

# Methylselenol producing selenocompounds enhance the efficiency of mammaglobin-A peptide vaccination against breast cancer cells

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**Abstract.** Previous phase I DNA-vaccine based clinical trials using Mammaglobin-A (Mam-A), a human breast tumor associated antigen (TAA), demonstrated that this agent was safe and efficient at treating patients with stage IV breast cancer. The long-term success of cancer vaccines is limited by the diminished expression of human leukocyte antigen (HLA) class I molecules in the tumor microenvironment. The current study assessed the impact of various selenocompounds on the expression of HLA class I molecules in THP-1 cells, an apparent proficient antigen that presents a human monocyte-like cell line, and their eventual activation of MamA2.1 (HLA-A2 immunodominant epitope of Mam-A) specific cytotoxic CD8<sup>+</sup> T lymphocytes (CTLs). The results revealed that, following treatment with methylselenol producing compounds [methylselenic acid (MSA) and dimethylselenide (DMDSe)], the expression of HLA class-I was increased and components involved with the antigen presentation machinery of THP-1 cells were upregulated. Furthermore, CTLs activated by MamA2.1 peptide presenting THP-1 cells, pre-treated with MSA and DMDSe, demonstrated an enhanced cytotoxicity in HLA-A2<sup>+</sup>/Mam-A<sup>+</sup> AU565 and UACC-812 breast cancer cell lines when compared with CTLs activated by THP-1 cells without drug treatment. However, no significant cytotoxicity was observed under similar conditions in HLA-A2<sup>+</sup>/Mam-A<sup>-</sup> MCF-7 and MDA-MB-231 breast cancer cell lines. The results indicated that treatment with methylselenol producing compounds retained antigen-dependent activation of CD8<sup>+</sup> T cells. The data of the current study demonstrated that MSA and DMDSe potentiated effector cytotoxic responses following TAA specific activation of CTLs, indicating their future role as vaccine adjuvants in cancer immunotherapy.

## Introduction

Tumors adopt various immune escape strategies to avoid recognition and elimination by cytotoxic CD8<sup>+</sup> T lymphocytes (CTLs) (1). A major mechanism by which tumors escape immune recognition is by downregulating their surface expression of human leukocyte antigen (HLA) class I molecules. A major explanation for the disappointing outcome from DNA and peptide-based anti-cancer vaccines is that the minimal tumoral expression of HLA class I molecules do not allow for the vaccine activated CTLs to exert their cancer eliminating cytotoxic effect in the tumor microenvironment (2). Similarly, decreased HLA expression is also considered to play a deleterious role in the favorable clinical outcomes of immune checkpoint inhibitor based cancer immunotherapeutic strategies. A decreased HLA class I expression is noted in various solid organ tumors including breast cancers (3). Hence, there is an important need to develop novel approaches to enhance HLA class I expression to overcome tumor immune escape and promote tumor rejection. Further, potential HLA class I recovery in the tumor microenvironment will complement the clinical outcome of various anti-tumor immunotherapeutic strategies including vaccine based approaches.

Active metabolite derivatives of Selenium (Se) have been suggested to exert an anti-carcinogenic effect on many solid organ tumors such as prostate and breast cancer (4). Clinical interventional studies demonstrated that a supra-nutritive intake of Se has positive effects in the prevention of several solid organ cancers (5). The two key metabolites of Se (Fig. 1A), hydrogen selenide (H<sub>2</sub>Se), derived mainly from inorganic selenocompounds such as selenate or selenite, and methylselenol (CH<sub>3</sub>SeH), derived from organic selenocompounds, such as methyl selenic acid (MSA) and dimethylselenide (DMDSe), have been shown to be crucial for the biological function of these selenocompounds (6,7). While the exact mechanisms of action of this anti-carcinogenic effect of selenium derivatives are unknown, several studies have suggested that the Se metabolite, methylselenol, is the active Se compound for anti-carcinogenic effects while remaining non-cytotoxic on normal terminally differentiated cells. Murine tumor studies have shown that combined treatment of MSA with paclitaxel reduced tumor growth of tumor xenografts with triple negative

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breast cancer cells (TNBC) compared to paclitaxel treatment alone (8).

Mammaglobin-A (Mam-A) is a human breast tumor-associated antigen (TAA) expressed in 40-80% of primary and metastatic breast cancers (9-13). Previously, we have demonstrated that Mam-A based vaccination to stage-IV human breast cancer patients was safe and efficient in increasing progression free survival in vaccinated patients. Murine and *in vitro* studies have demonstrated that HLA-A2-restricted MamA2.1 peptide (amino acids 83-92 of Mam-A, LIYDSSLCDL) exerted specific immunodominance towards effector cytotoxic activation of naïve CD8<sup>+</sup> T lymphocytes (14,15). While we have shown that following Mam-A vaccination there was some increase in the frequency of MamA2.1+CD8 T cells, strategies to further enhance HLA class I expression will provide an additional adjuvant methodology to enhance vaccine efficiency. Therefore, in this communication, we studied the role of selenium compounds towards increasing the cytotoxic efficiency of HLA-A2 restricted Mam-A epitope (MamA2.1) activated CTLs on Mam-A expressing human breast cancer cells.

