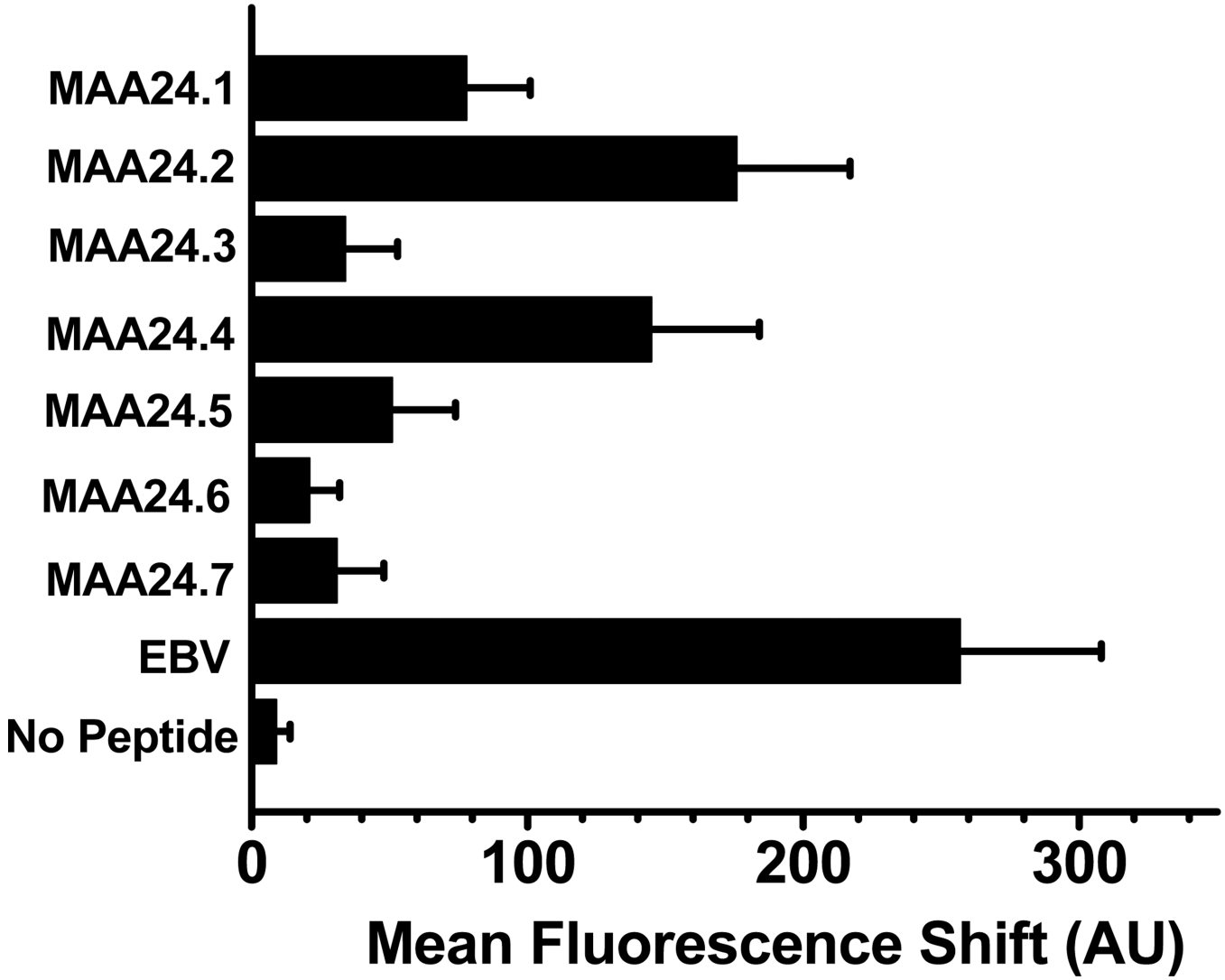


Figure 1.
HLA-24 membrane stabilization by Mam-A-derived candidate epitopes is a surrogate for binding affinity. Results are expressed as mean fluorescence shift ± standard deviation.

