Conjugation with S4 protein transduction domain enhances the immunogenicity of the peptide vaccine against breast cancer

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Abstract. Although peptide vaccines offer a novel venue for cancer immunotherapy, clinical success has been rather limited. Cell-penetrating peptides, due to their ability to translocate through the cell membrane, could be conjugated to the peptide vaccine to2 enhance therapeutic efficiency. The S4 transduction domain of the shaker-potassium channel was conjugated to mammaglobin-A (MamA) immunodominant epitope (MamA2.1) to verify its anticancer immunogenicity. S4-MamA2.1 peptide has demonstrated significantly higher epitope loading and stable membrane expression of HLA-A2 antigen-presenting molecules on T2 cell lines. Further, these S4-MamA2.1 treated T2 cells were able to activate naïve $CD8^+$ T cells to induce MamA-specific cytotoxicity against breast cancer cells. Conjugation of the S4 domain has also demonstrated a slight increase in immunogenicity of lesser immunodominant MamA epitopes. The conjugation of the S4 domain to N-terminus of MamA2.1 demonstrated significantly higher immunogenicity over C-terminus conjugation. Taken together, the results of the present study suggest that conjugation of the S4 cell-penetrating peptide domain to MamA2.1 epitope enhances the peptide vaccine immunogenicity against MamA-expressing breast cancers.

Introduction

Breast cancer is the second leading cause of cancer-related mortality accounting for up to one-third of newly diagnosed cancers in the women from USA (1). Despite the availability of advanced surgical tools and chemo/radiation treatment, the long-term therapeutic success of stage IV metastatic breast cancer is low (2). This raises a need for immune-targeted therapies that can provide improved long-term response along with reduced treatment resistance and side-effect profile. Advances in the understanding of tumor immunology enabled the development of anticancer vaccines as a viable immunotherapeutic approach (3). Tumor-associated antigens (TAAs), either expressed exclusively in the tumor tissue or selectively overexpressed by the oncogenes, offer unique molecular targets that can be utilized for vaccine development (4). Mammaglobin-A (Mam-A), a 10-kDa human breast cancer-associated antigen almost exclusively expressed in 40-80% of primary and metastatic breast cancers, is an attractive vaccine target (5). There is conflicting literature evidence on the correlation between MamA expression and hormonal status (6). While some studies have suggested a positive correlation between MamA expression and estrogen receptor expression in breast tumors (7), other studies have revealed an association between MamA expression and ER- breast tumors (8). The previous phase I clinical trials performed by the authors have revealed that the MamA-based cDNA vaccine was safe and could elicit modest $CD8^+$ and $CD4^+$T lymphocyte immune responses in patients with breast cancer (9). A previous study by Jaramillo *et al* (10) investigated seven human leukocyte antigen (HLA)-A2 restricted MamA immunodominant epitopes and demonstrated that MamA2.1 (83-92 amino acid sequence of MamA) induced highest cytotoxic $CD8^+$T lymphocyte (CTL) responses. Combination of MamA2.1 peptide vaccine with immune adjuvants such as methylselenol or CpG oligodeoxy-nucleotides (ODN2006 and M306) has further enhanced peptide-mediated CTL responses, thus offering potential peptide-based cancer immunotherapy against breast cancer (11,12).

Peptide vaccine-based active immunotherapy offers a novel tool to combat cancer. In this form of vaccination, immunodominant epitope sequences from TAAs are utilized to amplify host antitumor adaptive immune responses. As cancer cells selectively upregulate the expression of certain tumor-associated and mutant proteins that are otherwise not expressed in normal cells, these unique amino acid sequences could be utilized to develop peptide vaccines (13). Specificity, which is the hallmark of human adaptive immune response, represents the ability of adaptive immune cells (CD8 and CD4 T lymphocytes) to preferentially recognize TAAs on cancer cells and destroy them. TAAs are generally presented by

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antigen presentation molecules expressed on all nucleated cells to activate $CD8^+$T lymphocytes from naïve to cytotoxic phenotype by the HLA class I pathway (14). It has been revealed that in the HLA class I pathway the antigen TAA is intracellularly processed by proteasome to 8-10 amino acid-epitope for further presentation to $CD8^+$T lymphocytes (15).

The initial excitement in cancer vaccines is significantly dampened by the minimal potency of this strategy to elucidate anticancer CTL responses. This is particularly true with peptide vaccines which have demonstrated poor uptake into the lymphatics and eventual delivery to lymph nodes (16). Furthermore, peptides should be actively transported (ATP-dependent) into the antigen-presenting cells (APCs) for internalization and major histocompatibility complex loading to be presented for priming of naïve $CD8^+$T lymphocytes to induce a cytotoxic anticancer effect (17). However, this challenge also offers new venues of research to explore complementary strategies to enhance the efficacy of peptide vaccines. Novel design of peptide antigens to enhance their delivery through the hydrophobic cell membrane of APCs could enhance the HLA class I presentation of this peptide to $CD8^+$T lymphocytes resulting in antitumor CTL responses (18). Cell-penetrating peptides (CPPs) are a unique class of delivery tools that aid with the translocation of the macromolecular cargo bound to them (19). Previous studies have revealed that the protein transduction domain of human immunodeficiency virus TAT protein (Tat47-60) and S4 domain of Shaker-Potassium channel protein (S4) interact with the cell membrane leading to translocation through the hydrophobic core of the cell membrane in an ATP energy-independent manner (20,21). The ability of CPPs to translocate the peptide antigen target could, in principle, cause enhanced intracellular delivery and eventual downstream CTL responses. In the present study, the novel applicability of the S4 protein transduction domain was reported as a potent CPP conjugate for peptide vaccine delivery.