## Materials and methods

**Cell lines and healthy human CD8<sup>+</sup> T lymphocytes.** The human breast cancer cell lines were selected based on the specific expression of antigen presenting class I HLA-A2 molecule and expression of tumor specific antigen, mammaglobin-A. The following cell lines: MAM-A<sup>+</sup>/HLA-A2<sup>+</sup> (AU565 and UACC-812) and MAM-A<sup>+</sup>/HLA-A2<sup>+</sup> (MCF-7 and MDA-MB-231), and human monocyte-like HLA-A2<sup>+</sup> cell line, THP-1 cells, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human CD8<sup>+</sup> T cells from HLA-A2+ healthy subjects were obtained from StemCell Technologies (Cambridge, MA, USA). All cell cultures and incubations were performed as per provider's recommendations and described by us before (16). Briefly, cell were cultured in RPMI-1640 medium at 37°C in 5% CO<sub>2</sub> incubator until they were 80% confluent. The presence of Mam-A and HLA-A2 expression in the breast cancer cell lines was confirmed by western blot analysis (data not shown). The selenocompounds, methylseleninic acid (MSA), dimethylselenide (DMDSe) and selenomethionine (SeMet) were obtained from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany. The THP-1 cells were cultured in 24 well plates, 1x10<sup>5</sup> per well and stimulated with respective selenocompounds (5 µM) for 24 h. These cells were later used for various experiments detailed below. For MamA2.1 peptide stimulation (Peptide 2.0 Inc, Chantilly, VA), CD8<sup>+</sup> T lymphocytes (1x10<sup>6</sup>) were cultured in 2 ml of supplemented RPMI-1640 media in 24-well plates in the presence of irradiated (5,000 rads) THP-1 cells (1x10<sup>6</sup>) loaded with Mam-A2.1 in the presence of β2 m (3 µg/ml), CD3 (500 ng/ml), CD28 mAb (500 ng/ml) and recombinant human IL-2 (20 U/ml). The CD8<sup>+</sup> T lymphocytes were isolated by immunomagnetic separation (MACS Miltenyi Biotec, San Diego, CA) and the resulting purity was verified to be >95%. The MamA2.1 peptide was custom synthesized by Peptide 2.0 Inc. (Chantilly, VA) and purified on HPLC column to >95% purity.

**High-performance liquid chromatography.** The supernatant from cell cultures were treated with methanol (1:1 final concentration)

and injected into the HPLC system. The Agilent 1100 HPLC system was comprised of isocratic pump (G1310A) and an auto sampler (G1313A). The Gemini C18 (3 µM, 110 Å, 50x1 mm inner diameter) columns were utilized for chromatography. The mobile phase was 0.1% formaldehyde in 40% methanol. The flow rate was 100 µl/min. Injection volumes were 10 µl. Data were analyzed with Chemstation software.

**Cytotoxicity assay.** The cytotoxic efficiency of peptide-activated CD8<sup>+</sup> T cells was investigated by its ability to lyse the target breast cancer cells by non-irradiative LDH release assay (Promega Corporation, Madison, WI, USA) and and MTT assay (Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The breast cancer cells (1x10<sup>4</sup> cells) in 100 µl of complete medium at were plated in triplicate cultures in round bottom 96-well plates in the presence of varying numbers of CD8<sup>+</sup> T cells (6.25:1 to 50:1) and incubated at 37°C. The percentage specific lysis was calculated as follows: [(experimental LDH release-spontaneous LDH release)/(maximum LDH release-spontaneous LDH release)] x100.

**Flow cytometry.** The HLA-A2 expression in cells were analyzed by flow cytometry using appropriated primary (BB7.2, BioLegend, San Diego, CA, USA) and FITC-labelled secondary antibody. Samples were analyzed using a FACS Calibur/LSRII flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Data were analyzed using BD FACSDiva software. Gates were set according to isotype controls.

**ELISA.** The secretory extracellular IFNγ in the cell supernatant was quantitated by ELISA as per the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA; catalog# DY285B). Quantification was performed with a standard curve using the manufacturer provided standards. Detection at 450 nm was performed using EMax Plus spectrophotometer and data analysis was carried out using software provided by the manufacturer (Molecular Devices, Sunnyvale, CA, USA).

**Western blotting.** Total proteins were extracted from cells with lysis buffer for western blot analysis as previously described. Total proteins were separated on a 4-12% sodium dodecyl sulfate-polyacrylamide gradient gel and transferred onto a nitrocellulose membrane. All primary and secondary Abs were obtained from Abcam (Cambridge, MA, USA) or Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The following specific primary antibodies to TAP-1 (sc-376796, Santa Cruz Biotechnology, Inc., 1:200), TAP-2 (sc-515576, Santa Cruz Biotechnology, Inc., 1:200), LMP-2 (ab3328, Abcam, 1:500), LMP-7 (ab3329, Abcam, 1:500), tapasin (ab196764, Abcam, 1:500), β2-microglobulin (sc-515576, Santa Cruz Biotechnology, Inc., 1:200) and β-actin (sc-8432, Santa Cruz Biotechnology, Inc., 1:200). The following specific secondary antibodies were used based the species of the primary antibody: Goat anti-mouse-HRP (ab205718, Abcam, 1:2,500) and goat anti-rabbit-HRP (ab205718, Abcam, 1:2,500). The transferred and probed nitrocellulose membranes were developed using the chemiluminescence kit (EMD Millipore, Billerica, MA, USA) and analyzed on using Bio-Rad Universal Hood II (Hercules, CA, USA). Morphometric analysis was done using the software provided by the company.

Table I. Primer sequences used for reverse transcription-quantitative PCR.

