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Selective Targeting of Multiple Myeloma by B cell Maturation Antigen (BCMA)-specific Central Memory CD8+ Cytotoxic T Lymphocytes: Immunotherapeutic Application in Vaccination and Adoptive Immunotherapy

Jooeun Bae[1,2], **Mehmet Samur**[1,2], **Paul Richardson**[1,2], **Nikhil C. Munshi**[1,2,3], **Kenneth C. Anderson**[1,2]

[1]Dana-Farber Cancer Institute, Boston, Massachusetts, USA

[2]Harvard Medical School, Boston, Massachusetts, USA

[3]VA Boston Healthcare System, Boston, Massachusetts, USA

Abstract

To expand the breadth and extent of current multiple myeloma (MM)-specific immunotherapy, we have identified various antigens on CD138+ tumor cells from newly diagnosed MM patients (n=616) and confirmed B-cell Maturation Antigen (BCMA) as a key myeloma-associated antigen. The aim of this study is to target the BCMA, which promotes MM cell growth and survival, by generating BCMA-specific memory $CD8^+$ CTL that mediate effective and long-lasting immunity against MM. Here we report the identification of novel engineered peptides specific to BCMA, $BCMA_{72–80}$ (**Y**LMFLLRKI) and $BCMA_{54–62}$ (**Y**ILWTCLGL), which display improved affinity/stability to HLA-A2 compared to their native peptides and induce highly functional BCMA-specific CTL with increased activation (CD38, CD69) and co-stimulatory (CD40L, OX40, GITR) molecule expression. Importantly, the heteroclitic $BCMA_{72–80}$ specific CTL demonstrated poly-functional Th1-specific immune activities [IFN-γ/IL-2/TNF-α production, proliferation, cytotoxicity] against MM, which were correlated with expansion of $Tetramer^+$ and memory $CD8^+$ CTL. Additionally, heteroclitic $BCMA_{72–80}$ specific CTL treated with anti-OX40 (immune agonist) or anti-LAG-3 (checkpoint inhibitor) display increased immune function, mainly by central memory CTL. These results provide the framework for clinical application of heteroclitic $BCMA_{72–80}$ peptide, alone and in combination with anti-LAG3 and/or anti-OX40 therapy, in vaccination and/or adoptive immunotherapeutic strategies to generate long-lasting anti-tumor immunity in patients with MM or other BCMA expressing tumors.

INTRODUCTION

Despite recent advances in treatment of multiple myeloma (MM) including incorporation of novel therapies into the stem cell transplantation paradigm, ongoing DNA damage and

Corresponding Author and Address Jooeun Bae, Ph.D. Dana-Farber Cancer Institute, Harvard Medical School, 450 Brookline Avenue, Boston, MA 02115, Phone: 617-632-5908, Fax: 617-632-2140, jooeun_bae@dfci.harvard.edu.

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genomic evolution underlie relapse in many patients.[1,2] Novel therapeutic approaches with distinct mechanisms of action are therefore needed. The constitutive evolving genetic complexity, coupled with immune responsiveness of B cell malignancies, has stimulated the development of immunotherapeutic options in MM including monoclonal antibodies, bispecific antibodies, immunotoxins, and chimeric antigen receptor T cell (CAR-T).[3,4] Although MM patient-specific CAR-T therapy has achieved remarkable deep responses, durability of responses is not establishes and they are labor-intensive, time-consuming, and expensive.[5–8] To overcome these limitations, we have developed immunogenic peptides-based cancer vaccines as an off-the-shelf immunotherapy for treating patients more widely and efficiently.[9,10] Our peptide-based therapeutic approach does not have limitations of recombinant proteins, mRNA, or DNA-based vaccines, which require the processes of internalization, degradation of protein into optimal immunogenic peptides to HLA, along with additional steps required for suitable translation (for mRNA) or transcription (for DNA). To overcome MHC restriction and treat a more diverse patient population using our well-defined immunogenic peptide vaccine approach, we have pooled peptide cocktails to include major HLA subtypes.[11–13] Moreover, we have already shown that lenalidomide can augment peptide vaccine specific immune responses and memory cytotoxic T cell (CTL) activities, setting the stage for combination approaches with checkpoint inhibitors and/or immune agonists. In addition, anti-tumor efficacy triggered by immunogenic peptides can be enhanced by their ability to induce “epitope spreading” upon the generation of effector cells, whereby targeted lysed cancer cells release new antigenic epitopes which are subsequently taken up, processed, and presented by antigen-presenting cells to a new repertoire of CTLs.[14,15]

B cell maturation antigen (BCMA) is a member of the TNF receptor superfamily 17 (TNFRSF17) and is characterized as a type III trans-membrane protein containing cysteine-rich extracellular domains with a central role in regulating B-cell maturation and differentiation into plasma cells.[16–18] As a receptor for the MM cell growth and survival factors, B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL), BCMA is required for the survival of MM cells, making it a promising therapeutic target.[19,20] Nearly all MM tumor cells express BCMA, and it has been proposed as a marker for identification of tumor cells.[21–26] Its selective expression on a subset of mature B and long lived plasma cells further suggest a favorable therapeutic index for BCMA directed treatment approaches. At present BCMA is being targeted by several immunotherapeutic strategies including antibodies (naked antibodies, antibodies-drug conjugates, and bispecific antibodies) and cellular therapies (chimeric antigen receptor T-cells), with promising clinical results even in relapsed refractory MM.[27–31] In addition, serum soluble BCMA is elevated among patients with MM and chronic lymphocytic leukemia and can served as a prognostic marker and monitor of clinical response. Finally, most recent studies indicate that BCMA is expressed in non-hematopoietic tissue: BCMA is abnormally expressed in non-small cell lung cancer cell lines and may play a role in the tumors through the ERK1/2 signaling pathway.[32,33] These data support targeting BCMA in immunotherapeutic strategies in MM and potentially BCMA expressing solid tumors as well.

In the present study, we developed a peptide-based immunotherapeutic approach targeting BCMA that induces development of antigen-specific $CD8^+$ CTL with effective and long-

lasting immunity against MM cells. The identified novel immunogenic native and heteroclitic HLA-A2specific BCMA peptides are capable of eliciting MM-specific immune responses with highly effective anti-tumor activities. Importantly, the heteroclitic $BCMA_{72–80}$ (**Y**LMFLLRKI) peptide demonstrated the highest level of immunogenicity, with the greatest affinity/stability to HLA-A2 molecule and robust induction of BCMA-specific memory CTL with poly-functional activities against HLA-$A2^+$ patients' MM cells and MM cell lines. Our studies provide the framework for clinical application of this novel engineered immunogenic $BCMA_{72–80}$ (**Y**LMFLLRKI) peptide in cancer vaccine and adoptive immunotherapeutic protocols to provide long lasting memory antitumor immunity in patients with MM or BCMA expressing cancers.

MATERIALS AND METHODS

Cell lines

The MM cell lines, MM1S, OPM2, OPM1, H929, OCIMY5, RPMI, U266, KMS1, HSB2, McCAR and ANBL6, and a breast cancer cell line MDA-MB-231 were obtained from ATCC (Manassas, VA). The T2 cell line, a human B and T cell hybrid expressing HLA-A2 molecules, was provided by Dr. J. Molldrem (University of Texas M. D. Anderson Cancer Center, Houston, TX). The cell lines were cultured in DMEM (for MM and T2 cells; Gibco-Life Technologies, Rockville, MD) or Leibovitz's L-15 (for MDA-MB231; ATCC, Manassas, VA) media supplemented with 10% fetal calf serum (FCS; BioWhittaker, Walkersville, MD), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco-Life Technologies).

Reagents

Fluorochrome conjugated anti-human monoclonal antibody (mAb) specific to BCMA, HLA-A2, CD3, CD8, CD38, CD40L, CD69, 41BB, CCR7, CD45RO, CD107a, IFN-γ, IL-2, TNF-α, PD1, LAG3, OX40 or GITR was purchased from Becton Dickinson (BD) (San Diego, CA), LifeSpan Bioscience (Seattle, WA) or BioLegend (San Diego, CA). Live/Dead Aqua stain kit was purchased from Molecular Probes (Grand Island, NY). Recombinant human GM-CSF was obtained from Immunex (Seattle, WA); and human IL-2, IL-4, IFN-α, and TNF-α were purchased from R&D Systems (Minneapolis, MN). Heteroclitic $hBCMA_{72–80}$ (**Y**LMFLLRKI) peptide-specific Tetramer-PE was synthesized by MBL International Corporation (Woburn, MA). Clinical grade mAb to LAG3 or OX40 was provided by Bristol-Myers Squibb (New York, NY).

Synthetic peptides

Native BCMA peptides [$BCMA_{64–72}$ (LIISLAVFV), $BCMA_{69–77}$ (AVFVLMFLL), $BCMA_{9–17}$ (SQNEYFDSL), $BCMA_{72–80}$ (VLMFLLRKI), $BCMA_{54–62}$ (AILWTCLGL), $BCMA_{114–120}$ (ILPRGLEYT)], heteroclitic BCMA peptides [$hBCMA_{72–80}$ (**Y**LMFLLRKI), $hBCMA_{54–62}$ (**Y**ILWTCLGL), $hBCMA_{9–17}$ (**Y**QNEYFDSL)] and HIV-$Gag_{77–85}$ (SLYNTVATL) were synthesized by standard fmoc (9-fluorenylmethyl-oxycarbonyl) chemistry, purified to >95% using reverse-phase chromatography, and validated by mass-spectrometry for molecular weight (Biosynthesis, Lewisville, TX).

HLA-A2 affinity and stability Assays

T2 cells were pulsed overnight with various doses of peptide plus β2-microglobulin (3 μg/ml) (Sigma, St Louis, MO). Following overnight incubation, the cells were stained with HLA-A2-PE mAb and analyzed by flow cytometry. Peptide/HLA-A2 complex stability was measured on peptide loaded T2 cells at 0, 2, 4, 6 and 14 hours post-Brefeldin A treatment by staining with HLA-A2-PE mAb and flow cytometry analyses and shown as an increase in specific HLA-A2 median fluorescence intensity (MFI).