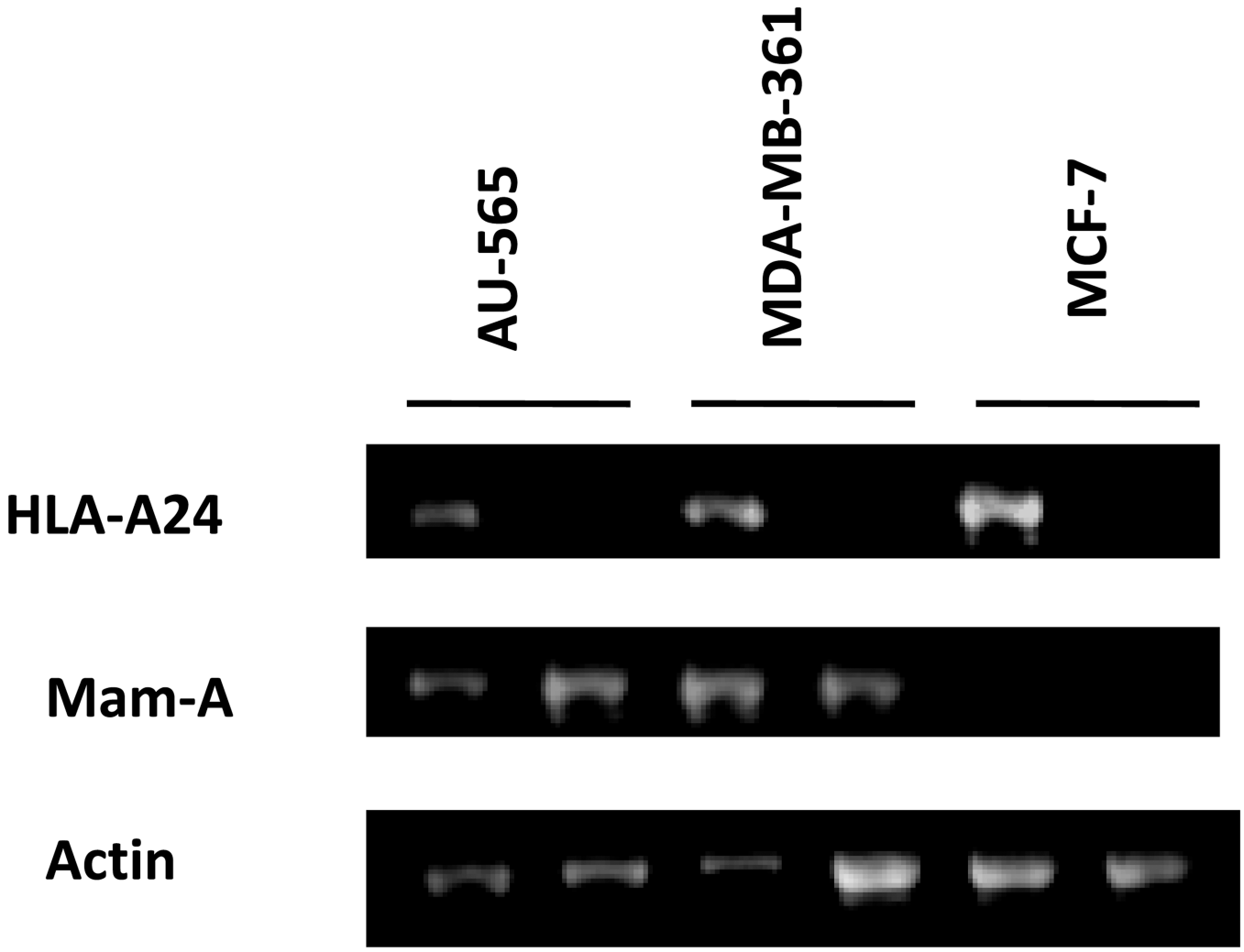