Materials and methods

Cell lines and reagents. The T2 cell line ($HLA\text{-}A2^+$) and two human breast cancer cell lines [MCF-7 (cat. no. HTB-22; $HLA\text{-}A2^+$/MamA-, ER^+, PR^+ and $HER2^+$/mild)] and AU565 (cat. no. CRL-2351; $HLA\text{-}A2^+$/$MamA^+$, ER-, PR- and $HER2^+$) were obtained from the American Type Culture Collection. Pooled primary human $CD8^+CD45RA^+$ naïve T lymphocytes collected from healthy donors were obtained from StemCell Technologies (cat. no. 70030). All cell cultures were performed under aseptic conditions and as aforementioned (12). All cells were confirmed to be free of mycoplasma contamination before experiments. The expression of HLA-A2 and MamA was confirmed by mRNA analysis. All synthetic peptides (Table I) were obtained from Peptide2.0, Inc. and confirmed by the manufacturer to have >95% purity. Unless mentioned otherwise, all other chemicals were obtained either from MilliporeSigma or Thermo Fisher Scientific, Inc. The present study was approved (approval no. HS2018-4093) by the Tennessee State University (Nashvile, USA) institutional review board.

HLA-A2 membrane stabilization assay. The peptide binding to HLA-A2 was assessed using transporter associated with antigen processing (TAP) protein-deficient T2 cells (22). Towards this, T2 cells ($0.5x10^5$/ml) were incubated for 24 h in 96-well cell culture plates with 200 μl of complete RPMI-1640 cell culture media (Thermo Fisher Scientific, Inc.) at 37˚C along with human β2-microglobulin (3 μg/ml) and various individual peptides (50 μg/ml) mentioned below in the results section (Fig. 1). The T2 cells were then washed and incubated for 30 min at 4˚C with FITC labeled BB7.2 anti-HLA-A2 monoclonal antibody (10 μg/ml; BioLegend, Inc., cat. no. 343304) and fixed with 0.5% paraformaldehyde (cat. no. F8775; MilliporeSigma). FITC-labeled mouse monoclonal IgG2b/κ (clone: MG2b-57; 1:20; cat. no. 401206; BioLegend, Inc.) was used as an isotype control and stained at 4˚C. The cells were analyzed with a FACS Calibur™/LSRII flow cytometer (Becton, Dickinson and Company) and analyzed by FlowJo v10 software (FlowJo, LLC). Results were expressed as the median channel fluorescence shift corresponded to the difference between the median fluorescence obtained with peptide-pulsed T2 cells and empty (no peptide) T2 cells.

Immunogenic peptide stimulation and generation of MamA-specific $CD8^+$T lymphocytes. The human naïve $CD8^+$T lymphocytes ($0.5x10^5$) were cultured in 2-ml complete RPMI-1640 media in 6-well plates in the presence of irradiated (5,000 rads) peptide loaded T2 cells ($1x10^6$, 20:1) in the presence of β2m (3 μg/ml), CD3 (500 ng/ml), CD28 mAb (500 ng/ml) and recombinant human IL-2 (20 U/ml) for 5 days per stimulation cycle and repeated for three stimulation cycles. Following this, the activated $CD8^+$T lymphocytes were isolated by immunomagnetic separation (MACS® cell separation; Miltenyi Biotec, Inc.) with >95% purity.

Cytotoxicity assay. The MamA-specific cytotoxicity of activated $CD8^+$T lymphocytes [referred to as effector cells (E)] was analyzed by calorimetric lactate dehydrogenase (LDH) release assay (Abcam) following the manufacturer's protocol (23). The breast cancer cells [$1x10^4$ cells, referred to as target cells (T)] were co-cultured with activated $CD8^+$T lymphocytes with an E:T ratio of 20:1. The percentage specific lysis was calculated as follows: [(experimental LDH release-spontaneous LDH release)/(maximum LDH release-spontaneous LDH release)] x100.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Expression profiles of genes in the activated human $CD8^+$T lymphocytes were analyzed using the FAM-labeled Taqman gene expression RT-qPCR primers (Thermo Fisher Scientific, Inc.) for interferon-γ (IFNγ, Hs00989291_m1), granzyme B (Hs00188051_m1), and β-actin (Hs01060665_g1) (12). The total RNA obtained by TRIzol® extraction protocol was utilized to perform RT-qPCR (iTaq one step kit; cat. no. 1725151; Bio-Rad Laboratories, Inc.) with thermocycling conditions consisting of an initial denaturation of 95˚C for 15 min, followed by 40 cycles of 95˚C for 30 sec, followed by 61˚C for 1 min; and analyzed by $2^{-\Delta\Delta Cq}$ method in a final reaction volume of 50 μl using Bio-Rad CFX connect system (Bio-Rad Laboratories, Inc.) (24).

Statistical analysis. Data are expressed as the mean ± SEM from four independent studies. For multiple groups, significant

Table I. List of cell-penetrating peptides and HLA-A2 restricted MamA epitopes.