Gene	Forward (5'-3')	Reverse (5'-3')	Acc. no.	Product length (bp)
Tap1	TGCCTAAGAAGCTGGGAAAA	GTAAGCCAAGGCCTCCTTCT	NM_013683.2	203
Tap2	CGGTGCTAAAGGAGATCCAG	CCATCACCCCTCCGTATGACT	NM_011530.3	204
LMP2	TCTTCTGTGCCCTCTCAGGT	TGGTCCCAGCCAGCTACTAT	NM_013585.2	193
LMP7	GGAACGCATCTCCGTGTCTG	CTGCCGGTAACCACTGTCCA	NM_010724.2	223
Tapasin	ACACTGCGAGATGAGCCGCTTC	TGAGGACGGTCAGCACTACTGT	NM_009318.2	221
B2m	GCCGAACATACTGAACTGCT	GCCATACTGGCATGCTTAAC	NM_009735.3	207
Jak1	CCGCATGAGGTTCTACTTTACC	TCAAATCATACTGTCCCTGTGC	NM_146145.2	179
Jak2	GCAGATTCATTCAGCAATTCAG	CGTCCTGTTCTGTCACTGTCTC	NM_008413.3	240
Stat1	GACACCTGCAACTGAAG	CACCAGCATGTTGTACC	NM_001205313.1	239
Stat2	CTGAAGGAGATGAGTCACATGC	GGTGAACCTGTTCCCAGTCTTC	NM_019963.1	189
Stat3	ACAACGCTGGCTGAGAAGCTCC	TTGTGCTTAGGATGGCCCGCTC	NM_213659.3	226
Irf1	GAAGATAGCCGAAGACCT	CTTCATCTCCGTGAAGAC	NM_001159396.1	205
Irf2	GGTCCTGACTTCAGCTAT	TTCTGCGTAGGAAGACAG	NM_008391.4	220
Irf5	GTCAAGACGAAGCTCTTTAGCC	CTGCTCTACCATGTGGTCTTTG	NM_001252382.1	279
Irf7	GTTTACGAGGAACCTATGCAG	GAAGCGTCTCTGTGTAGTGCAG	NM_001252601.1	277
Irf9	GCGTTGTAAACCACTCAGACAG	CATAGATGAAGGTGAGCAGCAG	NM_001159417.1	204
GAPDH	TTGTGCAGTGCCAGCCTCGT	TCGGCCTTGACTGTGCCGTT	NM_008084.3	214
$\beta$ -actin	ACTGTTCGAGTCGCGTCC	ATGGCTACGTACATGGCTGG	NM_007393.5	487

**Reverse transcription-quantitative PCR (RT-qPCR).** Expression profiles of genes at mRNA level in the breast cancer cell lines were analyzed using the SyBr-green detection based RT-qPCR primers (Table I), obtained from Integrated DNA Technologies (San Jose, CA, USA). Briefly, total RNA was extracted from  $10^6$  cells using TRIzol reagent (Sigma-Aldrich; Merck KGaA) and analyzed as mentioned previously. The cycling conditions consisted 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. The final reaction volume of 50  $\mu$ l using BioRad CFX96 (Hercules) and analyzed by  $2^{-\Delta\Delta C_q}$  method (17).

**Statistical analysis.** Data are expressed as mean  $\pm$  SEM from four independent studies. Significant differences between groups were assessed using Tukey HSD pair-wise comparisons for two groups and one-way ANOVA for multiple comparisons. A P-value of  $<0.05$  was considered significant.

## Results

**Enhanced HLA class I expression following treatment with methylselenol producing selenocompounds.** As upregulation of HLA class I molecules in the tumor-infiltrating immune cells is considered critical for cancer immunotherapy, we first examined the potential impact of the three selenocompounds, namely methylseleninic acid (MSA), dimethyldiselenide (DMDSe) and selenomethionine (SeMet), towards modulation of the surface expression of HLA class I molecules on THP-1 cells. As the metabolite methylselenol is volatile, we investigated the production of dimethylselenol (DMSe; Fig. 1A) following treatment with various selenium compounds (18,19). We first tested for DMSe production by

THP-1 cells following treatment with selenocompounds for 72 h. As shown in Fig. 1B, there was an enhanced DMSe production following treatment with MSA and DMDSe, while there was no production of DMSe following treatment with SeMet. We next tested the cell surface expression of HLA-A2 on THP-1 cells following treatment with selenocompounds. As shown in Fig. 1C-F, DMDSe (2.5  $\mu$ M) induced a 2.4-fold increased expression of HLA-A2 (from  $29.8 \pm 4.3\%$ , without treatment, to  $71.7 \pm 9.1\%$  following DMDSe treatment,  $P < 0.05$ ) on THP-1 cells. Further, MSA (2.5  $\mu$ M) induced a 2.3-fold increased expression of HLA-A2 molecules, while SeMet (1-50  $\mu$ M) did not induce any change in HLA-A2 expression on THP-1 cells. These data suggest that MSA and DMDSe induced enhanced expression of HLA class I molecules which could have a critical adjuvant role in antigen presentation, eventually leading to potential immune mediated elimination of tumor cells following anti-tumor vaccination.