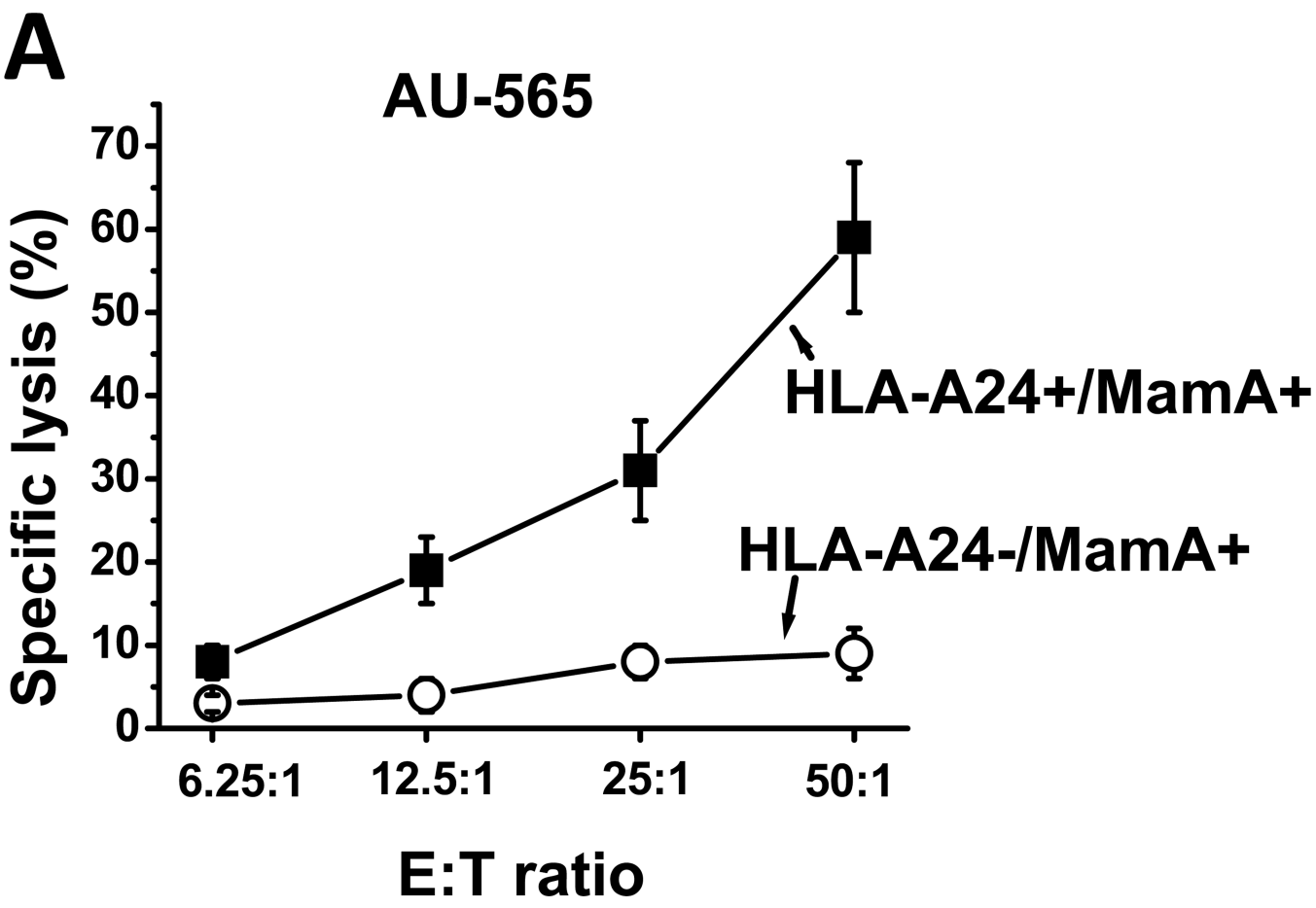