Peptide	Sequence
TAT	YGRKKRRQRRRPPQ
AntP	RQIKIWFQNRRJKWKK
S4	RVIRLVRVFRIFKLSR
R8	RRRRRRRRR
TP10	AGYLLGKINLKALAALAKKIL
MPG	GLAFLGFLGAAGSTMGAWSQPKKKRKV
A9	AAAAAAAAA (negative control)
MamA2.1	LIYDSSLCDL
MamA2.2	KLLMVLMLA
MamA2.3	LMVLMLAAL
MamA2.4	FLNQTDETL
MamA2.5	TLSNVEVFM
MamA2.6	MQLIYDSSL
MamA2.7	TINPQVSKT

S4, shaker-potassium channel protein; MamA2.1, mammaglobin-A.

differences between the groups were assessed using Tukey HSD pair-wise comparisons for two groups and one-way ANOVA for multiple comparisons. For two groups, unpaired t-test was used. $P<0.05$ was considered to indicate a statistically significant difference. All data analysis was carried out using GraphPad Prism 8 software (Dotmatics) or SPSS software version 21 (IBM Corp.).

Results

Enhanced HLA-A2 membrane stabilization of S4 conjugated MamA2.1 peptide. The T2 cells, due to their deficiency of TAP protein cannot load peptides from endogenous proteasome processed external antigenic proteins onto HLA-A2 molecules (22). This restricts the stable translocation of peptide-loaded HLA-A2 to the extracellular surface of cell membranes needed for eventual activation of naive $CD8^+$T lymphocytes to cytotoxic effector phenotype. However, external peptides that can optimally bind to the 3-dimensional peptide binding groove structure of HLA-A2 will circumvent the TAP-dependent pathway and induce stable membrane expression of HLA-A2. Therefore, T2 cell lines are widely utilized for peptide-HLA-A2 binding assay (25). To determine the HLA-A2 binding capability of CPP-conjugated MamA2.1 peptide (Fig. 1), membrane stabilization studies were performed utilizing T2 cells. In order to achieve this, each conjugated peptide (10 μg/ml) was incubated with T2 cells for 24 h, following which, HLA-A2 membrane expression was analyzed by median fluorescence channel shift (MFCS). As demonstrated in Fig. 1, MamA2.1 conjugated with AntP (221±35 AU, $P<0.05$) and S4 (326±43 AU, $P<0.05$) peptides demonstrated significantly increased MFCS as compared with unconjugated MamA2.1 (157±22 AU). Understandably, poly-alanine (A9) conjugated MamA2.1 (MamA2.1-A9), negative control, did not induce any surface expression of HLA-A2. All other conjugated peptides demonstrated a similar MCFS as compared with unconjugated MamA2.1. These data strongly suggested that S4-conjugated MamA2.1 induced the highest surface expression of HLA-A2 with a possibly enhanced potential to induce cytotoxic activation of naïve $CD8^+$T lymphocytes.

Cytotoxic efficiency of MamA2.1-S4 peptide-restricted $CD8^+$T lymphocytes. Next, it was determined if S4 conjugated MamA2.1 peptide-pulsed T2 cells could activate naïve $CD8^+$T lymphocytes. Towards this, naïve $CD8^+$T cells obtained from HLA-$A2^+$ healthy donors were stimulated *in vitro* by various peptide-pulsed T2 cells for four cycles followed by isolation of activated $CD8^+$T cells by magnetic beads. These HLA-A2 restricted and activated $CD8^+$T cells were co-cultured with human breast cancer cells. Two breast cancer lines, UACC812 (HLA-$A2^+$$MamA^+$) and MCF7 (HLA-$A2^+$$MamA^-$), were utilized in the present study. As demonstrated in Fig. 2A, $CD8^+$T cells activated by MamA2.1-S4-pulsed T2 cells demonstrated highest cytotoxicity (51.9±7.2%, $P<0.05$) followed by MamA2.1-AntP mediated activation (36.2±6.3%, $P<0.05$), compared with unconjugated MamA2.1 peptide (22.7±3.4%). As expected, no cytotoxicity was observed against the MCF7 breast cancer cell line which does not express MamA protein. Gene expression analysis of inflammatory cytokines (Fig. 2B and C) demonstrated enhanced expression of IFNγ and granzyme B in $CD8^+$T cells activated by MamA2.1-S4 pulsed T2 cells. These data specifically indicate that S4 conjugation of MamA2.1 peptide enhanced the potential vaccine efficiency of HLA class I restricted anticancer activation of $CD8^+$T cells.

S4 conjugation enhances the immunodominance of other HLA-A2 restricted MamA epitopes. A previous study by Jaramillo *et al* (10) , have identified seven candidate HLA-A2 restricted MamA epitopes (MamA2.1-2.7) based on an *in silico* binding algorithm. S4 conjugation to these seven epitopes was tested if it would enhance the immunodominance as measured by membrane stabilization and $CD8^+$T cell activation. As demonstrated in Fig. 3A, along with MamA2.1 (as aforementioned), S4 conjugation to HLA-A2 restricted MamA epitopes and further enhanced the membrane stabilization of MamA2.4 (303±38 AU, $P<0.05$), as compared with unconjugated MamA2.4 (169±24 AU). The other five epitopes (namely, MamA2.2, 2.3, 2.5, 2.6 and 2.7), while they demonstrated slightly increased membrane stabilization, did not achieve statistical significance (Fig. S1). Similarly, stimulation of $CD8^+$T cells by T2 cells pulsed with S4 conjugated MamA2.4 (in addition to MamA2.1) enhanced the cytotoxicity against UACC812 breast cancer cells (Fig. 3B). Furthermore, $CD8^+$ T cells stimulated by S4 conjugation of MamA2.2 and 2.3 epitopes also demonstrated significant cytotoxicity. As expected, none of the epitopes induced cytotoxicity of $CD8^+$T cells against MamA non-expressing MCF7 breast cancer cells (Fig. 3C). In line with membrane stabilization and cytotoxicity data, $CD8^+$T cells stimulated by MamA2.4-S4 and 2.1-S4 have demonstrated the highest expression of inflammatory cytokines IFNγ and granzyme B (Fig. 3D and E). Taken together, these data clearly suggested that conjugation of MamA immunodominant epitopes with S4-CPP, enhanced the cellular internalization and presentation of the antigenic