**Upregulation of antigen presenting machinery following treatment with methyl selenol producing selenocompounds.** To determine the molecular mechanisms leading to the enhanced upregulation of the HLA-A2 surface expression following treatment with selenocompounds, we analyzed the changes in the expression of molecules involved in the surface expression of HLA class I molecules, also known as antigen presentation machinery (APM). As shown in Fig. 2, treatment with MSA and DMDSe increased the protein and mRNA transcript levels of key APM components, namely, TAP-1, TAP-2, LMP-2, LMP-7, tapasin and  $\beta$ 2-microglobulin in THP-1 cells. However, SeMet treatment did not induce the expression of the APM components. These data demonstrate that the MSA and DMDSe induced the APM which has a potential to induce

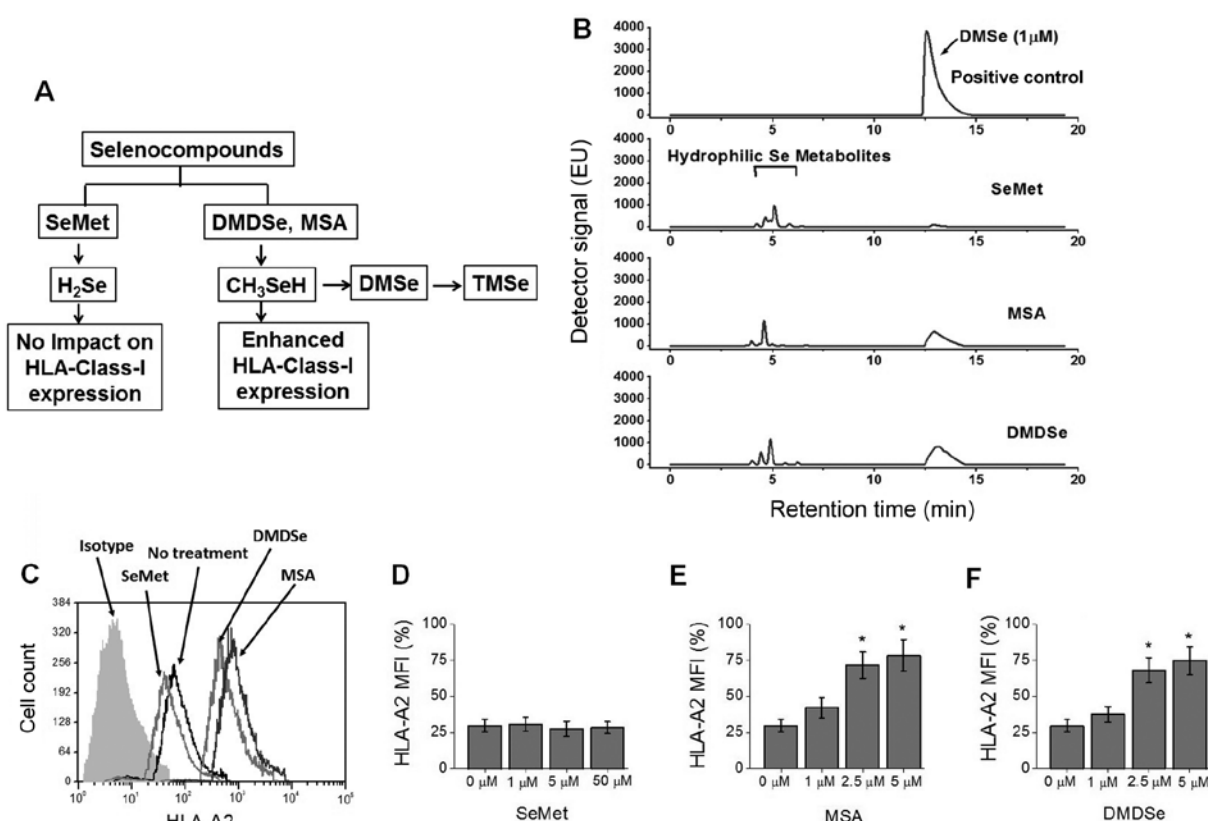


Figure 1. HLA-A2 expression in THP-1 cells following treatment with selenocompounds. (A) Schematic of selenocompound metabolism. (B) Production of DMSe by THP-1 cells following treatment with SeMet (50  $\mu$ M), MSA (2.5  $\mu$ M) and DMDSe (2.5  $\mu$ M). DMSe could be further methylated to unstable TMSe. Pure DMSe at 1  $\mu$ M in cell culture media was analyzed and used as positive control. (C) HLA-A2 surface expression on THP-1 cells following treatment with SeMet (50  $\mu$ M), MSA (2.5  $\mu$ M) or DMDSe (2.5  $\mu$ M). Percent expression was analyzed via isotype labelled negative control antibody staining. (D-F) Dose response following treatment (D) SeMet, (E) MSA and (F) DMDSe to assess the expression of HLA-A2. Data are presented as the mean  $\pm$  SEM (n=4). Statistical significance for C, D and E were analyzed via one-way ANOVA. \*P<0.05 vs. 0  $\mu$ M. HLA, human leukocyte antigen; SeMet, selenomethionine; MSA, methylselenic acid; DMDSe, methylselenol derivative dimethyl selenol; TMSe, trimethyl selenol; MFI, mean fluorescence intensity.

tumor associated antigen specific effector CD8<sup>+</sup> T cell immune responses against cancer cells.

