Breast Cancer Res Treat (2014) 147:527–537
DOI 10.1007/s10549-014-3129-x

PRECLINICAL STUDY

Identification and translational validation of novel mammaglobin-A CD8 T cell epitopes

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Received: 1 May 2014 / Accepted: 6 September 2014 / Published online: 12 September 2014
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Abstract Mammaglobin-A (MAM-A) is a secretory protein that is overexpressed in 80 % of human breast cancers. Its near-universal expression in breast cancer as well as its exquisite tissue specificity makes it an attractive target for a breast cancer prevention vaccine, and we recently initiated a phase 1 clinical trial of a MAM-A DNA vaccine. Previously, we have identified multiple MAM-A CD8 T cell epitopes using a reverse immunology candidate epitope approach based on predicted binding, but to date no attempt has been made to identify epitopes using an unbiased approach. In this study, we used human T cells primed in vitro with autologous dendritic cells expressing MAM-A to systematically identify MAM-A CD8 T cell epitopes. Using this unbiased approach, we identified three novel HLA-A2-restricted MAM-A epitopes. CD8 T cells specific for these epitopes are able to recognize and lyse human breast cancer cells in a MAM-A-specific, HLA-A2-dependent fashion. HLA-A2$^+$/MAM-A$^+$ breast cancer patients have an increased prevalence of CD8 T cells specific for these novel MAM-A epitopes, and vaccination with a MAM-A DNA vaccine significantly increases the number of these CD8 T cells. The identification and translational validation of novel MAM-A epitopes has important implications for the ongoing clinical development of vaccine strategies targeting MAM-A. The novel MAM-A epitopes represent attractive targets for epitope-based vaccination strategies, and can also be used to monitor immune responses. Taken together these studies provide additional support for MAM-A as an important therapeutic target for the prevention and treatment of breast cancer.

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Electronic supplementary material The online version of this article (doi:10.1007/s10549-014-3129-x) contains supplementary material, which is available to authorized users.

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Keywords Mammaglobin A · T cell epitopes · Cancer vaccine · Immune therapy

Introduction

Cancer vaccination has broad appeal because of the potential for selective tumor destruction, minimal systemic toxicity, and sustained benefit secondary to immunologic memory. Preclinical studies and human clinical trials have demonstrated that CD8 T cells are key effectors in mediating antitumor immunity. The choice of antigen to be targeted by cancer vaccination is critical, since the antigen can influence not only the efficacy of the antitumor immune response, but also the potential for autoimmunity [30].

Mammaglobin-A (MAM-A) is a 10 kD secretory protein that was first identified by Watson and Fleming using a differential screening approach [39]. In normal tissues, MAM-A expression is restricted almost exclusively to normal breast epithelium, where it is expressed at low levels [15]. MAM-A is overexpressed in 80 % of human breast cancers, and expression is observed in all stages of disease (ductal carcinoma in situ, invasive breast cancer, and metastatic disease) [9, 38]. The consistent and dramatic expression of MAM-A in breast cancer, as well as its exquisite tissue specificity makes it an attractive target for breast cancer vaccine therapy. In proof-of-concept studies, we demonstrated the safety and potential efficacy of a MAM-A DNA vaccine in a preclinical model [2, 25]. Based on these studies, we recently initiated a phase 1 clinical trial of a MAM-A DNA vaccine in breast cancer patients with metastatic disease [34].

CD8 T cells recognize antigen as peptide fragments presented at the cell surface by HLA class I molecules. While we have previously identified MAM-A-derived epitopes restricted by HLA-A2 and other class I alleles [2, 17, 18, 23, 32, 36, 37], and validated the translational relevance of these epitopes in breast cancer patients [15, 17, 18, 35], these epitopes were identified through a reverse immunology candidate epitope approach [19]. This means that candidate epitopes were initially identified based on predicted binding using a computer algorithm. The candidate epitopes were then validated by peptide binding assays in vitro, by generating CD8 T cell lines in vitro, and by determining the frequency of epitope-specific CD8 T cells in MAM-A$^+$ breast cancer patients.

In the present study we used an unbiased approach to systematically identify potential MAM-A-derived CD8 T cell epitopes. We generated MAM-A-specific T cells in vitro by priming naïve T cells with autologous dendritic cells transduced to express MAM-A. With this unbiased approach, MAM-A-derived epitopes are naturally processed, presented, and recognized by functional T cell repertoires. The same techniques have been used successfully to identify novel RVFV epitopes [40]. We identified three novel HLA-A2-restricted MAM-A epitopes. Of note, some of the most robust responses observed in the initial cultures were directed at these epitopes, and CD8 T cells specific for these epitopes were able to recognize and lyse breast cancer cells in a MAM-A-specific, HLA-A2-dependent manner. In addition, we show that CD8 T cell immunity to these epitopes can be enhanced by vaccination with a MAM-A DNA vaccine in the context of a phase 1 clinical trial. These studies highlight the advantages of an unbiased approach to epitope identification and confirm the potential of MAM-A as a breast cancer antigen.

Materials and methods

Study subjects

Healthy donor PBMC specimens were obtained from human platelet apheresis specimens. The specimens were isolated by density gradient centrifugation and stored in liquid nitrogen until evaluation. Peripheral blood was obtained from breast cancer patients after informed consent in accordance with a protocol approved by the Institutional Review Board at Washington University School of Medicine (WUSM).