Generation of dendritic Cells

Peripheral blood mononuclear cells (PBMC) were isolated by standard density gradient centrifugation over Ficoll-Paque™ Plus (Amersham Pharmacia Biotech AB, Uppsala Sweden) from leukopaks obtained from multiple HLA-$A2^+$ normal donors. Monocytes isolated from the PBMC were cultured for 7 days in the presence of 1,000 units/ml GM-CSF and 1,000 units/ml IL4 in RPMI-1640 medium (Gibco-Life Technologies) supplemented with 10% FCS. Fresh media plus GM-CSF and IL-4 was added to the cultures every other day. Mature DC (mDC) were obtained on day 7, following 3 additional days incubation with 1,000 units/ml IFN-α plus 10 ng/ml TNF-α.

Induction of heteroclitic BCMA peptide-specific CTL

$CD3^+$ T cells were obtained by negative selection from the HLA-$A2^+$ non-adherent cell fraction post monocyte separation using the EasySep® magnet and Robosep® from StemCell Technologies. Heteroclitic BCMA peptide-specific CTL (hBCMA-CTL) were generated *ex vivo* by repeated stimulation of the $CD3^+$ T cells with antigen-presenting cells (APC) pulsed with a heteroclitic BCMA peptide. In brief, heteroclitic $BCMA_{72–80}$ (**Y**LMFLLRKI) or $BCMA_{54–62}$ (**Y**ILWTCLGL) peptide (50 μg/ml)-pulsed APC were irradiated (10 Gy) and used to stimulate T cells at a 1 APC/peptide: 20 T cell ratio. The T cell cultures were restimulated every 7 days and maintained in AIM-V medium supplemented with 10% human AB serum (BioWhittaker) in the presence of IL-2 (50 units/ml).

Phenotypic analysis of BCMA peptide-specific CTL or tumor Cells

Phenotypic characterization was performed on BCMA-CTL after staining with Live/Dead Aqua stain kit and fluorochrome conjugated anti-human mAbs and Tetramer-PE. Alternatively, the MM and breast cancer cell lines were stained with fluorochrome-conjugated BCMA or HLA-A2 mAb to confirm antigen expression on target cells. After staining, the cells were washed, fixed in 2% paraformaldehyde, and analyzed by flow cytometry.

Cell proliferation by Carboxy Fluorescein Succinimidyl Ester (CFSE) tracking

BCMA-CTL were labeled with CFSE (Molecular Probes) and co-incubated with irradiated (10 Gy) tumor cells or peptide-pulsed APC in the presence of IL-2 (10 units/ml). On day 4, 5, 6 or 8 of co-culture, cells were harvested and stained with Live/Dead Aqua stain kit and CD3/CD8/CD45RO/CCR7 mAbs. The level of $CD3^+CD8^+$ CTL proliferation was determined as a reduction in CFSE fluorescence intensity, as measured by flow cytometry.

CD107a degranulation and intracellular IFN-γ/IL-2/TNF-α cytokines production

The functional immune activities of BCMA-CTL were measured by CD107a degranulation and Th1-specific cytokines production by flow cytometry. In brief, BCMA-CTL were co-incubated with tumor cells or peptide loaded T2 cells in the presence of CD107a mAb. After 1-hour incubation, CD28/CD49d mAb, Brefeldin A, and Monensin (BD) were added for an additional 5 hours. Cells were harvested, washed in PBS, and incubated with mAbs specific to T cell antigens. After surface staining, cells were washed, fixed/permeabilized, stained with anti-IFN-γ/IL-2/TNF-α mAbs, washed with Perm/Wash solution (BD), fixed in 2% paraformaldehyde, and analyzed by flow cytometry.

Statistical Analysis

Summary results are presented as the mean ± SE. Groups were compared using unpaired Student's t-test. Differences were considered significant when $*p < 0.05$.

RESULTS

Heteroclitic $BCMA_{72–80}$ peptide shows the highest affinity and stability to HLA-A2 molecules.

The full length BCMA protein sequence was evaluated to predict epitopes with HLA-A2 affinity, extended half-time disassociation rates, proteasome C terminal cleavage, and TAP transport using various search software programs. Among the six native peptides selected [$BCMA_{64–72}$ (LIISLAVFV), $BCMA_{69–77}$ (AVFVLMFLL), $BCMA_{9–17}$ (SQNEYFDSL), $BCMA_{72–80}$ (VLMFLLRKI), $BCMA_{54–62}$ (AILWTCLGL), $BCMA_{114–120}$ (ILPRGLEYT)], $BCMA_{72–80}$ (VLMFLLRKI) and $BCMA_{54–62}$ (AILWTCLGL) showed the highest HLA-A2 binding affinity in a dose-dependent manner, indicated as a significant increase in HLA-A2-specific MFI from baseline (n=3, $*p < 0.05$) (Figure 1A). Furthermore, the native $BCMA_{72–80}$ (VLMFLLRKI) and $BCMA_{54–62}$ (AILWTCLGL) peptides with highest HLA-A2 binding affinity were chosen for modification of their anchor residues to further enhance HLA-A2 affinity. The HLA-A2 nonspecific $BCMA_{9–17}$ (SQNEYFDSL) peptide was also modified to evaluate whether a change in an anchor motif alters HLA-A2 affinity. Among the engineered peptides designed, heteroclitic $BCMA_{72–80}$ (**Y**LMFLLRKI) and heteroclitic $BCMA_{54–62}$ (**Y**ILWTCLGL) peptides displayed a significant increase in HLA-A2 affinity than their native peptides (n=3, $*p < 0.05$). The highest affinity was detected by heteroclitic $BCMA_{72–80}$ (**Y**LMFLLRKI) in both doses (50 μg/ml, 100 μg/ml) tested, which was close to the binding affinity of the HLA-A2-specific HIV-$Gag_{77–85}$ (SLYNTVATL) peptide (n=3, $*p < 0.05$) (Figure 1B). In contrast, replacing the anchor motif in the non-HLA-A2 specific $BCMA_{9–17}$ (SQNEYFDSL) to heteroclite $BCMA_{9–17}$ (**Y**QNEYFDSL) did not alter its HLA-A2 affinity status, indicating that improved HLA-A2 affinity is only achieved in HLA-A2-specific peptides. In addition, native $BCMA_{72–80}$ and $BCMA_{54–62}$ peptides displayed extended HLA-A2 stability for greater than 6 hours, which was further enhanced in the engineered heteroclitic $BCMA_{72–80}$ (**Y**LMFLLRKI) and $BCMA_{54–62}$ (**Y**ILWTCLGL) peptides, close to the stability of the HLA-A2-specific HIV-$Gag_{77–85}$ peptide (n=3, $*p < 0.05$) (Figure 1C). In summary, the heteroclitic $BCMA_{72–80}$ peptide had the highest level of HLA-A2 affinity and stability, which was close to the HLA-A2 positive control HIV-$Gag_{77–85}$ peptide.

BCMA-specific CTL generated with heteroclitic $BCMA_{72–80}$ (YLMFLLRKI) or $BCMA_{54–62}$ (YILWTCLGL) peptide have increased T cell activation and co-stimulatory molecule expression.

Phenotypic characterization of heteroclitic $BCMA_{72–80}$ peptide-specific CTL ($hBCMA_{72–80}$ CTL) or heteroclitic $hBCMA_{54–62}$ peptide-specific CTL ($hBCMA_{54–62}$ CTL) was performed after the fourth round of respective peptide stimulation. Both $hBCMA_{72–80}$ CTL and $hBCMA_{54–62}$ CTL displayed increased levels of T cell activation markers (CD69, CD38) expression with the highest upregulation on the $hBCMA_{72–80}$ CTL [Baseline vs. $hBCMA_{72–80}$ CTL vs. $hBCMA_{54–62}$ CTL: $CD69^+$ cells – 0% vs. 50% vs. 39%, $CD38^+$ cells – 0% vs. 85% vs. 80%] (Figure 2A). In addition, the $hBCMA_{72–80}$ CTL showed distinctly higher expression of CD40L, OX30, GITR and 41BB costimulatory molecules than $hBCMA_{54–62}$ CTL [Baseline vs. $hBCMA_{72–80}$ CTL vs. $hBCMA_{54–62}$ CTL: $CD40L^+$ cells – 1% vs. 26% vs. 16%, $OX40^+$ cells – 1% vs. 54% vs. 43%, $GITR^+$ cells – 0% vs. 24% vs. 14%, $41BB^+$ cells – 1% vs. 20% vs. 2%] (Figure 2B). In summary, $hBCMA_{72–80}$ CTL and $hBCMA_{54–62}$ CTL generated from three $HLA\text{-}A2^+$ individuals demonstrated similar patterns of T cell activation and co-stimulatory molecule expression, with the highest upregulation seen on the $hBCMA_{72–80}$ CTL (n=3, $*p < 0.05$) (Figure 2C).

Heteroclitic $BCMA_{72–80}$ specific CTL display BCMA antigen-specific immune activities in response to MM cell lines.