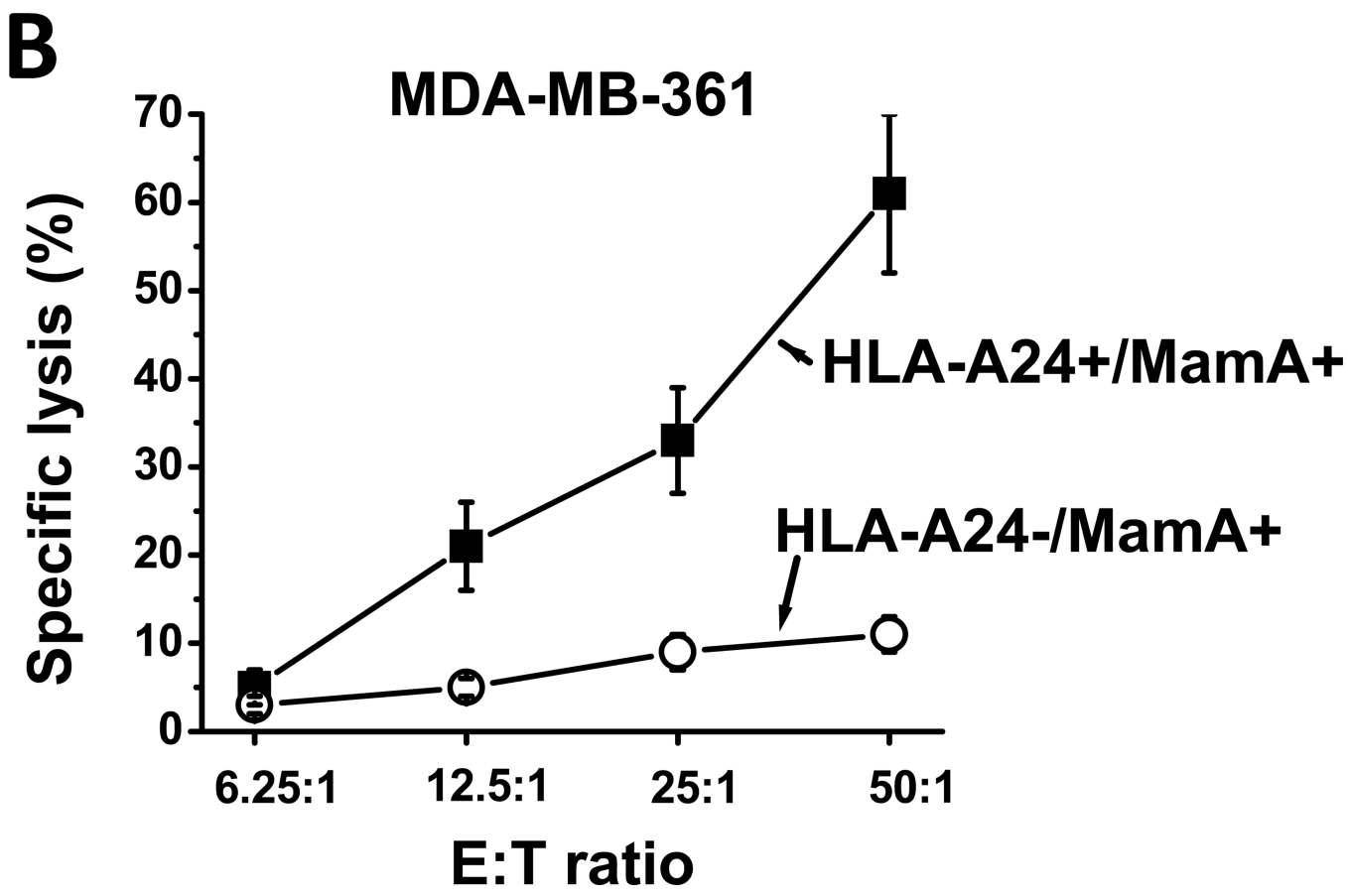