Peptides

A library of 21 overlapping peptides (P1–P21) spanning the entire MAM-A protein was synthesized. Each MAM-A peptide was 15 amino acids in length, and peptides overlapped by 4 amino acids. The MAM-A peptide library and a Flu-M1 control peptide [13] were synthesized by Biosynthesis (Lewisville, TX). A pool of 23 viral-derived peptides (including known HLA class I-restricted T cell epitopes from cytomegalovirus, Epstein-Barr virus, and influenza virus, [6]) was obtained from CTL Technologies (Shaker Heights, OH).

In vitro generation of MAM-A-specific CD8 T cells

For initial studies, PBMC from two healthy donors were used to generate monocyte-derived dendritic cells, as described [40]. Dendritic cells from each donor were transduced with a lentiviral vector expressing full length MAM-A and co-cultured at a dendritic cell to T cell ratio of 1:10 with autologous CD8 T cells (2×10^6/mL) purified by magnetic bead separation (Miltenyi Biotec; Auburn, CA). T cells were cultured in RPMI-1640 medium, supplemented with 5 % human AB serum (Sigma; St. Louis, MO) in 24-well plates. 50 U/mL recombinant IL-2 was

added to the cultures after 24 h, and then every 48 h. T cells were restimulated after 7–9 days using autologous, irradiated monocytes pulsed with the peptide library. In subsequent cultures, total PBMC were cultured in the presence of 20 μg of an individual peptide. PBMC stimulated with the immunodominant peptide (GILVFTFTL) of the influenza matrix protein (Flu-M1) were included as positive control.

Breast cancer cell lines

All breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). Breast cancer cell lines were cultured in RPMI-1640 medium (Gibco; Grand Island, NY) supplemented with 10 % fetal bovine serum, 1 % L-glutamine, and 1 % penicillin/streptomycin at 37 °C in a 5 % CO2 incubator.

ELISPOT assay

PBMC and CD8 T cell cultures were tested using an IFNγ-based enzyme-linked ImmunoSpot (ELISPOT) assay kit (Mabtech; Stockholm, Sweden). Briefly, 96-well filtration plates (Millipore; Darmstadt, Germany) were pre-coated with 12 μg/mL of the anti-IFNγ monoclonal antibody 1-DIK (Mabtech; Nacka Strand, Sweden) and kept at 4 °C until use. A total of 1.25×10^5 or 6.25×10^5 PBMC or T cells as well as 5×10^4 antigen-presenting cells (APC), HeLa or T2 cells, were then plated in duplicate in 100 μL of culture medium (RPMI-1640, 5 % fetal bovine serum, 1 % L-glutamine, 1 % penicillin/streptomycin) per well, plus peptide at a final concentration of 40 μg/mL. For blocking studies, stimulator cells were pulsed with peptide for 1 h and subsequently incubated with the anti-MHC class I mAb W6/32 (Bio Legend; San Diego, CA) or the anti-HLA-A2 mAb BB7.2 (Abcam; Cambridge, UK) for 30 min. Additional antibody (1 μg) was added to each well. Mouse Ig (BD Bioscience) was used as a negative control. Cells were cultured for 20 h at 37 °C in a humidified 5 % CO_2 atmosphere. Plates were then washed with phosphate buffered saline (PBS), and 100 μL of PBS containing 1 μg/mL of biotinylated anti-IFNγ monoclonal antibody, 7-B6-1-biotin (Mabtech) was added to each well. After 2 h, plates were washed and horseradish peroxidase streptavidin (Mabtech) was added at a dilution of 1:1,000 in PBS for 1 h. Spots were revealed by incubation with BCIP/NBT substrate for 3 min. The spots were then counted with a Cellular Technology Limited (CTL) ELISPOT analyzer. The average number of spots per well was used to express each experimental value as spot-forming cells (SFCs) per 10^6 PBMC or T cells. All assays included positive (Flu-M1, CEF) and negative (PBMC/T cells alone and stimulator cells without peptide) controls.

Flow cytometry based killing assay (FloKa)

A flow-based killing assay [14] was used to measure in vitro cellular cytotoxicity of $CD8^+$ T cells. Breast cancer cell lines (AU-565 and MCF-7) were washed with PBS, re-suspended at 1×10^7 cells/mL, and then labeled at room temperature for 15 min with 125 nM final concentration of 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen; Carlsbad, CA). Labeling reactions were stopped with complete RPMI media. Labeled target cells (2.5×10^4) were added to 96-well V-bottom tissue culture-treated plates along with T cells in complete RPMI media containing 50 U/mL of rhIL-2 and incubated at 37 °C for 4 h. The effector-target (E/T) ratio was 40:1 for all time points shown. Immediately before analysis, 5 μL of 7-aminoactinomycin D (7-AAD) (Calbiochem; La Jolla, CA) was added to each sample. 7-AAD incorporation is associated with cell death/apoptosis, as it intercalates with DNA in cells that have lost membrane integrity. The samples were then analyzed by flow cytometry on a FACScalibur flow cytometer (BD Biosciences; San Jose, CA) and $CFSE^+7\text{-}AAD^+$ tumor cells were counted. The acquired data were analyzed with Flowjo software. All cytotoxicity assays were performed in duplicate or triplicate. Data presented are representative of four individual cytotoxicity experiments.