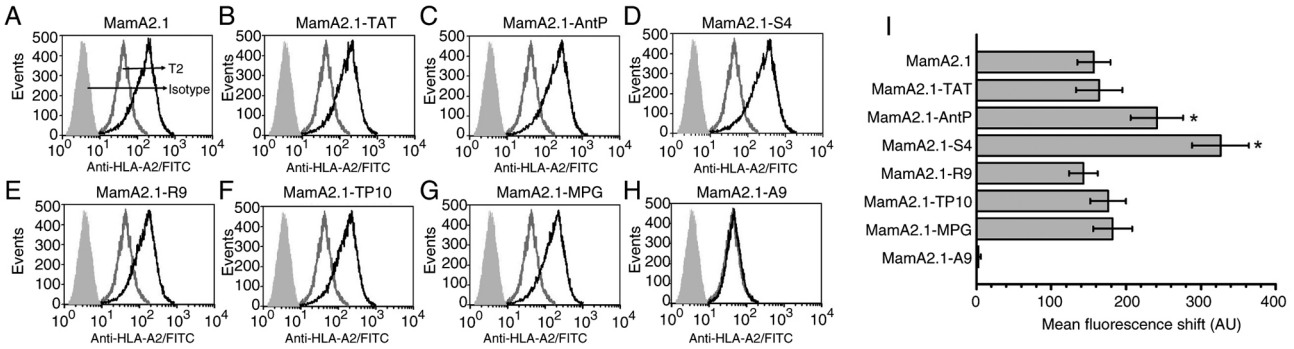

Figure 1. Membrane stabilization assay on T2 cells following treatment with various CPP-conjugated MamA2.1 epitope. (A-H) Various CPP conjugated MamA2.1 epitopes were incubated with T2 cells for 24 h. The membrane expression of HLA-A2 was determined by the shift in mean channel fluorescence of FITC labelled anti-HLA-A2 monoclonal antibodies (clone: BB7.2, demonstrated in unfilled bold black). The isotype control antibody (clone: MG2b-57) which has no binding affinity to HLA-A2 molecules is demonstrated in a filled grey histogram. The HLA-A2 membrane expression on empty T2 cells untreated with aforementioned epitopes is demonstrated in unfilled grey histogram. (I) Median fluorescence channel shift following treatment with various CPP conjugated MamA2.1 epitopes. *P<0.05 compared with the median fluorescence channel shift of MamA2.1 peptide. CPP, cell-penetrating peptides; MamA2.1, mammaglobin-A; HLA, human leukocyte antigen.

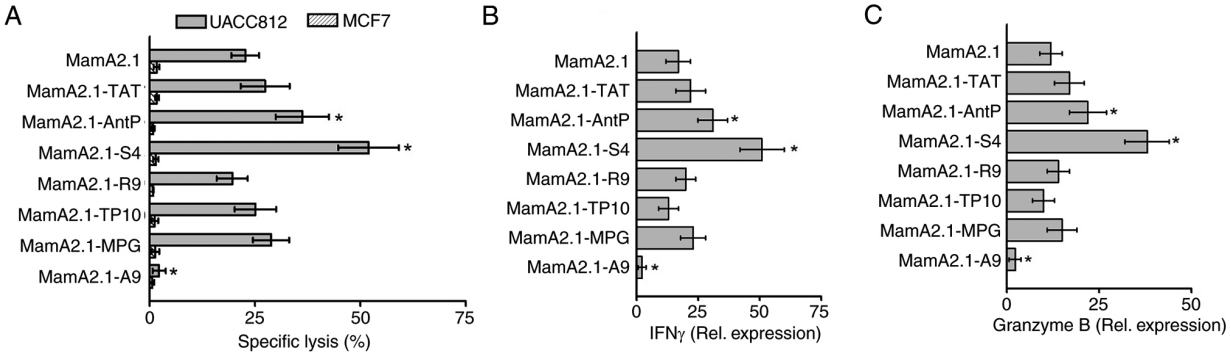

Figure 2. Activation of CD8+T lymphocytes following antigenic presentation of various cell-penetrating peptides -conjugated MamA2.1 epitope. (A) Cytotoxicity of activated CD8+T lymphocytes against human breast cancer cell lines, UACC812 (HLA-A2+MamA+, demonstrated in grey) and MCF-7 (HLA-A2+MamA-, demonstrated in crossed lines). (B and C) Gene expression of (B) IFNγ and (C) granzyme B in activated CD8+T lymphocytes. *P<0.05, as compared with the unconjugated MamA2.1 epitope. MamA2.1, mammaglobin-A; IFNγ, interferon-γ.