Figure 2.
Expression of Mam-A and HLA-A24 in stable transfected and wild type breast cancer cell lines.





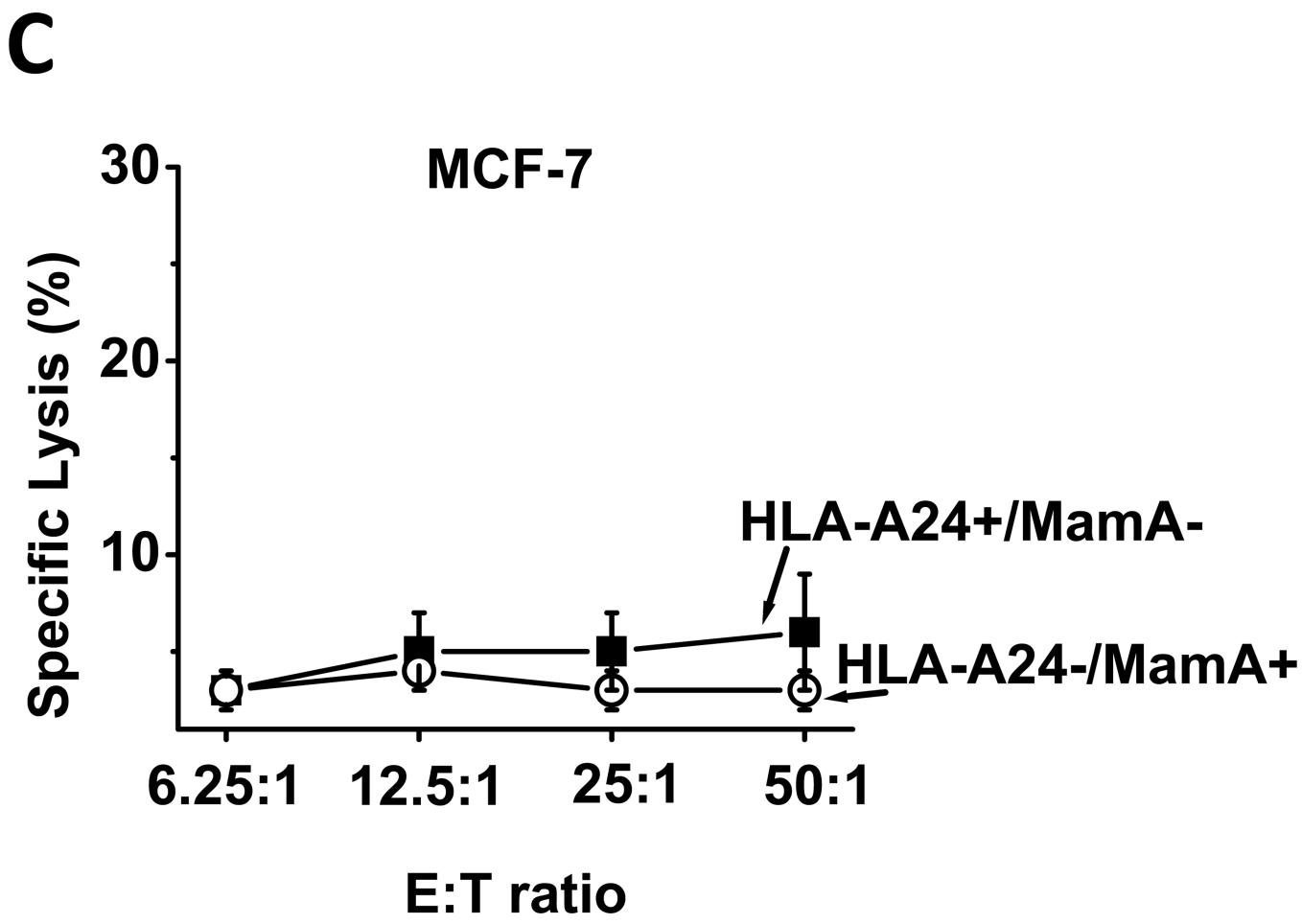


Figure 3.

CD8$^+$ CTL lines generated *in vitro* with candidate epitope-pulsed T2.A24 cells recognize breast cancers in a Mam-A-specific and HLA-A24-restricted fashion. Two CD8$^+$ CTL lines were generated from HLA-A24$^+$ healthy females by means of four weekly *in vitro* stimulations with pooled T2.A24 cells individually loaded with MAA24.1–7. The Mam-A specificity, and HLA-A24-restriction of the resulting CD8$^+$ CTL lines were evaluated against the AU-565 (transfected: HLA-A24$^+$/Mam-A$^+$; wild type: HLA-A24$^-$/Mam-A$^+$), MDA-MB-361 (transfected: HLA-A24$^+$/Mam-A$^+$; wild type: HLA-A24$^-$/Mam-A$^+$) and MCF-7 (transfected: HLA-A24$^+$/Mam-A$^-$; wild type: HLA-A24$^-$/Mam-A$^-$) breast cancer cell lines by means of a standard LDH release assay. Results obtained in triplicate cultures are expressed as mean ± standard deviation.