Peptide-binding assay

To determine whether the epitopes identified can bind to HLA-A2, a MHC stabilization assay was performed using T2 cells that are deficient in presentation of endogenous peptides, as described previously [4, 7, 25, 31, 33]. T2 cells (1×10^6/well) were incubated with different concentrations of individual peptide in 24-well plates for 18 h at 25 °C. A melanoma-associated epitope (G280-9 V) was used as a positive control [21]. The cells were washed with FACS buffer (1 % BSA and 0.1 % sodium azide in PBS) and stained with a FITC-conjugated mAb specific for HLA-A2 (BB7.2, BD Biosciences) at 4 °C for 30 min. After incubation, the cells were washed with FACS buffer, fixed with 1 % paraformaldehyde in PBS, and analyzed by flow cytometry using a FACSCalibur device (BD Biosciences) and mean fluorescence intensity (MFI) was recorded. The acquired data was analyzed with Flowjo software. The percent MFI increase was calculated as follows: percent MFI increase = (MFI with the given peptide−MFI without peptide)/(MFI without peptide) × 100.

Phase 1 clinical trial of a MAM-A DNA vaccine

We initiated a phase 1 clinical trial of a MAM-A DNA vaccine at WUSM to evaluate the safety and immunogenicity of a plasmid MAM-A DNA vaccine [34]. Fourteen

HLA-A2$^+$ and/or HLA-A3$^+$ patients with metastatic breast cancer were treated with the MAM-A DNA vaccine. Patients had stable disease for at least 30 days after chemotherapy, or for at least 30 days on hormonal therapy. The vaccine was administered intramuscularly using the Biojector 2000 Needle-Free Injection Management System on days 0, 28, and 56. Peripheral blood specimens were obtained before and after vaccination as indicated. PBMC were isolated from heparinized blood by density gradient centrifugation using Ficoll-Hypaque and stored in liquid nitrogen until evaluation.

Results

Unbiased identification of novel MAM-A candidate T cell epitopes

We used autologous DC transduced with a lentiviral vector to express MAM-A to identify MAM-A-derived CD8 T cell epitopes. Circulating CD8 T cells from two healthy HLA-A2-positive donors were primed and expanded by autologous DC prepared as noted above. The ability of the stimulated CD8 T cells to produce cytokines in response to each individual MAM-A peptide, or a pool of all peptides, was then determined by IFNγ ELISPOT and flow cytometry analysis (intracellular cytokine staining) (Fig. 1). Representative ELISPOT and intracellular cytokine data are shown in Fig. 1a, b, and show IFNγ production in response to P2, P12, P13, and the peptide pool. Cytokine production was not limited to IFNγ, as IL-2 and TNFα were detectable as well. In addition to cytokine production, CD107a/b, a surrogate marker for cytolytic activity, was upregulated after peptide stimulation. Of note, both donors responded to P2, P12, and P13, whereas one of two donors responded to P5, P16, P20, and P21 (data not shown). As HLA-A2 CD8 T cell epitopes have previously been identified in P16, P20, and P21 [18, 32], they were not characterized further.

Generation of CD8 T cell lines specific for candidate MAM-A-derived peptides

CD8 T cell lines specific for the P2, P5, P12, and P13 peptides were established using PBMC from both HLA-A2-positive healthy donors and breast cancer patients. In initial studies CD8 T cell lines were established from two additional healthy donors, and the activity and specificity was assessed by IFNγ ELISPOT. CD8 T cell lines showed specific recognition for the peptide used for stimulation when presented by peptide-pulsed HeLa or T2 cells, but not for irrelevant HLA-A2-binding peptides ($p < 0.05$, Fig. 1c, d). Unstimulated PBMC typically did not yield sufficient cells for testing, whereas PBMC stimulated with Flu-M1 peptide typically induced >500 SFU/10^6 cells for the Flu-M1 peptide but not the MAM-A-derived peptides (data not shown). CD8 T cell lines specific for the P2, P5, P12, and P13 peptides were also generated from patients with breast cancer. These cultures also showed peptide specificity by ELISPOT (Supplementary Fig. 1a, b). In cultures from some individuals we observed the response to P5 was lower than the response to P2 and/or P12. Recognition of peptides was restricted by HLA-A2, as evidenced by the ability of an anti-HLA-A2 mAb, and/or an anti-HLA class I mAb to significantly block IFNγ release by T cells ($p < 0.05$, Fig. 1e). In contrast, a mouse Ig control antibody had no effect on IFNγ release.

Identification of minimal peptide epitopes

The minimal epitopes corresponding to P2, P5, P12, and P13 were predicted using the NetMHC 3.2 prediction algorithm [22] (Fig. 2). The minimal epitopes for the P12 and P13 peptides are identical. Binding of synthetic peptides encoding these minimal epitopes to HLA-A2 was confirmed using T2 cells (Fig. 2). The CD8 T cell response observed to each of the minimal epitopes was comparable to the response observed to the corresponding parent peptides, confirming that the minimal epitopes identified by the prediction algorithms are likely the native epitopes.

The novel MAM-A epitopes are processed and presented by breast cancer cells

To assess whether the MAM-A epitopes are naturally processed and presented by breast cancer cells, we determined if the CD8 T cell lines specific for P2, P5, and P12/13 could recognize breast cancer cells. The ability of two human breast cancer cell lines, AU565 (MAM-A$^+$, HLA-A2$^+$) and MCF-7 (MAM-A$^-$, HLA-A2$^+$), to be recognized by MAM-A-specific CD8 T cell lines from four breast cancer patients was tested by ELISPOT. The CD8 T cell lines were able to recognize the MAM-A$^+$ AU565 breast cancer cells, but not the MAM-A$^-$ MCF-7 breast cancer cells (Fig. 3a). Addition of anti-HLA class I mAb or anti-HLA-A2 mAb significantly impaired recognition of AU565 breast cancer cells, confirming that recognition of the MAM-A$^+$ tumor cells is HLA-A2 restricted (Fig. 3a). Of note, CD8 T cells were able to recognize MCF-7 cells pulsed with either the P2 or P12 peptides, but not an irrelevant peptide, demonstrating that MCF-7 cells can present antigen effectively (Supplementary Fig. 2). Recognition of MCF-7 cells pulsed with MAM-A peptides was almost completely abrogated by anti-HLA-A2 mAb (Supplementary Fig. 2b).