**Enhanced activation of Mam-A specific effector CD8<sup>+</sup> T lymphocyte mediated cytotoxic responses following treatment with selenocompounds.** As our data demonstrated that following MSA and DMDSe treatment there was enhanced HLA-A2 expression on THP-1 cells, we next determined if this enhanced expression enabled HLA class-I immunodominant MamA2.1 peptide presentation and eventual antigen-specific CD8<sup>+</sup> T cell activation leading to cell mediated cytotoxicity against breast cancer cells. We therefore stimulated naïve CD8<sup>+</sup> T lymphocytes collected from healthy HLA-A2<sup>+</sup> human subjects with MamA2.1peptide in the presence of antigen presenting HLA-A2<sup>+</sup> THP-1 mononuclear cells pre-treated with selenocompounds (Fig. 3A). We collected these activated CD8<sup>+</sup> T cells by magnetic beads isolation and ascertained the purity (>95%) by flow cytometry. We investigated if these MamA2.1 activated CD8<sup>+</sup> T cells exerted higher cytotoxicity against breast cancer cell lines compared to activation by THP-1 cells without selenocompound treatment. The breast cancer cell lines (referred to as target cells) and MamA2.1 activated CD8<sup>+</sup> T cells (referred to as effector cells) were co-cultured. The cytotoxic effect was analyzed by LDH release assay and measured at various effector to

target (E:T) ratios (6.25: 1 to 50:1). As shown in Fig. 3B-E, the MamA2.1-specific CD8<sup>+</sup> T lymphocytes exerted higher cytotoxicity on HLA-A2<sup>+</sup>/MamA<sup>+</sup> AU565 and UACC-812 breast cancer cell lines. The CD8<sup>+</sup> T cells activated by THP-1 cells pre-treated with MSA (72 $\pm$ 8%, P<0.05) and DMDSe (66 $\pm$ 11%, P<0.05) exerted higher cytotoxicity (E:T 50:1) compared to non-selenocompound pre-treatment (44 $\pm$ 9%). However, pre-treatment with SeMet could not induce any significant additional cytotoxicity (46 $\pm$ 8%, P>0.05). Furthermore, MamA2.1 activated CD8<sup>+</sup> T cells did not induce any cytotoxicity on HLA-A2<sup>+</sup>/MamA<sup>-</sup> MCF-7 (Fig. 3D) and MDA-MB-231 (Fig. 3E) breast cancer cell lines, thus suggesting that the MamA2.1 activated CD8<sup>+</sup> T cells exerted cytotoxic effector functionality on breast cancer cells in Mam-A antigen-specific manner. Taken together, these data demonstrated that MSA and DMDSe induced HLA class I expression leading enhanced antigen presentation of immunodominant antigenic peptides following potential anti-cancer vaccination leading to breast cancer cell elimination.

**Increased IFN $\gamma$  signaling in CD8<sup>+</sup> T cells following activation by methylselenol producing selenocompound pre-treated THP-1 cells.** As interferon (IFN)- $\gamma$  signaling plays a critical role in cytotoxic CD8<sup>+</sup> T lymphocyte responses, we determined the expression of IFN signaling molecules. Using methodologies

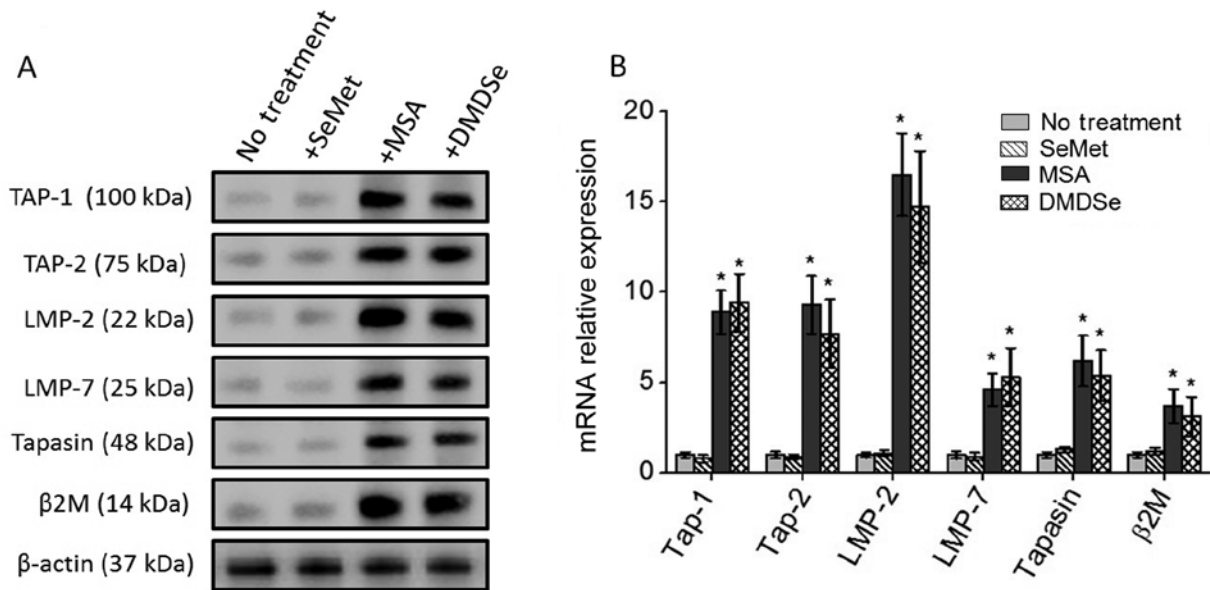


Figure 2. Expression of components involved in the APM of breast cancer cell lines following treatment with selenocompounds. (A) Western blot analysis and (B) mRNA levels of the APM components in THP-1 cells following treatment with SeMet (50  $\mu$ M), MSA (2.5  $\mu$ M) or DMDSe (2.5  $\mu$ M). Data are presented as the mean  $\pm$  SEM (n=4). Statistical significance was determined using a Tukey HSD test compared with non-treatment conditions. \*P<0.05 vs. 0  $\mu$ M. APM, antigen presentation machinery; SeMet, selenomethionine; MSA, methylselenic acid; DMDSe, methylselenol derivative dimethyl selenol; Rel, relative; TAP, transporter associated with antigen processing; β2M, β2-microglobulin.