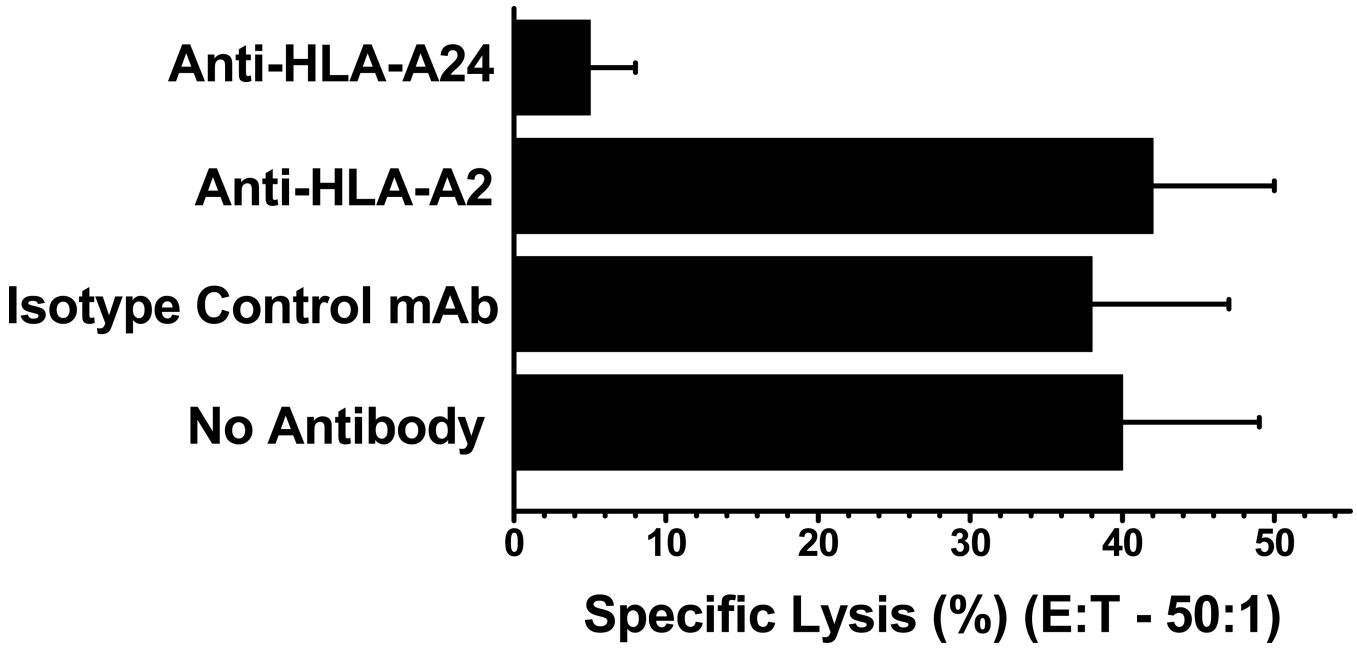


Figure 4.

Inhibition of the cytotoxic activity of the Mam-A-reactive CD8+ CTL line by anti-HLA-A24 Abs. T2.A24 cells individually loaded with the MAA24.2 and MAA24.4 peptides. The HLA class I-restriction of the resultingCD8+ T cell line was then evaluated against the AU-565 (HLA-A24+/Mam-A-A+) in the presence of the anti-HLA-A24 mAb, Anti-HLA-A2 mAb, MOPC-11 isotype control mAb or no Ab. The assay was carried out at an E:T ratio of 50:1 and are presented as mean ± standard deviation.