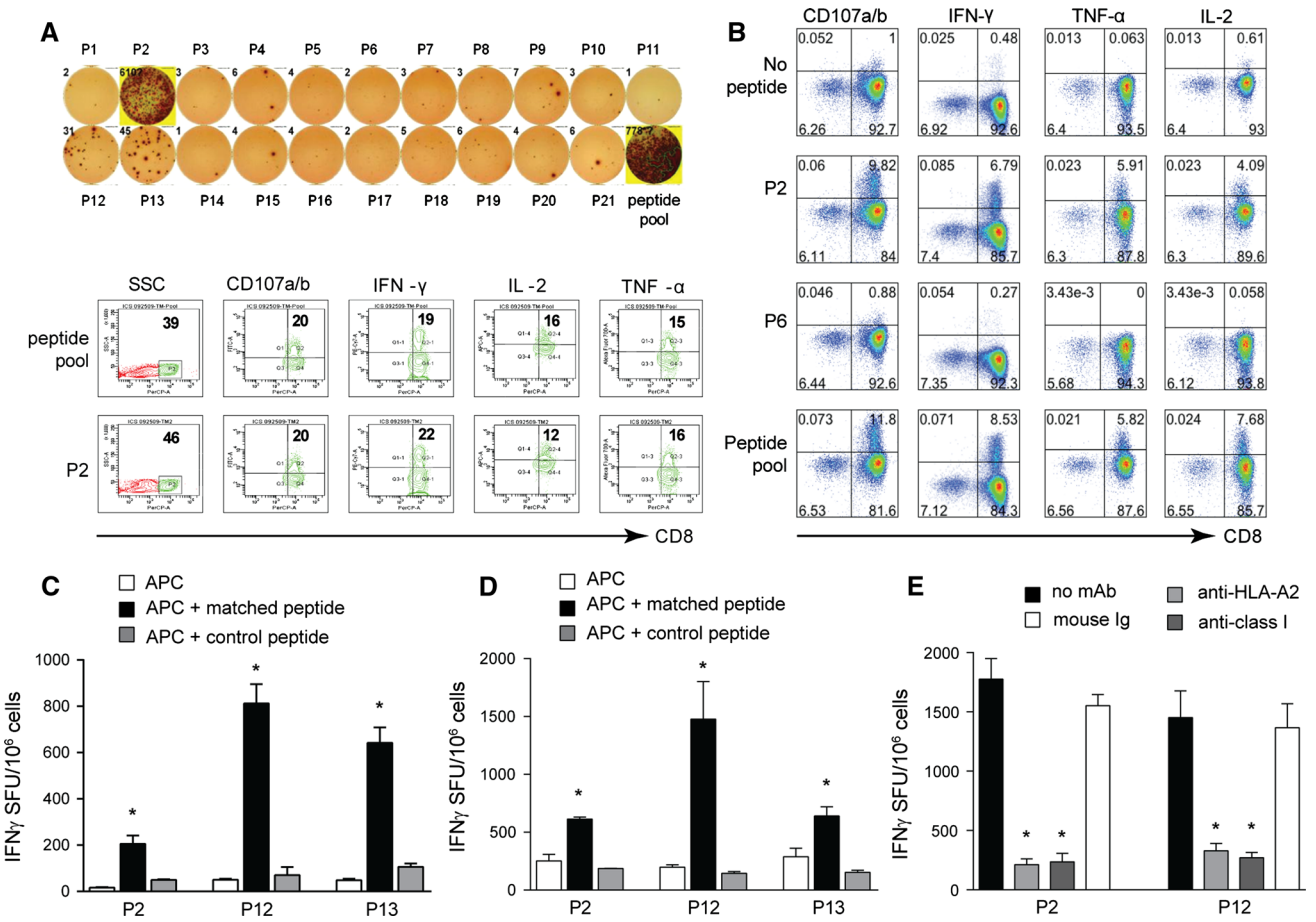

Fig. 1 Identification of novel CD8 T cell epitopes encoded by MAM-A. **a** Naïve CD8 T cells from two HLA-A2$^+$ healthy individuals were stimulated with autologous dendritic cells modified to express MAM-A. Primed T cells were tested for MAM-A reactivity using APC pulsed with a pool of 21 overlapping peptides (P1–P21) of 15 amino acids each, staggered by four residues across the MAM-A protein. IFNγ ELISPOT results from Donor 1 are displayed and show responses to P2, P12, P13, and a pool of all 21 peptides. Flow cytometry analysis shows degranulation (increased surface CD107a/b expression), and production of IFNγ, IL-2, and TNFα triggered by P2 or the peptide pool after a 6 h stimulation of CD8 T cells with peptide-pulsed C1R-A2 target cells. The *numbers in the upper right quadrants* indicate the percentage of dual positive cells. Gates were set using T cells cocultured with target cells in the absence of peptide (not shown) **b** Intracellular cytokine analysis of MAM-A-stimulated CD8 T cells from healthy donor 2 analyzed as above **c**, **d** Naïve PBMC from two additional HLA-A2$^+$ healthy individuals were cultured with MAM-A-derived peptides to generate epitope-specific CD8 T cell cultures. After in vitro culture, epitope specificity was assessed by IFNγ ELISPOT assay. ELISPOT data from two representative healthy individuals are shown. **e** CD8 T cell cultures specific for MAM-A epitopes are HLA-A2-specific. Epitope-specific CD8 T cells were tested for recognition of epitope-pulsed APC in the presence or absence of antibodies specific for HLA class I or HLA-A2. Error bars represent standard error, $*p < 0.05$