The phenotype and functional immune activities of $hBCMA_{72–80}$ CTL were assessed after each round of peptide stimulation. A gradual increase in the % $CD3^+CD8^+$ T cells (Supplemental Fig. 1) and a corresponding decrease in % $CD3^+CD4^+$ T cells (Supplemental Fig. 2) was observed after each round of $hBCMA_{72–80}$ peptide stimulation (N=3). In parallel, phenotype analyses showed a high expression of BCMA expression (mAb clones used to stain: ANC3B1, VICKY1, 19F2) on the H929, MMIS, U266 and OPM1 myeloma cell lines, but not on the MDA-MB231 breast cancer cell line (Supplemental Fig. 3). The $hBCMA_{72–80}$ CTL showed significantly ($*p < 0.05$) higher proliferation of $CD3^+CD8^+$ T cells in response to $HLA\text{-}A2^+$ $BCMA^+$ U266 (49%) compared to $HLA\text{-}A2^-$ $BCMA^+$ MM1S (7%), $HLA\text{-}A2^+$ $BCMA^-$ MDA-MB231 (9%), or media alone (6%) (Figures 3A, Histogram). This HLA-A2-restricted and BCMA antigen-specific $CD8^+$ CTL proliferation was consistently observed in $hBCMA_{72–80}$ CTL generated from three $HLA\text{-}A2^+$ individuals (Figure 3A, Bar graphs; n=3, $*p < 0.05$). In addition, $hBCMA_{72–80}$ CTL were further investigated for functional anti-tumor activities against myeloma cells by the upregulation of CD107a degranulation, which is widely accepted as a measure of cytotoxicity which directly correlates with tumor target cell killing by effector cells.[34,35] The $hBCMA_{72–80}$ CTL demonstrated robust CD107a degranulation (47%) and Granzyme B (33%)/Perforin (30%) upregulation in response to $HLA\text{-}A2^+$ $BCMA^+$ U266 cells, but not to mismatched $HLA\text{-}A2^+$ $BCMA^-$ MDAMB231 cells (Figure 3B). In additional experiments, $hBCMA_{72–80}$ CTL generated from multiple $HLA\text{-}A2^+$ individuals consistently demonstrated high levels of IFN-γ/IL-2/TNF-α production, 41BB upregulation, and CD107a degranulation against $BCMA^+$ MM cells in an HLA-A2 restricted manner (n=5, $*p < 0.05$) (Figure 3C). Thus, these data further demonstrate the MHC restricted and BCMA antigen-specific immune responses by heteroclitic $BCMA_{72–80}$ peptide generated CTL against myeloma.

Heteroclitic $BCMA_{72–80}$ specific CTL demonstrate functional immune responses to primary $CD138^+$ tumor cells from HLA-$A2^+$ patients' MM cells.

Next, we evaluated the functional activities of $hBCMA_{72–80}$ CTL against primary $CD138^+$ tumor cells isolated from MM patients. Compared to heteroclitic $BCMA_{54–62}$ CTL, heteroclitic $BCMA_{72}80$ CTL displayed more robust anti-MM activities against primary HLA-$A2^+$ MM cells, as measured by CD107a degranulation ($hBCMA_{72–80}$ CTL vs. $hBCMA_{54–62}$ CTL: 22 % vs. 14%) and IL-2 production (16% vs. 4%) (Figure 4A). In addition, $hBCMA_{72–80}$ CTL consistently demonstrated the highest CD107a degranulation, Granzyme B upregulation and IFN-γ/TNF-α Th1 cytokines production in an HLA-A2 restricted manner (Figure 4B). The $hBCMA_{72–80}$ CTL generated from other HLA-$A2^+$ individuals (n=3) displayed the same pattern of response and showed significant ($*p < 0.05$) increases in anti-tumor activities (CD107a degranulation, Granzyme B/Perforin upregulation and IFN-γ/IL-2/TNF-α production) against $CD138^+$ tumor cells from MM patients in an HLA-A2-restricted manner (Figure 4C). The robust functional activities by $hBCMA_{72–80}$ CTL were aligned with their increased upregulation of T cell activation and co-stimulatory molecule expression (Figure 2). Thus, these data provide additional evidence on the great immunogenicity of heteroclitic $BCMA_{72–80}$ peptide and support its potential clinical application in novel MM treatments.

Heteroclitic $BCMA_{72–80}$ specific CTL are enriched for $Tetramer^+$ $CD8^+$ T cells with robust anti-MM activities.

The $hBCMA_{72–80}$ CTL were further characterized to define the specific Naïve:Memory $CD8^+$ T cell subsets with anti-MM activities. The $Tetramer^+$ $hBCMA_{72–80}$ CTL population displayed significantly higher T cell activation and co-stimulatory molecule expression as compared to the non-specific $Tetramer^-$ cells ($Tetramer^+$ vs. $Tetramer^-$, $CD38^+$: 49% vs. 3%, $CD40L^+$: 38% vs. 1%, $41BB^+$: 25% vs. 2%, $OX40^+$: 46% vs. 2%, $GITR^+$: 35% vs. 2%) (Figure 5A). In addition, the $Tetramer^+$ $hBCMA_{72–80}$ CTL population consistently demonstrated high levels of CD107a degranulation against U266 MM cells ($Tetramer^+$ vs. $Tetramer^-$: Donor A, B, C - 83%, 97%, 97% vs. 6%, 18%, 13%) (Figure 5B). Finally, we evaluated the frequency of $Tetramer^+$ cells within $CD107a^+$ population of $hBCMA_{72–80}$ CTL. We observed that the majority of T cells inducing antitumor activities ($CD107a^+$: Donor A, B, C - 82%, 98%, 98%) were also within $hBCMA_{72–80}$ specific $Tetramer^+$ cells (Figure 5C). Therefore, these results confirm that anti-MM activities are indeed induced by the $BCMA_{72–80}$ peptide specific CTL, which also display high expression of key T cell activation markers and co-stimulatory molecules.

Heteroclitic $BCMA_{72–80}$ peptide induces MM-specific memory $CD8^+$ CTL with anti-tumor activities.

To characterize anti-tumor activities of BCMA peptide-specific CTL, we evaluated the BCMAspecific memory CTL development after each cycle of peptide stimulation. Gradual progressive phenotypic changes were detected within $CD8^+$ T cells of $hBCMA_{72–80}$ CTL; from naïve ($CD45RO^-CCR7^+$) at baseline [Donor 1: 83%, Donor 2: 84%] to central memory (CM; $CD45RO^+CCR7^+$) after 2 cycles of peptide stimulation [Donor 1: 32%, Donor 2: 50%], and then to an enriched effector memory (EM; $CD45RO^+CCR7^-$) CTL after

4 cycles of stimulation (Donor 1: 55%, Donor 2: 78%) (Figure 6A). Overall, memory $CD8^+$ CTL was gradually developed following each round of heteroclitic $BCMA_{72–80}$ peptide stimulation (Figure 6B), which was associated with a corresponding decrease in naïve T cells (Figure 6C). These results therefore demonstrate the induction and gradual expansion of memory CTL upon the stimulation of T cells with heteroclitic $BCMA_{72–80}$ peptide.

Central memory $CD8^+$ T cells specific to heteroclitic $BCMA_{72–80}$ demonstrate the greatest anti-MM activities.

Memory T cells specific to heteroclitic $BCMA_{72–80}$ peptide were characterized for their immune functional activities. The $CD45RO^+$ memory $hBCMA_{72–80}$-CTL (Donor A) demonstrated higher CD107a degranulation in response to U266 (CD45RO+ vs. CD45RO-: 28% vs. 7%) and McCAR (13% vs. 4%) (Figure 7A). The $hBCMA_{72–80}$ CTL displayed highly functional anti-MM activity against target cells, having both high BCMA (U266) and low BCMA expression (McCAR). Additionally, the $Tetramer^+$ cells were predominantly central memory subset, which was consistently seen in the $hBCMA_{72–80}$ CTL generated from different individuals (% CM of $Tetramer^+$ cells - Donor B: 88%, Donor C: 97%, Donor D: 100%) (Figure 7B). The $hBCMA_{72–80}$ specific CM subset also demonstrated highly functional immune activities against U266 cells. Importantly, the CD107a degranulation in the $hBCMA_{72–80}$ specific CTL was directly associated with the frequency of CM cells (% CM within $CD107a^+$ cells - Donor E: 81%, Donor F: 83%, Donor G: 67%, Donor H: 42%) (Figure 7C). Finally, the high responders (Donor E, Donor F) to the heteroclitic $BCMA_{72–80}$ peptide showed the greatest anti-MM activities and also displayed the highest CM subset frequency as compared to mid-level responder (Donor G) or low-level responder (Donor H). Thus, these results further indicate the distinct enrichment of effector cells with anti-MM activities are predominantly within $hBCMA_{72–80}$ specific central memory cell subset.

Inhibition of LAG3 or stimulation of OX40 enhances anti-MM activities of heteroclitic $BCMA_{72–80}$ peptide-specific central memory CTL.

Finally, we evaluated the potential impact of a checkpoint inhibitor (anti-LAG3) or immune agonist (anti-OX40) on the immune function of $hBCMA_{72–80}$ CTL. Treatment of $hBCMA_{72–80}$ CTL with either anti-LAG3 or anti-OX40 enhanced the specific cytotoxic activity, especially within the $CD45RO^+$ memory subset against U266 (untreated 28%, anti-LAG3 treated 36%, anti-OX40 treated 40%) and McCAR (untreated 13%, anti-LAG3 treated 17%, anti-OX40 treated 21%) (Figure 8A). The checkpoint inhibitor and immune agonist did not induce the anti-MM responses by $CD45RO^-$ non-memory cells within the BCMA-CTL. Lastly, the beneficial effect of antiLAG3 and anti-OX40 was further investigated within the specific central memory CTL subsets. Anti-LAG3 or anti-OX40 treatment had a greater impact on regulating the functional activity of CM subset compared to EM subset within the $hBCMA_{72–80}$ CTL, as evidenced by their higher CD107a degranulation against U266 MM cells (Figure 8B). Increased anti-tumor activities was observed consistently in the $hBCMA_{72–80}$ CTL generated from three different $HLA\text{-}A2^+$ individuals (Donor 1, Donor 2, Donor 3) upon anti-OX40 treatment, as demonstrated in % increase of $CD107a^+$ CTL, from each individual's baseline (no peptide stimulated), in HLA-A2 restricted manner (Figure 8C). Therefore, these results support the utility of anti-LAG3

or anti-OX40 to further enhance anti-MM activities of $hBCMA_{72–80}$ CTL induced from HLA-$A2^+$ individuals.