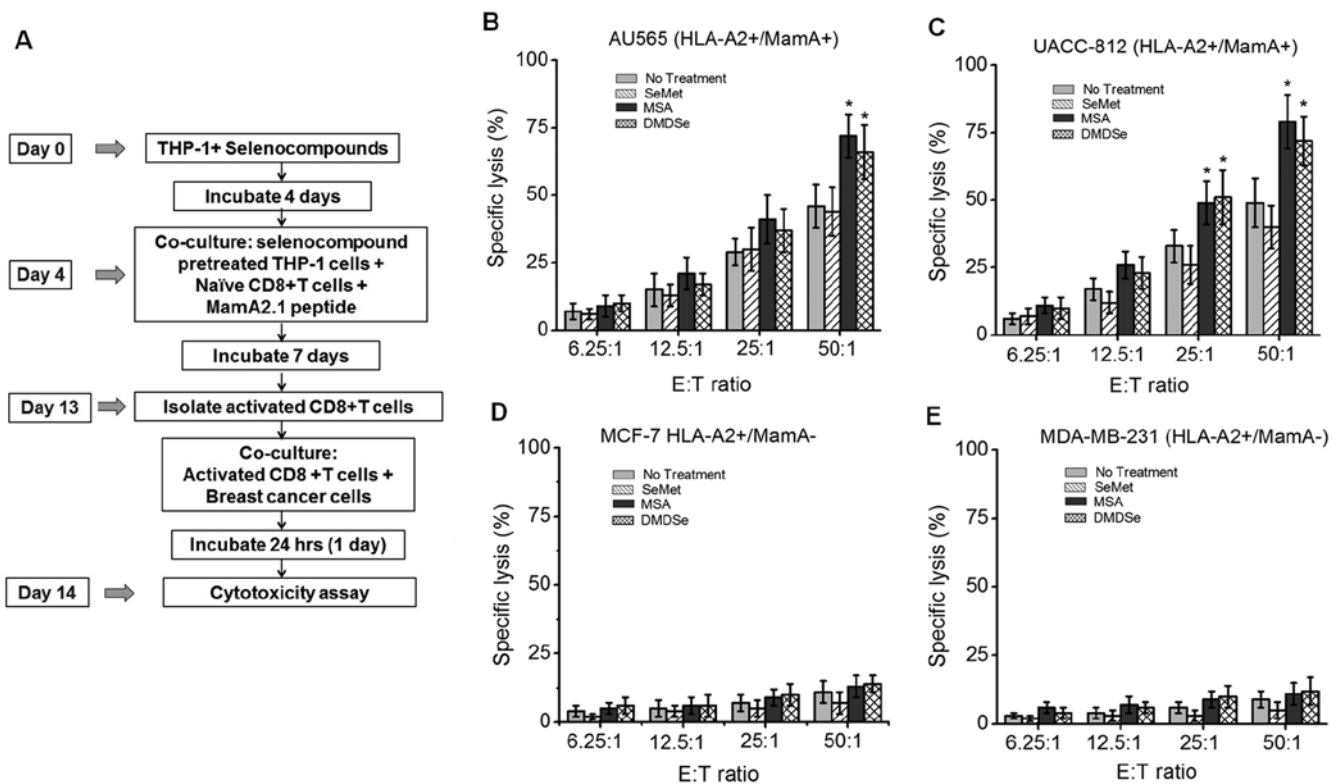


Figure 3. Cytotoxic potential of CTLs following activation by MamA2.1 peptide and co-stimulation with selenocompounds pre-treated THP-1 cells. (A) Experimental design schematic. Cytotoxicity of CTLs following activation by MamA2.1 peptide and THP-1 cells pre-treated with SeMet (50  $\mu$ M), MSA (2.5  $\mu$ M) or DMDSe (2.5  $\mu$ M) in (B) AU565 (HLA-A2+/MamA+), (C) UACC-812 (HLA-A2+/MamA+), (D) MCF-7 (HLA-A2+/MamA-) and (E) MDA-MB-231 (HLA-A2+/MamA-) cells. E:T ratios of 50:1, 25:1, 12.5:1 and 6.25:1 are presented. Data are presented as the mean  $\pm$  SEM (n=4). Statistical significance was determined via one-way ANOVA. \*P<0.05 vs. 0  $\mu$ M. CTLs, CD8+ T lymphocytes; SeMet, selenomethionine; MSA, methylselenic acid; DMDSe, methylselenol derivative dimethyl selenol; HLA, human leukocyte antigen; E:T, effector:target.

described above, we determined the transcript levels of IFN signaling molecules in MamA2.1 activated CD8+ T cells at

4 h following co-culture with AU565 cells at E:T ratio of 50:1. As shown in Fig. 4A, CD8+ T cell activation by THP-1 cells