To determine whether IFNγ secretion as measured by ELISPOT was associated with cytolytic activity, we assessed the ability of CD8 T cell lines to lyse human breast cancer cell lines using a flow-based killing assay (FloKA). CD8 T cell lines were able to lyse the MAM-A$^+$ AU565 breast cancer cell line (40–60 % specific lysis), but not the MAM-A$^-$ MCF-7 breast cancer cell line (<15 % specific lysis) (Fig. 3b, c). A control CD8 T cell line developed against Flu-M1 was able to lyse Flu-M1 peptide-pulsed HeLa cells (>90 % specific lysis) but showed minimal lysis of the two breast cancer cell lines (<10 % specific lysis). Thus, CD8 T cells specific for the novel MAM-A-derived epitopes specifically lyse breast cancer cells in an HLA-restricted fashion, indicating that these epitopes are naturally processed and presented by breast cancer cells.

Detection of pre-existing immunity against MAM-A-derived epitopes in breast cancer patients

To assess whether the novel MAM-A-derived epitopes are associated with pre-existing CD8 T cell responses, we obtained PBMC from breast cancer patients. PBMC from six HLA-A2$^+$ breast cancer patients (3 with MAM-A$^+$

Peptide	Amino acid sequence	Minimal epitope*
P2	MVLMLAALSQHCYAG	MLAALSQHCYA
P5	YAGSGCPLLENVISK	YAGSGCPLL
P12	LLQEFIDDNATTNAI	FIDDNATTNAI
P13	FIDDNATTNAIDELK	FIDDNATTNAI

* Based on netMHC 3.2

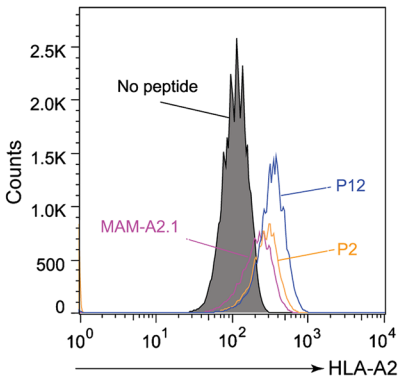

Fig. 2 Identification of candidate minimal MAM-A epitopes. *Left panel* The epitope prediction algorithm, Net-MHC3.2 (http://www.cbs.dtu.dk/services/NetMHC-3.2/) was used to define candidate minimal epitopes of P2 and P12/13 with the highest predicted binding to HLA-A2. *Right panel* The candidate minimal peptides were synthesized and tested for binding to HLA-A2 using T2 cells, as described [7, 33]. Binding of predicted minimal epitopes from both P2 and P12/13 was detected as an intensity similar to the reference mammaglobin-A peptide, MamA2.1 [18]

breast cancers, and 3 with MAM-A$^-$ breast cancers) were analyzed by ELISPOT in ex vivo analyses. Of note, specific activity against the MAM-A-derived epitopes was observed in the three patients with MAM-A$^+$ breast cancers, but little or no activity was observed in the three patients with MAM-A$^-$ breast cancers (Fig. 4). The response to P5 was consistently lower than the response to P2, P12, or P13. Reactivity against a pool of viral-derived peptides (CEF peptide) was observed among all six patients (data not shown).

MAM-A DNA vaccination enhances the CD8 T cell response to the novel MAM-A-derived epitopes

To assess the translational relevance of the MAM-A epitopes, we measured the immune response to these epitopes before and after vaccination with a MAM-A DNA vaccine. PBMC samples were collected from breast cancer patients enrolled in a phase 1 clinical trial of a MAM-A DNA vaccine. As shown in Fig. 5, four out of five HLA-A2$^+$ patients tested had markedly increased IFNγ responses specific for the novel epitopes following vaccination. The IFNγ responses against the P2, P5, and P12 peptides were similar to the responses to the minimal epitopes (data not shown). These results support the translational relevance of these epitopes for immune monitoring and/or epitope-specific vaccination strategies.

Discussion

MAM-A is a breast cancer-associated antigen that is an attractive target for cancer vaccine therapy. We have previously identified epitopes derived from MAM-A using a reverse immunology candidate epitope approach [12, 17, 18, 23, 32, 36, 37]. In this study we used an unbiased approach to identify three novel HLA-A2-restricted CD8 T cell epitopes derived from MAM-A. We confirmed the significance of these epitopes demonstrating that (1) the novel MAM-A epitopes are naturally processed and presented by human breast cancers; (2) MAM-A$^+$ breast cancer patients have a pre-existing CD8 T cell response to these epitopes; and (3) vaccination with a MAM-A DNA vaccine can enhance this pre-existing immune response.