DISCUSSION

Even in patients with refractory MM relapsing after allo-transplantation, long-lasting responses have been achieved with the infusion of donor lymphocytes (DLI).[36,37] These early encouraging results of DLI have provided the framework for evaluation of active-specific immunotherapy approaches to treat MM.[38–40] Cancer targeting vaccines, one such active-specific immunotherapy approach, have demonstrated the ability to induce highly effective $CD8^+$ CTL with anti-tumor activities.[41–43] The success of vaccination depends on selection of the appropriate patient population, targeting antigens expressed selectively on tumor, and utilizing combination approaches to effectively induce and maintain antigen-specific memory anti-tumor immune responses. We have recently reported on novel immunogenic HLA-A2 and HLA-A24 specific peptides derived from XBP1, CD138 and CS1 antigens, which are highly over-expressed in MM and solid tumors including breast, pancreatic, and colon cancers, and demonstrated their ability to induce the peptides-specific $CD8^+$ CTL with anti-tumor activities against HLA-$A2^+$ or HLA-$A24^+$ tumor cells both in preclinical and clinical studies.[11, 44–48] In addition, combination studies of peptide stimulation/vaccination with immune modulatory drugs such as lenalidomide or with histone deacetylase 6 inhibitor ACY241 enhanced the peptides-specific CTL activities against tumor cells. Our previous studies demonstrated that combinations of peptide stimulation with either Lenalidomide or ACY241 augmented antigens-specific $CD8^+$ CTL activity associated with upregulation of transcriptional regulators such as T-bet/Eomes and with activation of AKT, which links antigen-specific CTL differentiation to FOXO, mTOR and Wnt/β-catenin signaling pathways.[49,50] Importantly, these effects were confined primarily to antigen-specific $CD45RO^+$ memory CTL, with the most robust increases in IFN-γ and Granzyme B production and $CD8^+$ CTL proliferation in response to tumor cells observed mainly within the specific CM subset.

Due to our encouraging preclinical results, the XBP1/CD138/CS1 multipeptide vaccine has been evaluated, alone and in combination with lenalidomide, in clinical trials to treat patients with smoldering MM (SMM), as well as in combination with anti-PD1 in clinical trials to treat patients with triple negative breast cancer. In patients with SMM, the multipeptide vaccine was well tolerated and immunogenic as a monotherapy, evidenced by enhanced frequency of $Tetramer^+$ $CD8^+$ CTL with IFN-γ production; moreover, combination with lenalidomide triggered higher mean fold increases in $CD8^+$ T cells with tetramer-positivity and IFN–γ production. Importantly, $CD45RO^+$ memory CTL specific to the XBP1/CD138/CS1 peptides were induced by the peptide vaccine, and further enhanced in combination with lenalidomide.[51,52] Although stable disease and responses have been observed in SMM, randomized trials are needed to assess whether time to progression from SMM to active disease can be prolonged by the peptide vaccination.

To expand the MM-specific immunotherapy beyond XBP1/CD138/CS1 antigens, we have recently identified additional tumor associated antigens on $CD138^+$ tumor cells from newly diagnosed MM patients (N=616). Here we report on the identification and characterization

of a novel immunotherapeutic strategy targeting BCMA, selectively expressed on normal and malignant plasma cells and the target of several current immune treatments in MM. We report on highly immunogenic engineered BCMA-specific nanomers, heteroclitic $BCMA_{72–80}$ (**Y**LMFLLRKI) and $BCMA_{54–62}$ (**Y**ILWTCLGL), with highly improved HLA-A2 affinity/stability from their native BCMA peptides. These peptides evoke BCMA-specific CTL, increased BCMAspecific Tetramer$^+$ cells, enhanced CD107 degranulation, Th1-type cytokines (IFN-γ/IL-2/TNF-α) production, and proliferation to MM cells in an HLA-A2-restricted manner. Most importantly, the increase of BCMA-specific memory CD8$^+$ CTL, both CM and EM cells, along with the capacity of self-renewal and response to MM cells, strongly support the potential of heteroclitic BCMA peptide in novel vaccination and/or immunotherapeutic approaches in MM. Indeed, we have planned a clinical protocol with heteroclitic $BCMA_{72–80}$ (**Y**LMFLLRKI) peptide vaccination, harvest and expansion of BCMA-specific CM cells *ex vivo*, reinfusion of these CM cells as adoptive immunotherapy, and then vaccination with the BCMA peptide as needed thereafter to assure their persistence to effectively treat MM patients. We observed that BCMA-specific memory CD8$^+$ CTL expressed key molecules modulating T cells function, both for co-stimulation and immune suppression. These results are consistent with our previous report of increased expression of these molecules on XBP1/CD138/CS1-specific CTL generated *ex vivo* by peptides stimulation.[11] Importantly, the highest induction of co-stimulatory and immune checkpoint molecules was detected on CM subset within $hBCMA_{72–80}$ peptide–specific CTL, which is the population that demonstrated the most highly effective poly-functional activities against MM. Importantly, these findings indicated the potential of combination therapy of $hBCMA_{72–80}$ peptide with checkpoint inhibitors or immune agonists to enhance the functional anti-MM activities of BCMA-specific CTL. This may be particularly relevant, given the recent concerns when combining pembrolizumab (an antibody targeting PD-1) with immunomodulatory drugs lenalidomide or pomalidomide or with daratumumab (anti-CD38 mAb), where toxicities have curtailed studies.[53–55] Here, we report on targeting alternative inhibitory receptors and suppressive mechanisms within the MM tumor microenvironment. In particular, LAG3 (CD223) is the third inhibitory receptor to be targeted in the clinic, following CTLA and PD1/PD-L1[56,57] and was expressed on BCMA-specific CM CTLs. In parallel, immune agonists, especially the costimulatory tumor necrosis factor receptors targeting OX40 (CD134), 41BB (CD137) and GITR (CD357), have received considerable attention for their therapeutic utility in enhancing anti-tumor immune responses; among these, anti-OX40 mAb has recently demonstrated encouraging efficacy in induction of tumor regression by boosting effector T cell expansion and functional anti-tumor activities in several pre-clinical studies.[58–60] Importantly, we used a clinical grade anti-LAG3 and anti-OX40 (provided by Bristol-Myers Squibb; New York, NY) to evaluate changes in the functional activities of heteroclitic $BCMA_{72–80}$ specific CTL to MM cells. Our *ex vivo* studies demonstrated that both anti-LAG3 (checkpoint inhibitor) and anti-OX40 (immune agonist) increased functional activity specifically of memory CTL within the BCMA-CTL against MM cells, without affecting the activity of non-memory CTL. The impact on function of BCMA-CTL generated from multiple HLA-A2$^+$ individuals' T cells was greater after treatment with anti-OX40 than anti-LAG3, and greater within their specific CM versus EM subset. These studies provide the framework for scientifically informed

combination clinical trials of BCMA peptide-specific immunotherapy with the immune agonist or checkpoint inhibitor.

In summary, we have identified and validated novel immunogenic HLA-A2-specific native and engineered BCMA peptides, which are capable of inducing antigen-specific $CD8^+$ CTL with functional anti-tumor activities against MM cells. These results provide the framework for therapeutic application of these highly immunogenic heteroclitic BCMA peptides in MM patients as vaccines and/or as stimuli for expansion of autologous antigen-specific memory CTL. They further support the potential utility of combinations incorporating BCMA peptide vaccine or BCMA-specific adoptive T cells immunotherapy with anti-OX40 and/or anti-LAG3 to enhance BCMA directed anti-MM responses. Based on the encouraging results presented here, we plan to vaccinate patients with BCMA peptide, treat with BCMA-specific memory T cells expanded *ex vivo* for adoptive immunotherapy, and then vaccinate again as needed to provide for long term BCMA directed anti-MM immunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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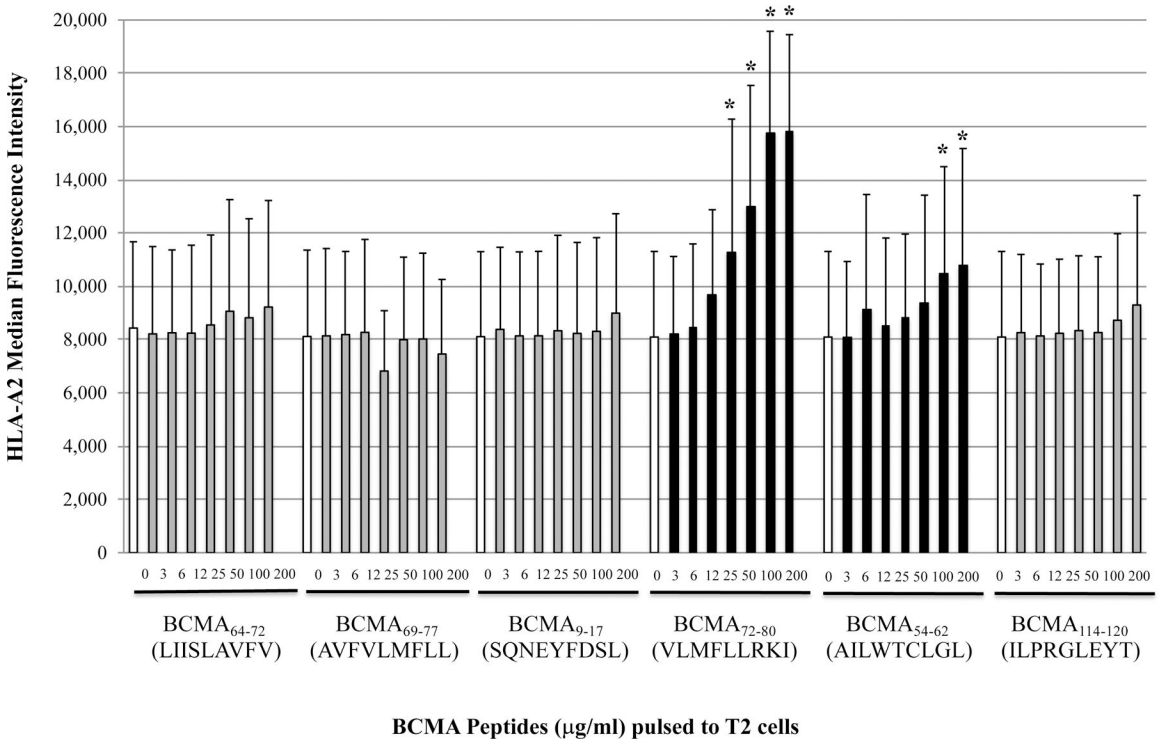
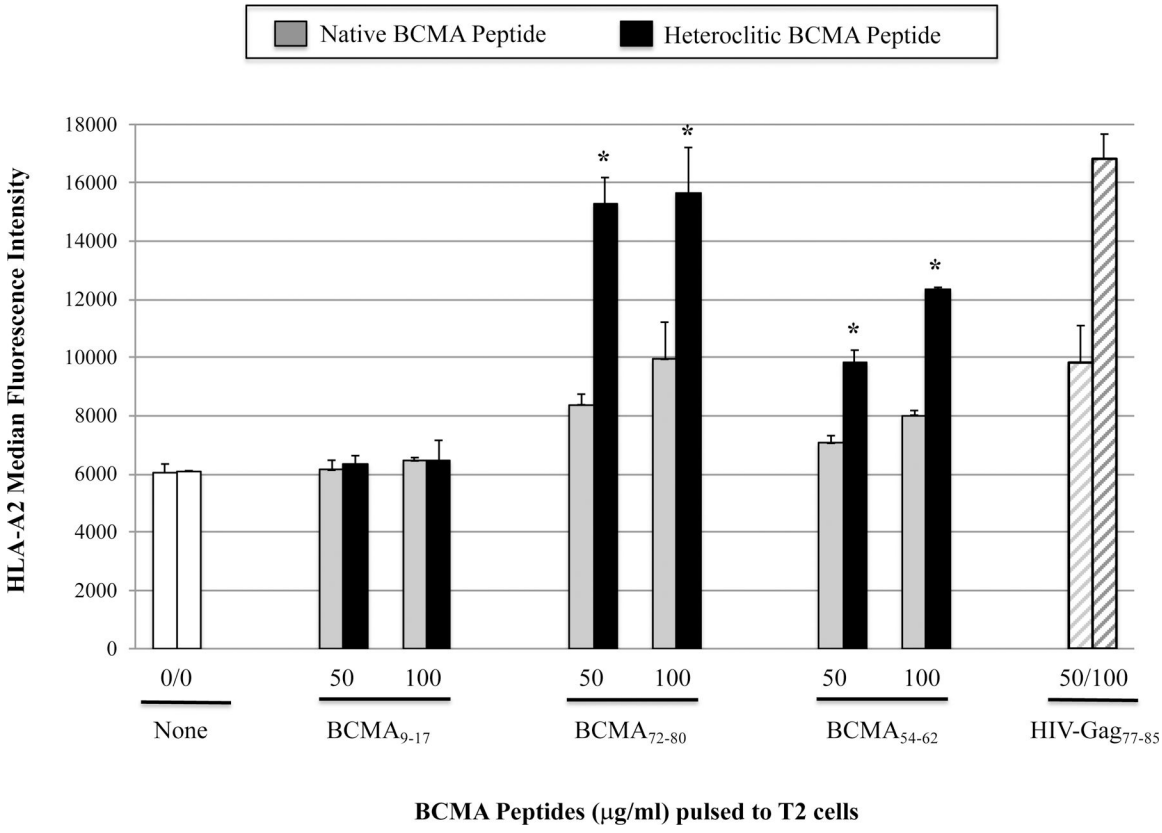
Key Points