MamA immunodominant epitopes to CD8+T cells, causing their inflammatory activation and anticancer efficiency.

Conjugation of S4 at the C-terminus of MamA2.1 enhanced its immunodominance. Next, it was verified whether immunodominance would be impacted by the preferential conjugation of S4-CPP at the N-terminus versus the C-terminus of the MamA2.1 epitope. As demonstrated in Fig. 4, conjugation of S4-CPP at the C-terminus over the N-terminus exerted higher immunodominance as demonstrated by increased membrane stabilization (Fig. S2), UACC cytotoxicity and gene expression of inflammatory cytokines IFNγ and granzyme B. These data demonstrated that the C-terminus conjugation of S4-CPP to MamA2.1 epitope enhanced the cellular internalization leading to the loading of this peptide to HLA-A2 and translocation to the cell membrane. This caused efficient presentation of the antigenic determinant to activate naïve CD8+T cells to induce anticancer cytotoxicity.

Discussion

Compared with other vaccine and immunotherapeutic strategies, peptide-based vaccination approaches offer major advantages by their ability to selectively target cancer cells, induce long-term memory CD8+T lymphocytes, minimal side-effect profile, along with low manufacturing costs and easy scalability (26). However, limited efficacy of peptide vaccines in clinical trials could be attributed to poor antigen presentation, low immunogenicity and immune suppressive tumor microenvironment (27). Conjugation of antigenic epitopes with CPPs has been considered to enhance the efficiency of peptide vaccination. Various clinical trials (NCT01396239) have been designed to study the application of CPPs as efficient peptide vaccine delivery agents (28). However, there has been significant debate regarding the universal choice of a particular CPP for its efficient translocation through the cell membrane for the eventual antigenic presentation of cancer epitopes to induce immune responses. In the current study, six different CPPs were tested for their ability to enhance the immunogenicity of the MamA epitopes. While various CPPs have been tested by several research groups (29), the CPP capability of S4 protein transduction domain has not been reported.

The immunogenicity of peptide vaccines is dependent upon the ability of this epitope to be loaded on HLA class I molecules for its eventual presentation to CD8+T lymphocytes (30). The present study revealed that conjugation of