Epitope identification is an important focus of human immunology research. Most studies have used a reverse immunology candidate epitope approach to identify epitopes. In this study we used an unbiased approach to identify MAM-A epitopes. We first developed and optimized this approach for the identification of epitopes within targeted regions of the HIV proteome [28]. Using combinatorial peptide libraries, we were able to successfully identify and validate novel HIV epitopes, even though the immunobiology of HIV has been studied extensively. These HIV epitopes are naturally processed and presented by HIV-infected cells, and recognized by precursor T cell repertoires in healthy donors and HIV-infected patients [29]. The ability to identify and validate epitopes derived from well-studied viral and shared tumor antigens underscores the translational relevance of this unbiased epitope identification strategy.

There are many potential factors that contribute to whether a candidate epitope is immunologically significant (immunodominant). The reverse immunology candidate epitope approach focuses on predicted binding as the key first step in prioritizing potential epitopes for further study. Although the reverse immunology approach has been used successfully, there are a number of potential limitations with this approach. First, predicted binding is highly dependent on sequences of known epitopes. For many

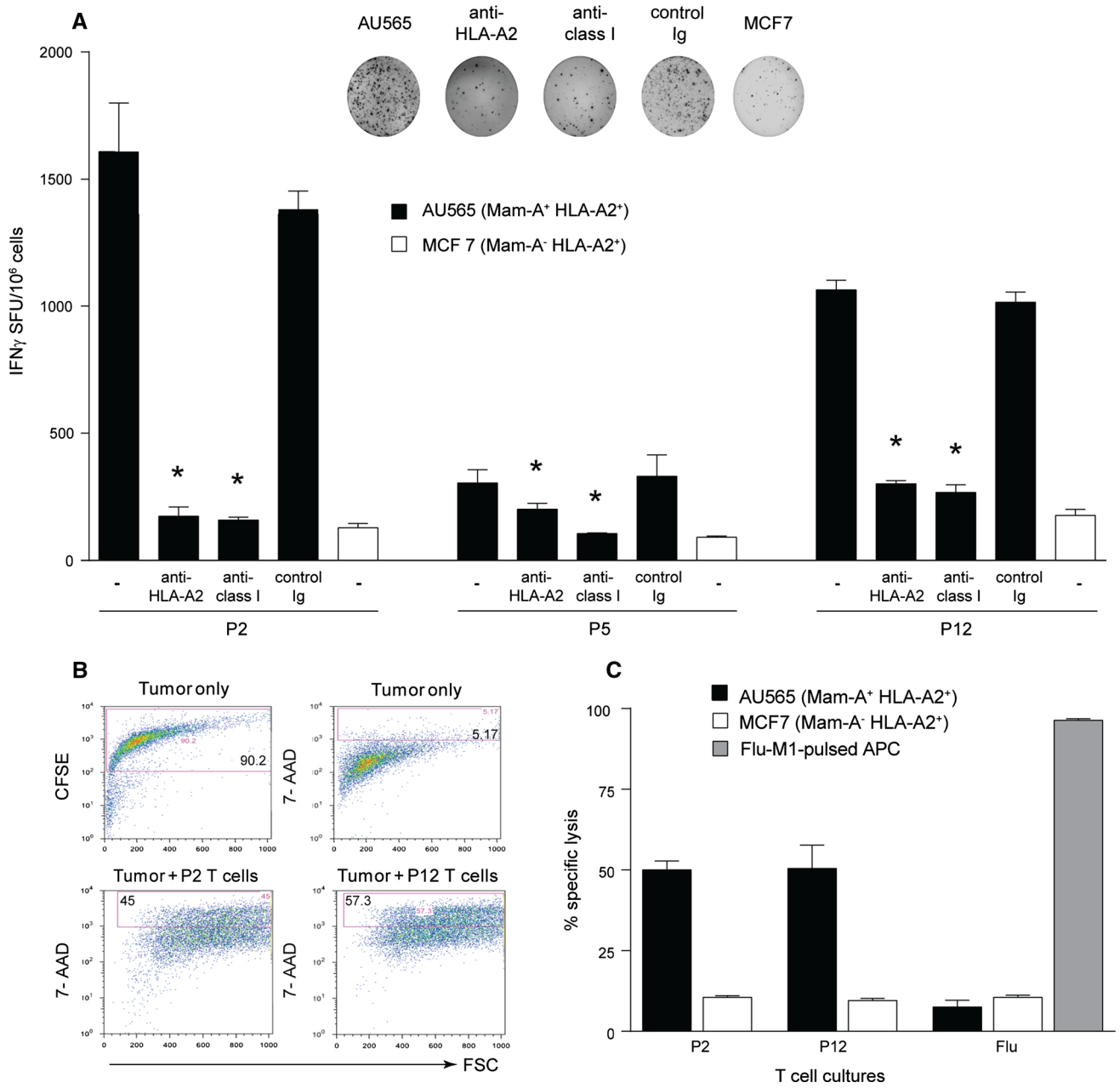

Fig. 3 CD8 T cells specific for novel MAM-A epitopes recognize and lyse breast cancer cells. **a** HLA-A2$^+$, MAM-A$^+$ breast cancer cells (AU565), but not HLA-A2$^+$, MAM-A$^-$ breast cancer cells (MCF-7) are recognized by peptide-specific CD8 T cells. PBMC from breast cancer patients ($n = 5$) were stimulated with P2, P5, and P12, and tested for recognition of breast cancer cells. Representative data show recognition of AU565 breast cancer cells was HLA-A2 restricted, as demonstrated by specific blocking with anti-HLA-A2 and anti-class I HLA antibodies, but not anti-mouse Ig control antibody (control Ig). Insert shows representative ELISPOT images of P12-stimulated T cells (1.25×10^5 T cells/well) **b** PBMC from breast cancer patients were stimulated with P2 or P12 and the ability to lyse MAM-A expressing breast cancer cells was assessed by FloKA. Representative flow cytometry analysis of T cell-mediated killing of the HLA-A2$^+$/MAM-A$^+$ breast cancer cell line AU565 is shown. **c** Lysis of CFSE-labeled breast cancer cells was determined using 7-AAD staining. The bar graph shows percent specific lysis of breast cancer cells (AU565, MCF-7) by epitope-specific T cells. Lysis of Flu-M1-pulsed APC by Flu-M1-specific T cells serves as a control. *Error bars* represent standard error, $*p < 0.05$