Engineered heteroclitic BCMA-specific peptides identified in this study has an immunotherapeutic potential as a vaccine and adoptive immunotherapy in myeloma patients by inducing the antigens-specific CTL.

Based on the encouraging results presented here, we plan to vaccinate patients with BCMA peptide, treat with BCMA-specific memory T cells expanded *ex vivo* for adoptive immunotherapy, and then vaccinate again as needed to provide for long term BCMA directed anti-MM immunity.





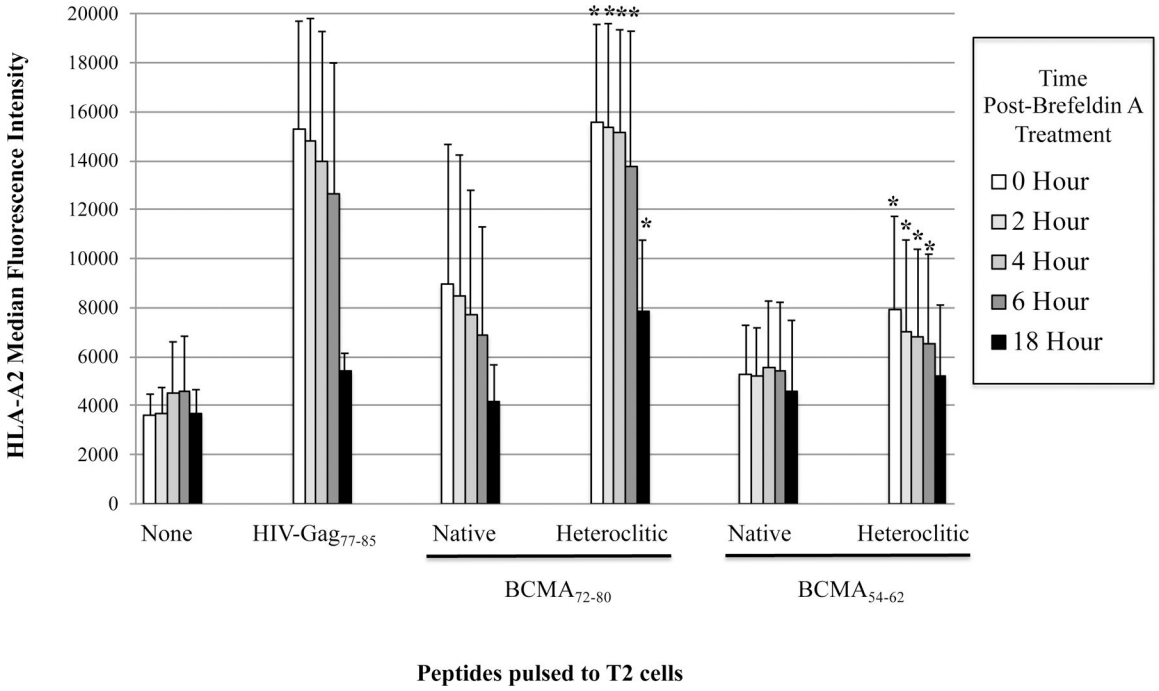
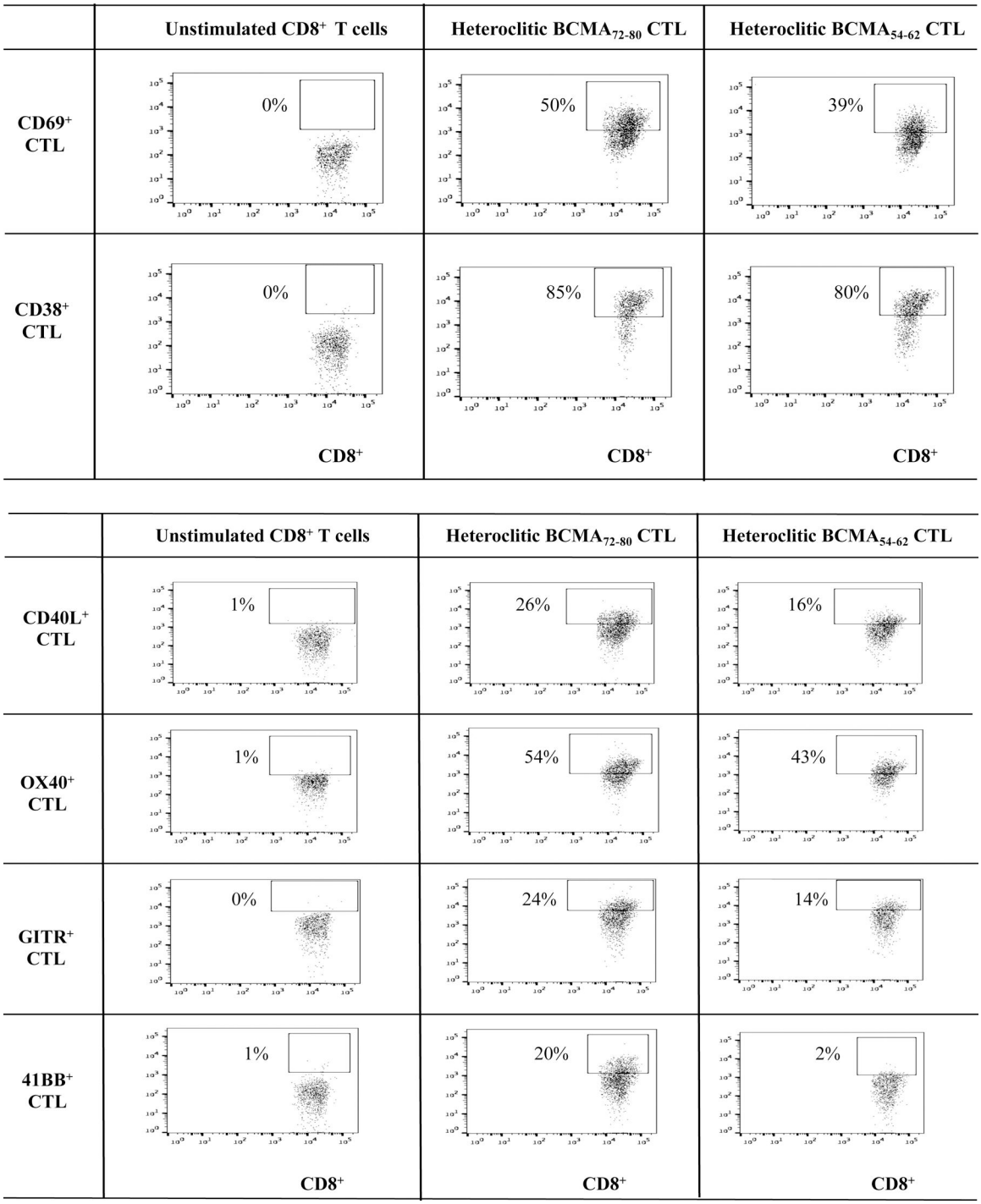


Figure 1. Characterization of native or heteroclitic BCMA peptide for their HLA-A2 binding affinity and stability.