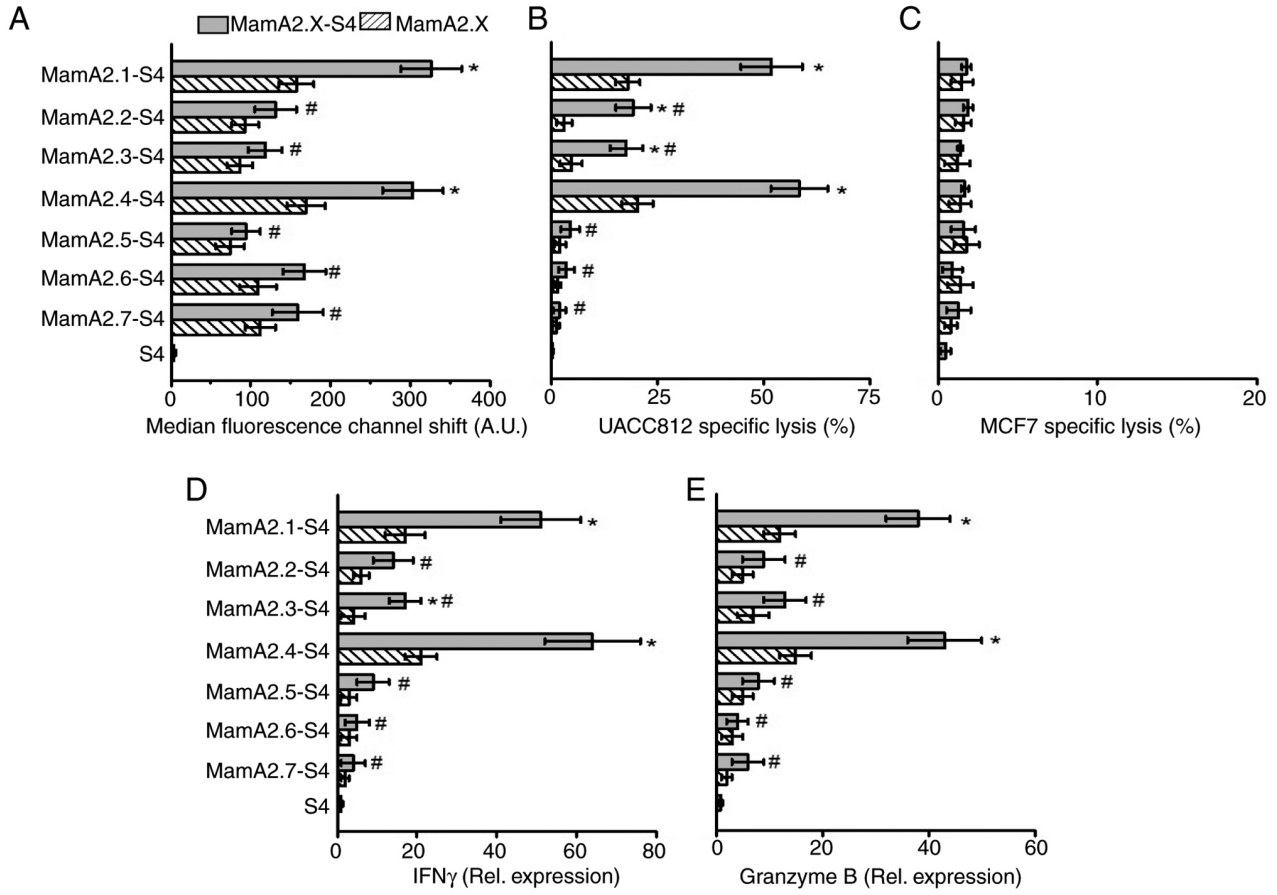

Figure 3. The N-terminus of S4 peptide is conjugated to various HLA-A2 restricted MamA antigenic epitopes. (A) Median fluorescence channel shift mediated by HLA-A2 expression on the cell surface T2 cells following treatment with S4 peptide conjugated HLA-A2 restricted MamA epitopes. (B and C) Cytotoxicity of these various peptide-activated CD8$^+$T lymphocytes against human breast cancer cell lines, (B) UACC812 and (C) MCF-7. (D and E) Gene expression of (D) IFNγ and (E) granzyme B in these various peptide-activated CD8$^+$T lymphocytes. *P<0.05, for conjugated MamA antigenic epitope as compared with the respective unconjugated epitope; $^\#$P<0.05, for S4 conjugated MamA2.X (X=2-7) as compared with S4 conjugated MamA2.1 antigenic epitope. S4, shaker-potassium channel protein; HLA, human leukocyte antigen; MamA2.1, mammaglobin-A; IFNγ, interferon-γ.

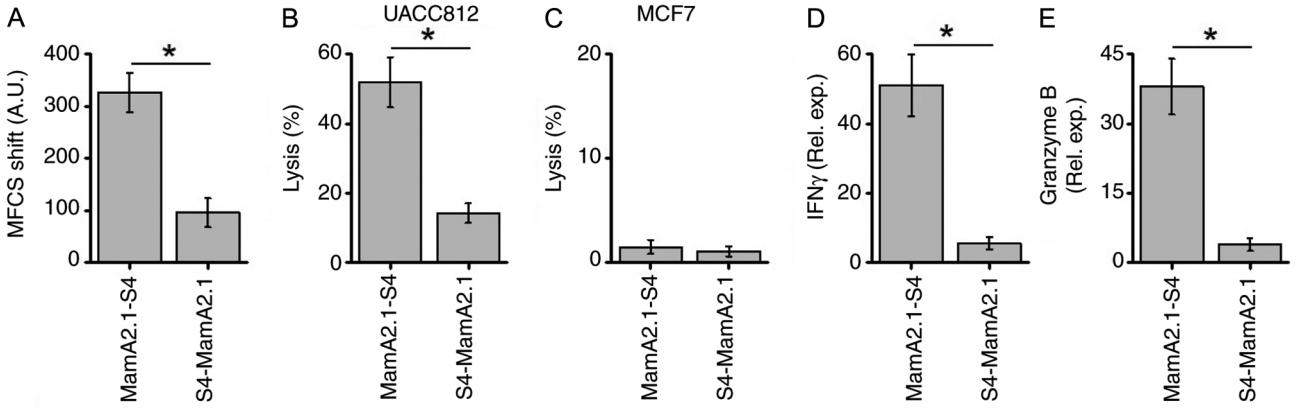

Figure 4. Comparison of MamA2.1 epitope conjugation to N-terminus versus C-terminus of S4 peptide. (A) Median fluorescence channel shift by HLA-A2 expression on the cell surface T2 cells following treatment with MamA2.1-S4 vs S4-MamA2.1 peptides. (B and C) Cytotoxicity of these CD8$^+$T lymphocytes individually activated by MamA2.1-S4 and S4-MamA2.1 against human breast cancer cell lines, (B) UACC812 and (C) MCF-7. (D and E) Gene expression of (D) IFNγ and (E) granzyme B in these activated CD8$^+$T lymphocytes. *P<0.05. MamA2.1, mammaglobin-A; S4, shaker-potassium channel protein; HLA, human leukocyte antigen; IFNγ, interferon-γ.

MamA2.1 and MamA2.4 epitopes with S4 domain enhances the stable membrane expression of HLA class I molecule. The S4-CPP with its ability to pass through the cell membrane, could enhance the cellular internalization of the HLA-A2 restricted MamA epitopes which in-turn enables the loading of this epitope onto HLA-A2 molecules. However, it may be unlikely that CPPs might impact other steps in antigen presentation, such as, proteasome-mediated proteolytic processing

for loading of epitopes onto HLA class I molecules (31). Enhanced fluorescence channel shift (Fig. 1) with CPP conjugated MamA2.1 and MamA2.4 epitopes suggested that there was an increased number of HLA-A2 molecules with stable membrane expression. Traditionally, TAP-deficient T2 cells were used to verify the activation of $CD8^+$T lymphocytes following appropriate recognition of antigenic determinants. The TAP protein is involved in translocation of endogenously processed antigenic determinants to endoplasmic reticulum for their eventual loading on HLA class I molecules in Golgi apparatus. The TAP-deficient T2 cells do not have stable surface membrane expression of HLA class I molecules. The exogenous antigenic determinant-pulsed T2 cells enable interaction of epitope-loaded HLA class I molecules to T-cell receptor (TCR) of naïve $CD8^+$T lymphocytes leading to their antigen specific cytotoxic activation.