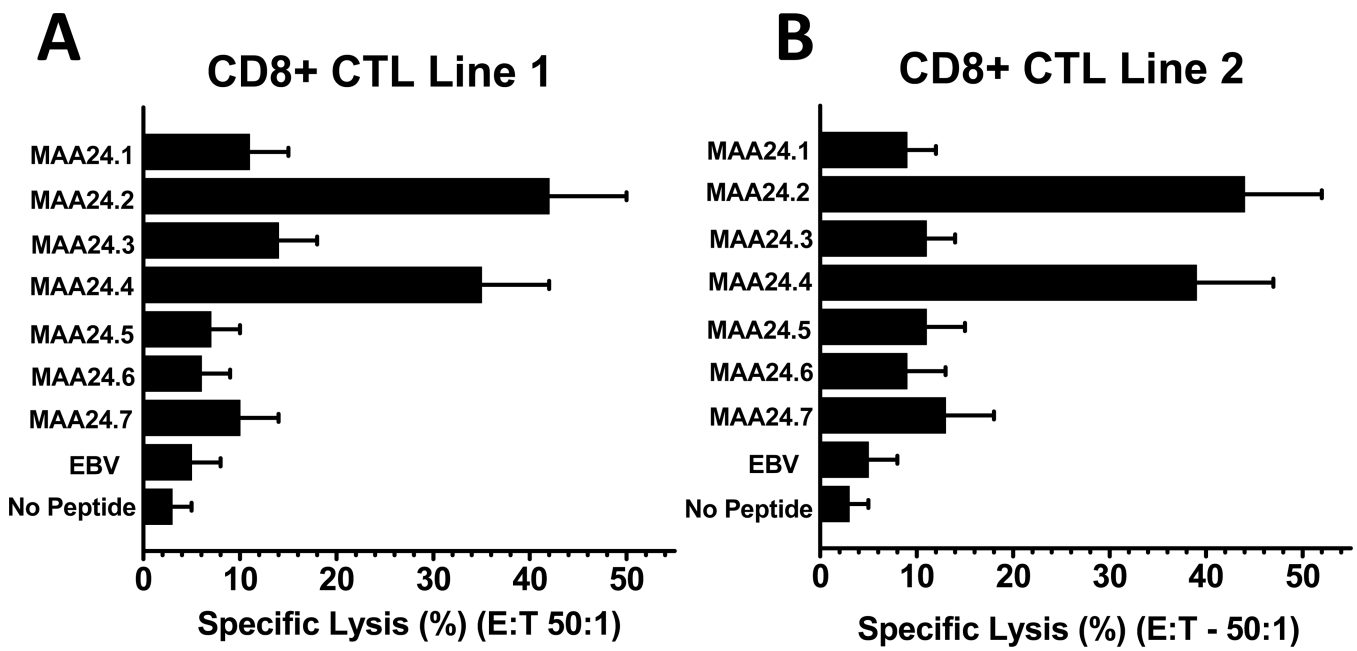


Figure 5.

Defining the $CD8^+$ CTL response to candidate Mam-A-derived HLA-A24-restricted epitopes. Two $CD8^+$ CTL lines were generated from individual HLA-$A24^+$ healthy individuals by *in vitro* stimulations with pooled T2.A24 cells individually loaded with candidate Mam-A-derived epitopes. The epitope specificity of the $CD8^+$ CTL lines was then evaluated for lysis against transfected AU-565 (HLA-$A24^+$/Mam-A^+) using T2.A24 cell lines individually loaded with candidate epitopes by means of a standard LDH release assay. T2 cells loaded with TYGPVFMSL (EBV-derived epitope) and empty T2.A24 cells (no peptide) were used as negative controls. The assay was performed at E:T ratios of 50:1, 25:1, 12.5:1 and 6.25:1 in triplicate cultures. Results obtained at E:T ratios of 50:1 are presented as mean ± standard deviation.

Table 1

HLA-A24-restricted Mam-A-derived candidate epitopes

Peptide Name	Start Position	Sequence	Score
MamA-A24.1	41	E Y K E L L Q E F	21
MamA-A24.2	16	C Y A G S G C P L	20
MamA-A24.3	84	I Y D S S L C D L	20
MamA-A24.4	72	E T L S N V E V F	14
MamA-A24.5	21	G C P L L E N V I	12
MamA-A24.6	6	V L M L A A L S Q	2
MamA-A24.7	61	E L K E C F L N Q	2
EBV	--	T Y G P V F M S L	