HLA alleles, predicted binding is based on limited information, and predicted binding may not identify all immunologically significant epitopes. Second, for an epitope to be immunologically significant, the T cell repertoire must be capable of recognizing the epitope. This is particularly important for candidate epitopes derived from shared tumor antigens. As shared tumor antigens are self-antigens, the T cell repertoire may be shaped/limited by central and

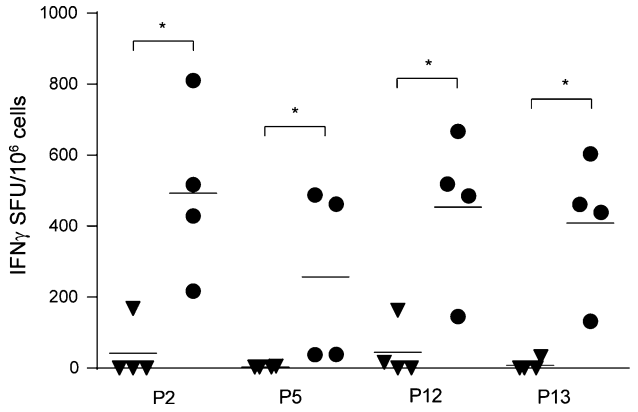

Fig. 4 Patients with MAM-A-expressing tumors have pre-existing immunity to the novel MAM-A epitopes. Pre-existing immunity to MAM-A epitopes in breast cancer patients was assessed directly ex vivo by IFNγ ELISPOT using 15-mer peptides. The results represent results from four patients with MAM-A^+ breast cancers (dots), and four patients with MAM-A^- breast cancers (triangles). The number of IFNγ SFU/10^6 PBMC in the absence of peptide was <15 (not shown)

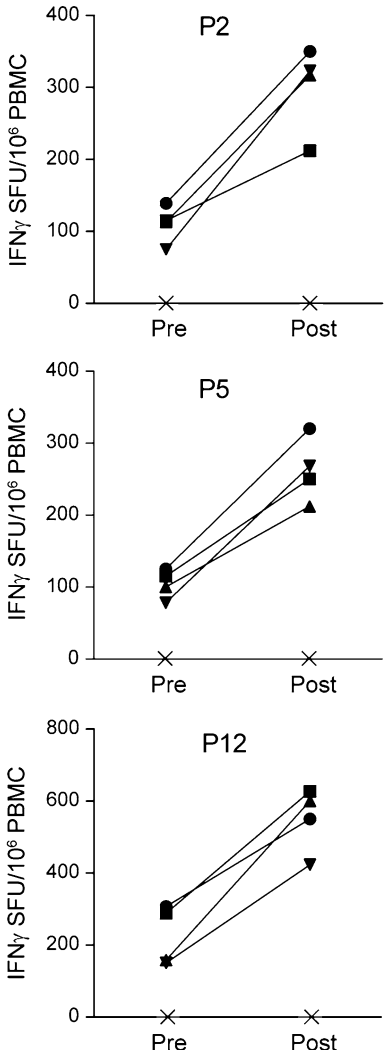

Fig. 5 MAM-A cDNA vaccination significantly enhances T cell responses to the novel MAM-A-derived peptides in breast cancer patients. Breast cancer patients were vaccinated three times with a MAM-A DNA vaccine in the context of a phase 1 clinical trial. The immune response to MAM-A epitopes was assessed before and after vaccination by IFNγ ELISPOT. The immune response (IFNγ SFU/10^6 PBMC) was significantly enhanced following vaccination in four of five patients evaluated. *Symbols* indicate individual patients

peripheral tolerance mechanisms. The unbiased approach to epitope identification addresses many of the potential limitations of the reverse immunology approach. Of note, all of the novel MAM-A epitopes identified in this study appear to be translationally significant, confirming the relevance of the unbiased approach.

Identification of novel MAM-A epitopes has significant translational implications. Translational applications include integration into epitope-based vaccination strategies, and immune monitoring. We are currently testing a MAM-A DNA vaccine in phase 1 clinical trials. This nucleic acid-based approach is not epitope-specific as the whole antigen is expressed and can be targeted by the immune system. However, several current cancer vaccine platforms are epitope-specific including the synthetic peptide vaccine platform [41] and selected dendritic cell vaccine platforms [27]. The synthetic peptide vaccine platform is perhaps the most common cancer vaccine platform given the remarkable safety and ease of manufacture of synthetic peptides. The development and translational success of synthetic long peptide vaccines has further increased the interest in the synthetic vaccine platform. The epitopes identified in this study are attractive candidates for integration into a synthetic peptide vaccine targeting MAM-A.