T2 cells were pulsed overnight with native or heteroclitic BCMA peptide in serum-fee AIM-V media and stained with HLA-A2-PE mAb for flow cytometry analyses. HLA-A2-specificity of the peptide is shown as an increase in HLA-A2 mean fluorescence intensity (MFI) on T2 cells. HLA-A2 specific HIV-$Gag_{77–85}$ peptide was used as the positive control. **Fig. 1A.** Native BCMA peptides (3 – 200 μg/ml) binding affinity to HLA-A2. **Fig. 1B.** Native and heteroclitic $BCMA_{9–17}$, $BCMA_{72–80}$ and $BCMA_{54–62}$ peptides (50 – 100 μg/ml) binding affinity to HLA-A2. **Fig. 1C.** Native and heteroclitic $BCMA_{72–80}$ and $BCMA_{54–62}$ peptides (50 μg/ml) binding affinity and stability to HLA-A2, post-Brefeldin A treatment (0 – 18 hours). Results represent the mean ± SE of three separate experiments.



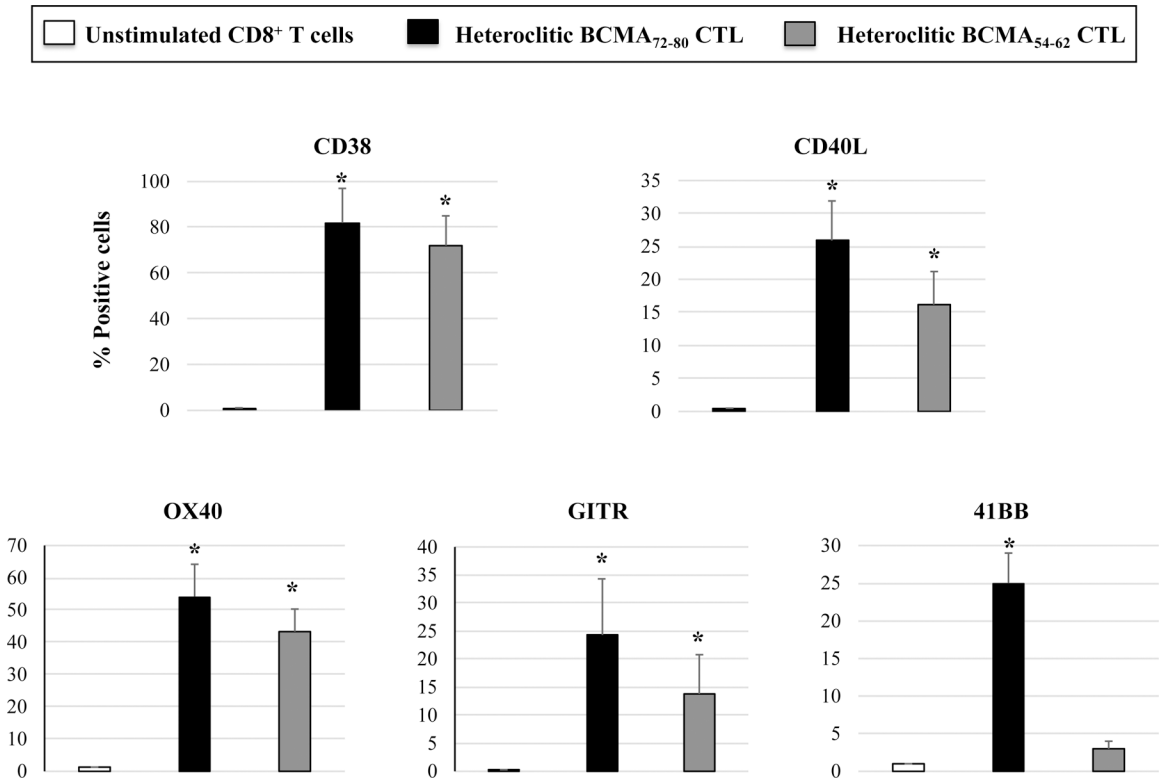
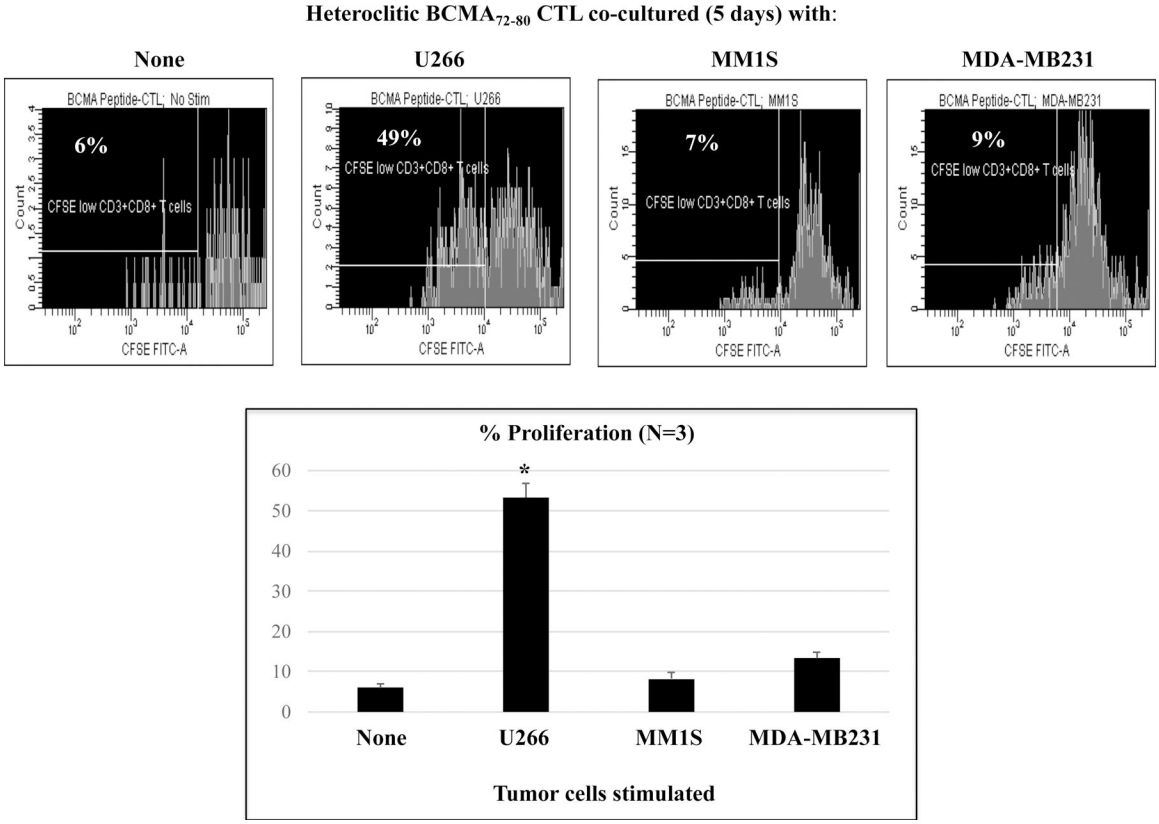
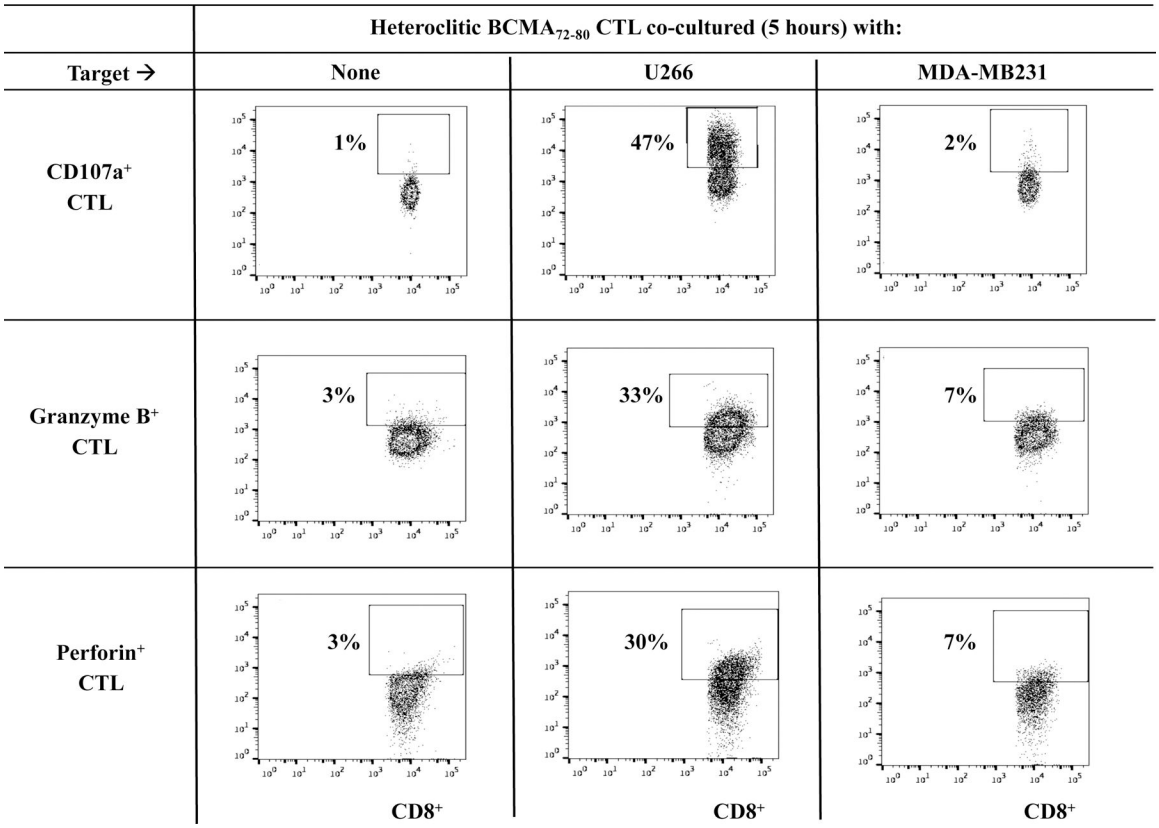


Figure 2. Upregulation of critical T cell activation and co-stimulatory markers on heteroclitic $BCMA_{72–80}$ peptide-specific CTL.