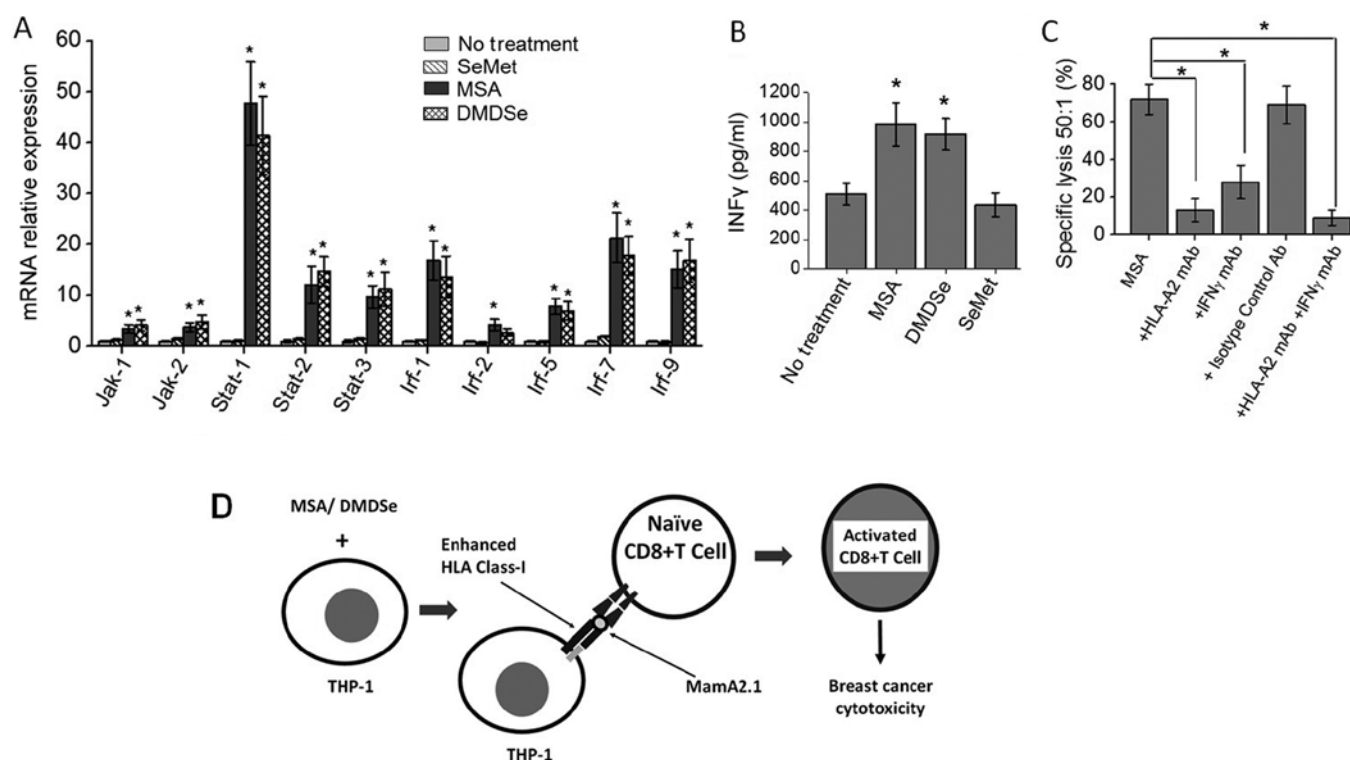


Figure 4. Upregulation of IFN $\gamma$  signaling in CTLs following activation by the MamA2.1 peptide and co-stimulation with selenocompound pre-treated THP-1 cells. (A) Expression of components involved in the IFN $\gamma$  signaling pathway molecules in CD8 $^{+}$  T cells following activation with MamA2.1 peptide, and in THP-1 cells pre-treated with SeMet (10  $\mu$ M), MSA (2.5  $\mu$ M) or DMDSe (2.5  $\mu$ M). (B) Quantity of IFN $\gamma$  protein secreted in the supernatant following the 4 h incubation of MamA2.1 activated CTLs and AU565 cells at an E:T ratio of 50:1, as determined via ELISA. (C) Cytotoxicity of CTLs on AU565 at an E:T ratio 50:1 upon blocking of IFN $\gamma$  and HLA-A2 by specific monoclonal antibodies. (D) Schematic of the mechanism of action of MSA and DMDSe towards CTL activation. Data are presented as the mean  $\pm$  SEM (n=4). Statistical significance between groups were assessed using Tukey HSD pair-wise comparisons (A) and one-way ANOVA for multiple comparisons (B and C) \*P<0.05 vs. 0  $\mu$ M. IFN $\gamma$ , interferon- $\gamma$ ; CTLs, CD8 $^{+}$  T lymphocytes; SeMet, selenomethionine; MSA, methylselenic acid; DMDSe, methylselenol derivative dimethyl selenol; HLA, human leukocyte antigen; Rel, relative; E:T, effector:target.