Immune monitoring is critical to assessing the efficacy of cancer vaccines. Although measurement of clinical outcomes such as overall and progression-free survival remains the benchmark for assessing efficacy of any therapeutic intervention particularly in late phase clinical trials, immune monitoring is a valuable surrogate for biologic efficacy, particularly in early phase clinical trials. Current immune monitoring techniques are epitope-based, including ELISPOT and multi-parameter flow cytometry. The MAM-A epitopes identified here represent attractive candidates for immune monitoring. CD8 T cell responses to these epitopes were particularly robust in vitro, suggesting that the epitopes are effectively processed. In addition, the response to these epitopes was consistent across multiple individuals, suggesting that the T cell repertoires to these epitopes remain intact. These observations suggest that the novel epitopes may be ideal for immune monitoring.

Of note, breast cancer patients with MAM-A^+ breast cancer have pre-existing CD8 T cell responses to the novel

MAM-A epitopes. These studies confirm and extend the results of Bharat et al., who demonstrated pre-existing CD8 T cell responses to the MAM-A2.1 epitope in breast cancer patients by tetramer and ELISPOT analysis [2]. Pre-existing CD8 T cell responses to shared tumor antigens have been previously described in melanoma patients [3, 11, 20, 26], and for the shared tumor antigens HER2 [8] and NY-ESO-1 [16]. Immunohistochemistry studies in breast and other solid malignancies [1] now suggest that endogenous antitumor CD8 T cell responses are associated with a favorable prognosis [3]. However, despite an improved prognosis, many patients with endogenous antitumor CD8 T cell responses ultimately progress [3], suggesting that the endogenous antitumor CD8 T cell response may ultimately become ineffective, either because of antigen loss, loss of class I HLA expression, or because of functional inactivation of tumor-specific CD8 T cells [3]. Functional inactivation of CD8 T cells is commonly associated with expression of programmed death-1 (PD-1) [10], and we recently demonstrated that PD-1 expression by breast cancer tumor infiltrating lymphocytes (TIL) is associated with larger primary tumor size, higher tumor grade, positive lymph node status, and an unfavorable prognosis [24]. Demonstration of pre-existing CD8 T cell responses to the novel MAM-A epitopes identified in this study confirms their translational significance.

A second confirmation of the relevance of the novel MAM-A epitopes is the demonstration that vaccination with a MAM-A DNA vaccine can significantly increase the pre-existing CD8 T cell response to these epitopes. As noted above, MAM-A is an attractive candidate for a breast cancer prevention vaccine. As the first step in the clinical translation of a MAM-A DNA vaccine, we recently initiated a phase 1 clinical trial in breast cancer patients with metastatic disease. Of note, MAM-A DNA vaccination significantly enhances the pre-existing immune response to the novel MAM-A CD8 T cell epitopes at 6 months following vaccination as measured by ELISPOT. This confirms and extends the study by Tiriveedhi et al. based on the same phase 1 clinical trial demonstrating that MAM-A DNA vaccination is able to successfully induce MAM-A-specific CD4 T cells following vaccination [34]. These results are also consistent with ELISPOT and tetramer analyses that we have performed based on previously identified MAM-A epitopes demonstrating a response to vaccination. A detailed analysis of the primary endpoints of the phase 1 clinical trial, including safety of the vaccine, clinical response, ELISPOT analyses, and peptide-MHC tetramer analyses documenting the safety and immunogenicity of the MAM-A cDNA vaccine is currently under review (Tiriveedhi et al., manuscript submitted).

We have successfully identified the minimal epitopes for each of the overlapping peptides in the present study. The identification of the minimal epitopes is important as the minimal epitopes represent what is actually presented by HLA-A2 molecules and recognized by CD8 T cells. This information is required for epitope-based vaccination strategies and immune monitoring. Identification of the minimal epitopes also provides the opportunity to engineer the epitopes to improve the immunogenicity of epitope-based vaccination strategies. For instance, epitopes can be modified to improve binding to HLA-A2, or to improve interaction with the TCR of responding T cells. In addition, identification and translational validation of multiple CD8 T cell epitopes provides the opportunity to combine these epitopes into a multi-epitope vaccine. Recent studies in preclinical models have shown that DNA vaccination with constructs containing multiple epitopes results in the induction of strong CD8 and CD4 T cell responses to all of the epitopes in the vaccines [5]. The results reported have the potential to influence epitope selection, and are likely to influence the future design of epitope-based MAM-A vaccines and strategies for immune monitoring.

In summary, we present a novel and broadly applicable approach to the identification of novel MAM-A CD8 T cell epitopes. CD8 T cells specific for these epitopes are able to specifically recognize and lyse human breast cancer cells in a MAM-A-specific, HLA-A2-dependent fashion. Patients with MAM-A$^+$ breast cancer have evidence of a preexisting CD8 T cell immune response specific to these epitopes, and this preexisting immune response is increased following vaccination with MAM-A DNA vaccine. These results support the use of an unbiased approach for the identification of novel epitopes from shared tumor antigens, and support our strategy to target MAM-A for breast cancer prevention and therapy.

Acknowledgments This work was supported by research Grants from the Swiss National Foundation (SNF) (PBBSP3-138709) (SM), DOD/CDMRP-BCRP W81XWH-06-1-0677 (WEG), and the Barnes Jewish Hospital Foundation (TM, SPG). We are also extremely grateful to George and Diana Holway, whose generous gift provided the resources necessary to help us realize the promise of new discovery.

Conflict of interest The authors declare that they have no conflict of interest. All the experiments conducted in this study comply with the current laws of the United States of America. Peripheral blood from patients was obtained after informed consent in accordance with a protocol approved by the Institutional Review Board at Washington University School of Medicine (WUSM).

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