$CD3^+$ T cells obtained from HLA-$A2^+$ individual donors were stimulated with heteroclitic $BCMA_{72–80}$ (**Y**LMFLLRKI) or $BCMA_{54–62}$ (**Y**ILWTCLGL) peptide loaded antigen-presenting cells and analyzed post-4 cycles of peptide stimulation (once/week) for activation and co-stimulatory molecules expression within the gated $CD3^+CD8^+$ T cells population by flow cytometry. **Fig. 2A.** Representative flow cytometry analyses for CD69 and CD38 expression. **Fig. 2B.** Representative flow cytometry analyses for CD40L, OX30, GITR and 41BB expression. **Fig. 2C.** Summary (mean ± SE; N=3) of activation and co-stimulatory molecule expression.





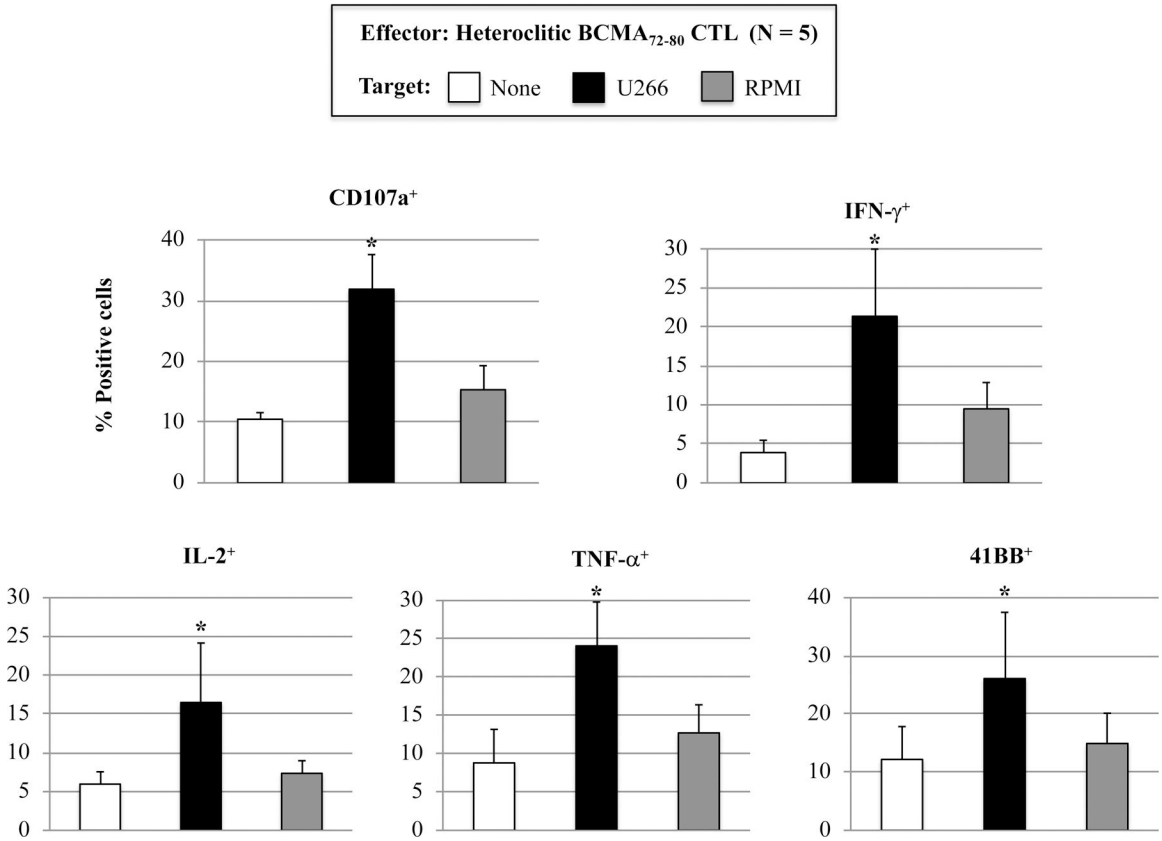
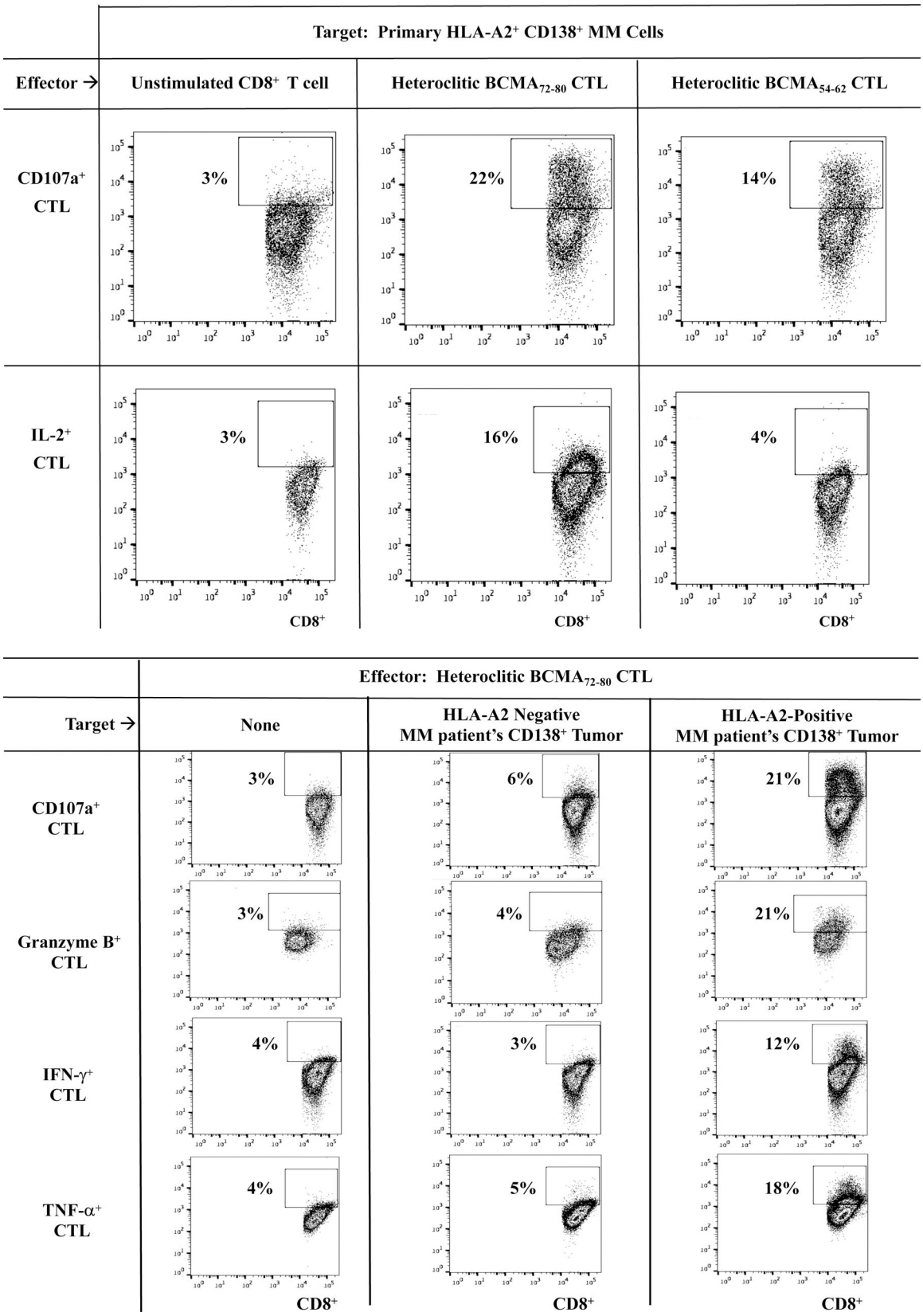


Figure 3. HLA-A2 restricted and BCMA antigen-specific immune responses by heteroclitic $BCMA_{72–80}$ specific CTL against cancer cell lines.

Heteroclitic $BCMA_{72–80}$ CTL were evaluated for their specific anti-tumor activities. **Fig. 3A.** Representative flow analyses and summary (mean ± SE; N=3) of $CD8^+$ T cells proliferation (day 5) of h against matched U266 ($HLA\text{-}A2^+$ $BCMA^+$) or mis-matcherd MM1S ($HLA\text{-}A2^-$ $BCMA^+$) and MDA-MB231 ($HLA\text{-}A2^+$ $BCMA^-$) cancer cell line. **Fig. 3B.** Representative flow analyses of CD107a degranulation and Granzyme B/Perforin upregulation by $hBCMA_{72–80}$ CTL. **Fig. 3C.** Summary (mean±SE; N=5) of CD107a degranulation, Th1 cytokine (IFN-γ/IL-2/TNF-α) production, and 41BB upregulation in $hBCMA_{72–80}$ CTL against matched U266 ($HLA\text{-}A2^+$ $BCMA^+$) or mis-matched RPMI ($HLA\text{-}A2^-$ $BCMA^+$) myeloma cell line.