The ability of S4-conjugated MamA epitope-pulsed T2 cells to prime CD8 T lymphocytes to activate their cytotoxic ability was identified (Fig. 2). Furthermore, conjugation of the S4 domain to less immunogenic MamA epitopes also revealed a marginal increase in HLA-A2 membrane expression and downstream T-cell activation (Fig. 3) suggesting that CPPs generally increase the final cytosolic availability of antigenic epitopes for loading onto HLA class I presentation. While it may be reasonable to expect that CPP-conjugation would directly increase the internalization and cytosolic abundance of antigenic epitope, studies by Backlund *et al* (32), revealed that enhanced immunogenicity of CPP-conjugation to a melanoma-associated antigenic epitope, gp100, requires Baft3-dependent cross presentation by professional APCs and not by direct CPP-mediated cytosolic internalization. They also revealed that enhanced immunogenicity following CPP-conjugation in their murine preclinical cancer model was dependent upon its ability to bind with lymph-tracking lipoprotein. Similarly, peptide loading capability of S4-conjugate epitopes to dendritic cells should be tested in murine models following the aforementioned MamA peptide vaccination. Therefore, future *in vivo* studies using dendritic cells are needed to validate the findings of the present study and to gain improved understanding of the molecular mechanisms impacting the vaccination efficiency of the S4-conjugated MamA epitopes. While the present study is limited to HLA-A2 subtype, future studies are needed to characterize the efficiency is the other HLA Class I subtypes for broader application among diverse HLA profiles.

In summary, the present study indicated that S4-domain conjugation enhances the anticancer immunogenicity of MamA epitope-based peptide vaccination. The present study is limited to assessing the *in vitro* efficiency of TAA-specific activation of $CD8^+$T lymphocytes. However, *in vivo* molecular mechanisms of antigen presentation are dependent upon vaccine bioavailability, tracking of vaccine to lymph nodes, cell type specific differences between various APCs and ability of T-cell receptor on $CD8^+$ T lymphocyte's binding ability to HLA molecules. Future studies are warranted in preclinical animal models to develop a detailed understanding of the efficiency of the S4-domain conjugation to immunodominant MamA epitopes. CPPs were considered to act as adjuvants to further enhance vaccine capability through binding with toll-like receptors (TLRs). The role of TLRs in CPP-conjugated peptide vaccination should also be analyzed. While the present study was designed to understand the priming of $CD8^+$T lymphocytes, future studies geared towards understanding $CD4^+$T lymphocyte adaptive immune responses will enable future applicability of CPP-conjugation for efficient peptide vaccination in cancer immunotherapy.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Authors' contributions

VT conceived the project and designed the experiments. JAM, UA, SV and VT performed the experiments. JAM, UA, SV, AYS and VT analyzed the data, and wrote and revised the paper. AYS and VT confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Healthy human tissue (including blood components) were obtained from external sources and commercial vendors. The present study was approved (approval no. HS2018-4093) by the Tennessee State University (Nashvile, USA) institutional review board.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Siegel RL, Giaquinto AN and Jemal A: Cancer statistics, 2024. CA Cancer J Clin 74: 12-49, 2024.
2. Brufsky A, Kwan ML, Sandin R, Stergiopoulos S, Karanth S, Cha-Silva AS, Makari D and Goyal RK: Trends in HR+ metastatic breast cancer survival before and after CDK4/6 inhibitor introduction in the United States: A SEER registry analysis of patients with HER2- and HER2+ metastatic breast cancer. Breast Cancer Res Treat 208: 223-235, 2024.
3. Sotirov S and Dimitrov I: Tumor-derived antigenic peptides as potential cancer vaccines. Int J Mol Sci 25: 4934, 2024.
4. Curigliano G, Bagnardi V, Ghioni M, Louahed J, Brichard V, Lehmann FF, Marra A, Trapani D, Criscitiello C and Viale G: Expression of tumor-associated antigens in breast cancer subtypes. Breast 49: 202-209, 2020.