pre-treated with MSA and DMDSe induced higher expression of IFN signaling molecules namely, Jak1, Jak2, Stat1, Stat2, Stat3, Irf-1, Irf-5, Irf-7 and Irf-9. As these data demonstrated there is increased transcript levels of IFN signaling molecules, we next determined the secretory IFN $\gamma$  protein concentration in the supernatant obtained from the co-cultures by ELISA. As shown in Fig. 4B, ELISA based analysis of the IFN $\gamma$  protein expression in the supernatant collected from the CD8 $^{+}$  T cells activated under MSA (1179 $\pm$ 127 Pg/ml, P<0.05) and DMDSe (983 $\pm$ 148 pg/ml, P<0.05) pre-treatment conditions resulted in enhanced IFN $\gamma$  expression compared to no selenocompound pre-treatment (511 $\pm$ 73 pg/ml). However, CD8 $^{+}$  T cells activated by SeMet pre-treatment conditions (549 $\pm$ 91 pg/ml, P>0.05) did not induce significant change in IFN $\gamma$  expression. Furthermore, we determine the direct effector role of the expressed IFN $\gamma$  on cytotoxic functionality by performing blocking studies. As shown in Fig. 4C, blocking with IFN $\gamma$  monoclonal antibodies (mAb) and HLA-A2 mAb significantly inhibited the cytotoxicity of MamA2.1 activated CD8 $^{+}$  T lymphocytes, thus suggesting that the cytotoxic effector role CD8 $^{+}$  T cells is dependent upon selenocompound mediated upregulation of IFN $\gamma$  signaling. Taken together, our current data demonstrated that MSA and DMDSe induced HLA class I expression and presentation of HLA-A2 restricted antigenic epitope for specific peptide vaccine-like activation of CD8 $^{+}$  T cell leading to enhanced breast cancer cell cytotoxicity.

## Discussion

Peptide and DNA based cancer vaccines have many advantages, including-being inexpensive, convenient acquisition of clinical-grade peptides, easy administration, higher specificity, potency due to their stronger compatibility with targeted proteins, and greater safety with few side effects (20,21). Previous studies from our laboratory have demonstrated safety and immune efficiency of mammaglobin-A, breast cancer specific tumor associated antigen, based DNA vaccine in breast cancer patients (16,22). The efficiency of these vaccination strategies have limited success as cancer cells downregulate surface expression of HLA class I molecules causing loss of identification of tumor cells by the host CTLs (23).

While the exact mechanism by which the trace element selenium (Se) exerts anti-cancer potential is unknown, our current study demonstrates that MSA and DMDSe, synthetic selenocompound and precursor of methylselenol, induced MHC class I surface expression on professional antigen presenting-like THP-1 cells. This data is in line with studies from other laboratories which have demonstrated enhanced HLA class I expression by these compounds in other cancer cell lines such as melanoma cells. In contrast, the SeMet based selenocompounds which do not produce methylselenol failed to induce HLA class I surface expression. This hypothesis is in accordance to results by Hagemann-Jensen *et al*, where in,



they have shown that MSA and DMDSe mediated upregulation of NKG2D receptor ligands (19).

The antigenic peptides loaded on HLA class I molecules and identified by CD8<sup>+</sup> T lymphocytes generally originate from the degradation of intracellular proteins by proteasomes and translocation to the lumen of the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP)1/2 heterodimeric complex. Defects in this antigen-processing machinery and, in particular, in TAP subunits, have been described as a major mechanism used by several tumors to escape from CTL attack (24). Evidence for antitumor CTL was provided by isolation of tumor-specific CTL from peripheral blood or tumor tissue of patients with diverse cancers, such as melanoma and lung carcinoma (25). The IFN $\gamma$  mediated pro-inflammatory *milieu* is critical for the priming and effector responses by CTLs following antigen presentation by HLA class I molecules (26). The CTLs can lyse target cells *via* the perforin granule exocytosis by the IFN $\gamma$  mediated JAK/STAT pathway. In our current study we demonstrated that the induction of HLA-A2 by MSA and DMDSe was associated with transcriptional upregulation of components of the APM, including proteasomal subunits and components of the peptide loading complex, along with upregulation of IFN $\gamma$  signaling, such as the upregulation of JAK/STAT/IRF molecules.

Our current study is limited to testing HLA class I expression in mononuclear tumor-infiltrating antigen presenting macrophage-like cells. However, the exact efficiency of seleno-compounds towards HLA expression on cancer cells could not be tested in our current *in vitro* system. In an *in vitro* system cancer cell lines already had enhanced cell surface expression of HLA class I molecules. To directly test this effect of seleno-compounds on cancer cells, would require *in vivo* based studies in murine or other small animal tumor models. Further we have currently utilized peptide-based model to study adjuvant effect of selenocompounds, however, future studies with Mam-A DNA-vaccine based model should be utilized to study the adjuvant effect of selenocompounds on DNA-based anti-cancer vaccines. Future studies utilizing *in vivo* model will provided a stronger evidence for human clinical application.

In conclusion, our data provides a new adjuvant approach to further potentiate anti-cancer vaccine strategies. Further, we have demonstrated that MSA and DMDSe could upregulate the MHC class I expression in cancer cells which would enable anti-cancer vaccine induced activated CTLs to inhibit tumor immune-evasion and promote cancer immune-elimination. Therefore, we think novel anti-cancer strategies utilizing inclusion of MSA and DMDSe will improve vaccine therapeutic outcomes by enhancing CTL mediated immune-surveillance and cancer cell elimination.

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## Availability of data and materials

The datasets used and/or analyzed are available from the corresponding author on reasonable request.

## Authors' contributions

VT conceived the present study. DB, MZ, MTI, RZ and VT designed the experiments. DB, MZ and VT performed the experiments. DB, MZ, RZ and VT analyzed the data. DB, MZ, MTI, RZ and VT wrote the manuscript. All authors read and approved the final version of the manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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