5. Liu Z, Yang X, Duan C, Li J, Tong R, Fan Y, Feng J, Cao R, Zhong W, Feng X, *et al*: Identification and characterization of mammaglobin-A epitope in heterogenous breast cancers for enhancing tumor-targeting therapy. Signal Transduct Target Ther 5: 82, 2020.
6. Milosevic B, Stojanovic B, Cvetkovic A, Jovanovic I, Spasic M, Stojanovic MD, Stankovic V, Sekulic M, Stojanovic BS, Zdravkovic N, *et al*: The enigma of mammaglobin: Redefining the biomarker paradigm in breast carcinoma. Int J Mol Sci 24: 13407, 2023.
7. Li G, Zhang J, Jin K, He K, Wang H, Lu H and Teng L: Human mammaglobin: A superior marker for reverse-transcriptase PCR in detecting circulating tumor cells in breast cancer patients. Biomark Med 5: 249-260, 2011.
8. O'Brien N, Maguire TM, O'Donovan N, Lynch N, Hill AD, McDermott E, O'Higgins N and Duffy MJ: Mammaglobin a: A promising marker for breast cancer. Clin Chem 48: 1362-1364, 2002.
9. Tiriveedhi V, Tucker N, Herndon J, Li L, Sturmoski M, Ellis M, Ma C, Naughton M, Lockhart AC, Gao F, *et al*: Safety and preliminary evidence of biologic efficacy of a mammaglobin-a DNA vaccine in patients with stable metastatic breast cancer. Clin Cancer Res 20: 5964-5975, 2014.
10. Jaramillo A, Narayanan K, Campbell LG, Benshoff ND, Lybarger L, Hansen TH, Fleming TP, Dietz JR and Mohanakumar T: Recognition of HLA-A2-restricted mammaglobin-A-derived epitopes by CD8+ cytotoxic T lymphocytes from breast cancer patients. Breast Cancer Res Treat 88: 29-41, 2004.
11. Babaer D, Amara S, McAdory BS, Johnson O, Myles EL, Zent R, Rathmell JC and Tiriveedhi V: Oligodeoxynucleotides ODN 2006 and M362 exert potent adjuvant effect through TLR-9/-6 synergy to exaggerate mammaglobin-A peptide specific cytotoxic CD8+T lymphocyte responses against breast cancer cells. Cancers (Basel) 11: 672, 2019.
12. Babaer D, Zheng M, Ivy MT, Zent R and Tiriveedhi V: Methylselenol producing selenocompounds enhance the efficiency of mammaglobin-A peptide vaccination against breast cancer cells. Oncol Lett 18: 6891-6898, 2019.
13. Nelde A, Rammensee HG and Walz JS: The peptide vaccine of the future. Mol Cell Proteomics 20: 100022, 2021.
14. Xie N, Shen G, Gao W, Huang Z, Huang C and Fu L: Neoantigens: Promising targets for cancer therapy. Signal Transduct Target Ther 8: 9, 2023.
15. Nguyen AT, Szeto C and Gras S: The pockets guide to HLA class I molecules. Biochem Soc Trans 49: 2319-2331, 2021.
16. Moynihan KD, Holden RL, Mehta NK, Wang C, Karver MR, Dinter J, Liang S, Abraham W, Melo MB, Zhang AQ, *et al*: Enhancement of peptide vaccine immunogenicity by increasing lymphatic drainage and boosting serum stability. Cancer Immunol Res 6: 1025-1038, 2018.
17. Ding Y, Li Z, Jaklenec A and Hu Q: Vaccine delivery systems toward lymph nodes. Adv Drug Deliv Rev 179: 113914, 2021.
18. Kim CG, Kye YC and Yun CH: The role of nanovaccine in cross-presentation of antigen-presenting cells for the activation of CD8(+) T cell responses. Pharmaceutics 11: 612, 2019.
19. Yang J, Luo Y, Shibu MA, Toth I and Skwarczynskia M: Cell-penetrating peptides: Efficient vectors for vaccine delivery. Curr Drug Deliv 16: 430-443, 2019.
20. Tiriveedhi V and Butko P: A fluorescence spectroscopy study on the interactions of the TAT-PTD peptide with model lipid membranes. Biochemistry 46: 3888-3895, 2007.
21. Tiriveedhi V, Miller M, Butko P and Li M: Autonomous transmembrane segment S4 of the voltage sensor domain partitions into the lipid membrane. Biochim Biophys Acta 1818: 1698-1705, 2012.
22. Bossi G, Gerry AB, Paston SJ, Sutton DH, Hassan NJ and Jakobsen BK: Examining the presentation of tumor-associated antigens on peptide-pulsed T2 cells. Oncoimmunology 2: e26840, 2013.
23. Tiriveedhi V, Ivy MT, Myles EL, Zent R, Rathmell JC and Titze J: Ex vivo high salt activated tumor-primed CD4+T lymphocytes exert a potent anti-cancer response. Cancers (Basel) 13: 1690, 2021.
24. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
25. Luft T, Rizkalla M, Tai TY, Chen Q, MacFarlan RI, Davis ID, Maraskovsky E and Cebon J: Exogenous peptides presented by transporter associated with antigen processing (TAP)-deficient and TAP-competent cells: Intracellular loading and kinetics of presentation. J Immunol 167: 2529-2537, 2001.
26. Zahedipour F, Jamialahmadi K, Zamani P and Jaafari MR: Improving the efficacy of peptide vaccines in cancer immunotherapy. Int Immunopharmacol 123: 110721, 2023.
27. Stephens AJ, Burgess-Brown NA and Jiang S: Beyond just peptide antigens: The complex world of peptide-based cancer vaccines. Front Immunol 12: 696791, 2021.
28. Bottens RA and Yamada T: Cell-Penetrating peptides (CPPs) as therapeutic and diagnostic agents for cancer. Cancers (Basel) 14: 5546, 2022.
29. Hasannejad-Asl B, Pooresmaeil F, Takamoli S, Dabiri M and Bolhassani A: Cell penetrating peptide: A potent delivery system in vaccine development. Front Pharmacol 13: 1072685, 2022.
30. Buonaguro L and Tagliamonte M: Peptide-based vaccine for cancer therapies. Front Immunol 14: 1210044, 2023.
31. Wu X, Li T, Jiang R, Yang X, Guo H and Yang R: Targeting MHC-I molecules for cancer: Function, mechanism, and therapeutic prospects. Mol Cancer 22: 194, 2023.
32. Backlund CM, Holden RL, Moynihan KD, Garafola D, Farquhar C, Mehta NK, Maiorino L, Pham S, Iorgulescu JB, Reardon DA, *et al*: Cell-penetrating peptides enhance peptide vaccine accumulation and persistence in lymph nodes to drive immunogenicity. Proc Natl Acad Sci USA 119: e2204078119, 2022.