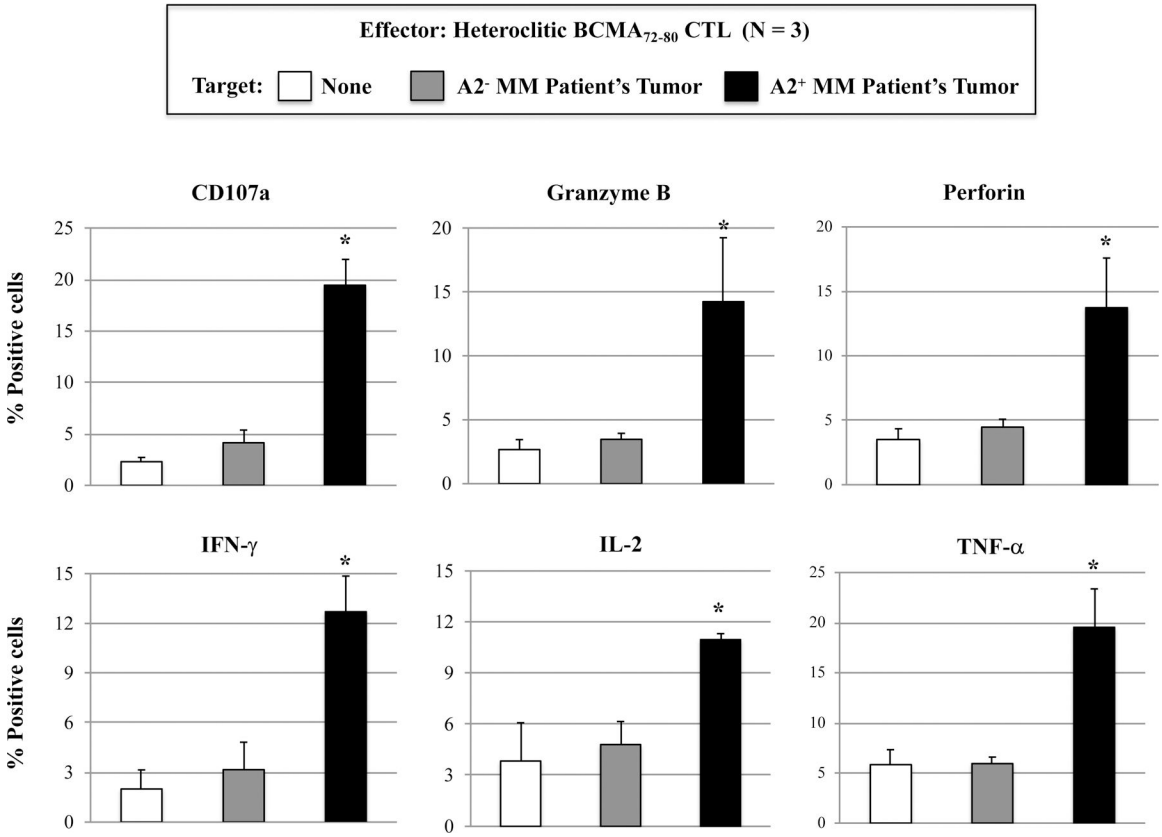
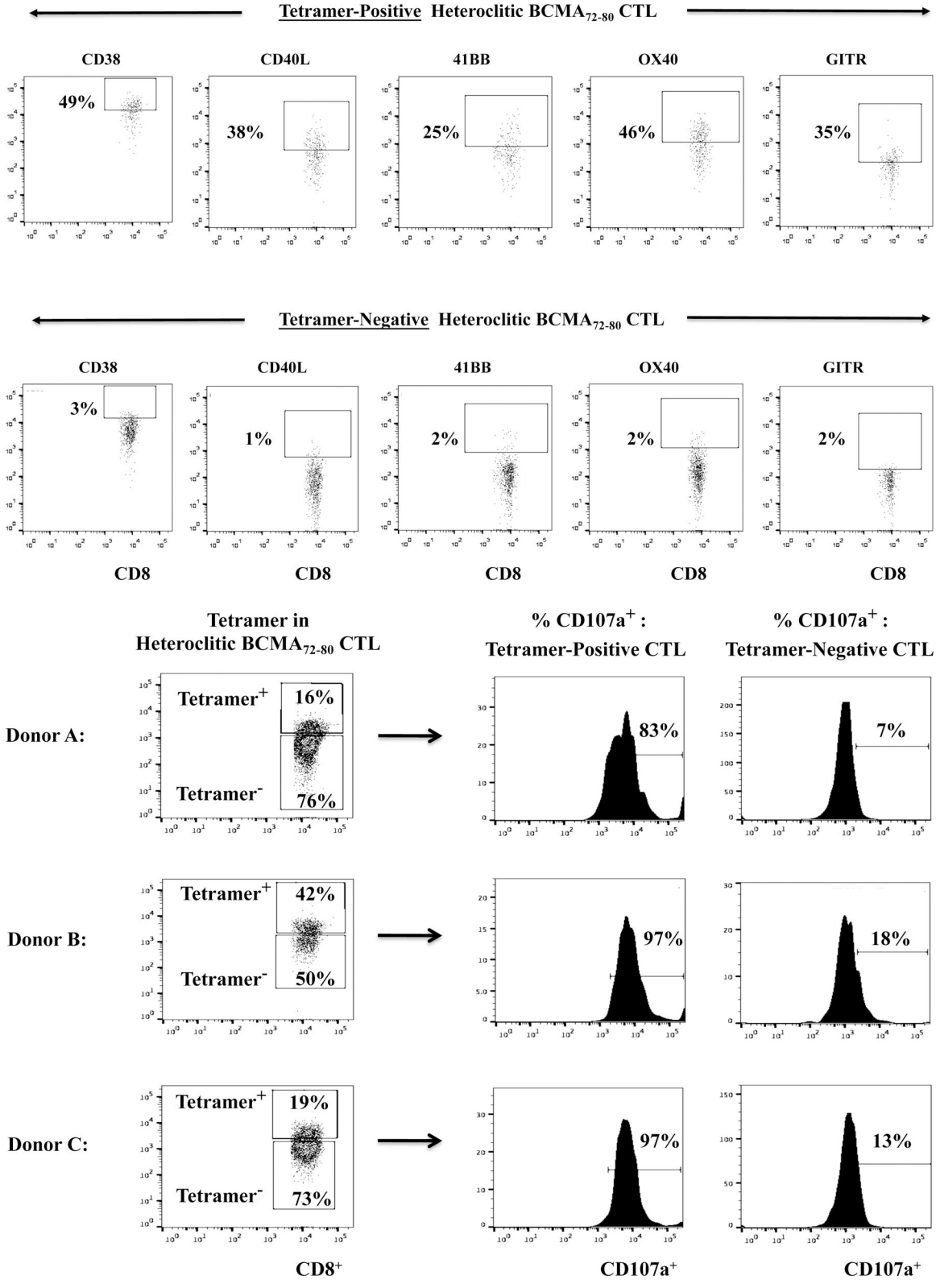


Figure 4. Functional anti-myeloma activities of heteroclitic hBCMA$_{54–62}$ specific CTL or heteroclitic hBCMA$_{72–80}$ specific CTL against primary CD138$^+$ MM tumor cells.

Heteroclitic BCMA peptide-specific CTL were evaluated for their functional activities against primary CD138$^+$ tumor cells obtained from HLA-A2 negative or HLA-A2 positive MM patients. **Fig. 4A.** Representative flow analyses of CD107a degranulation and IL-2 production by hBCMA$_{72–80}$ CTL and hBCMA$_{54–62}$ CTL against HLA-A2$^+$/CD138$^+$ MM cells. **Fig. 4B.** Representative flow analyses of HLA-A2 restricted CD107a degranulation, Granzyme B upregulation, and IFN-γ/TNF-α cytokines production by hBCMA$_{72–80}$ CTL in response to CD138$^+$ cells from MM patients. **Fig. 4C.** Summary (mean ± SE; N=3) of MHC-restricted poly-functional immune responses of hBCMA$_{72–80}$ CTL against primary CD138$^+$ cells from MM patients.



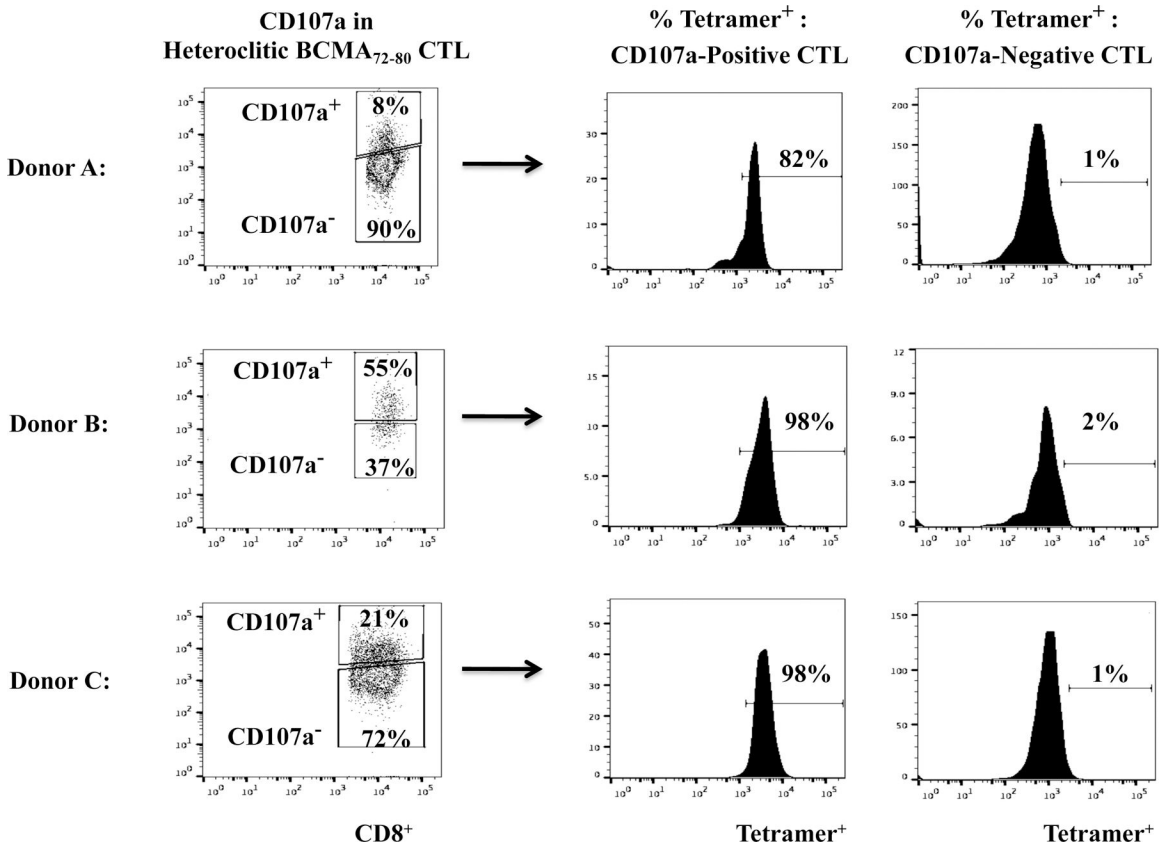
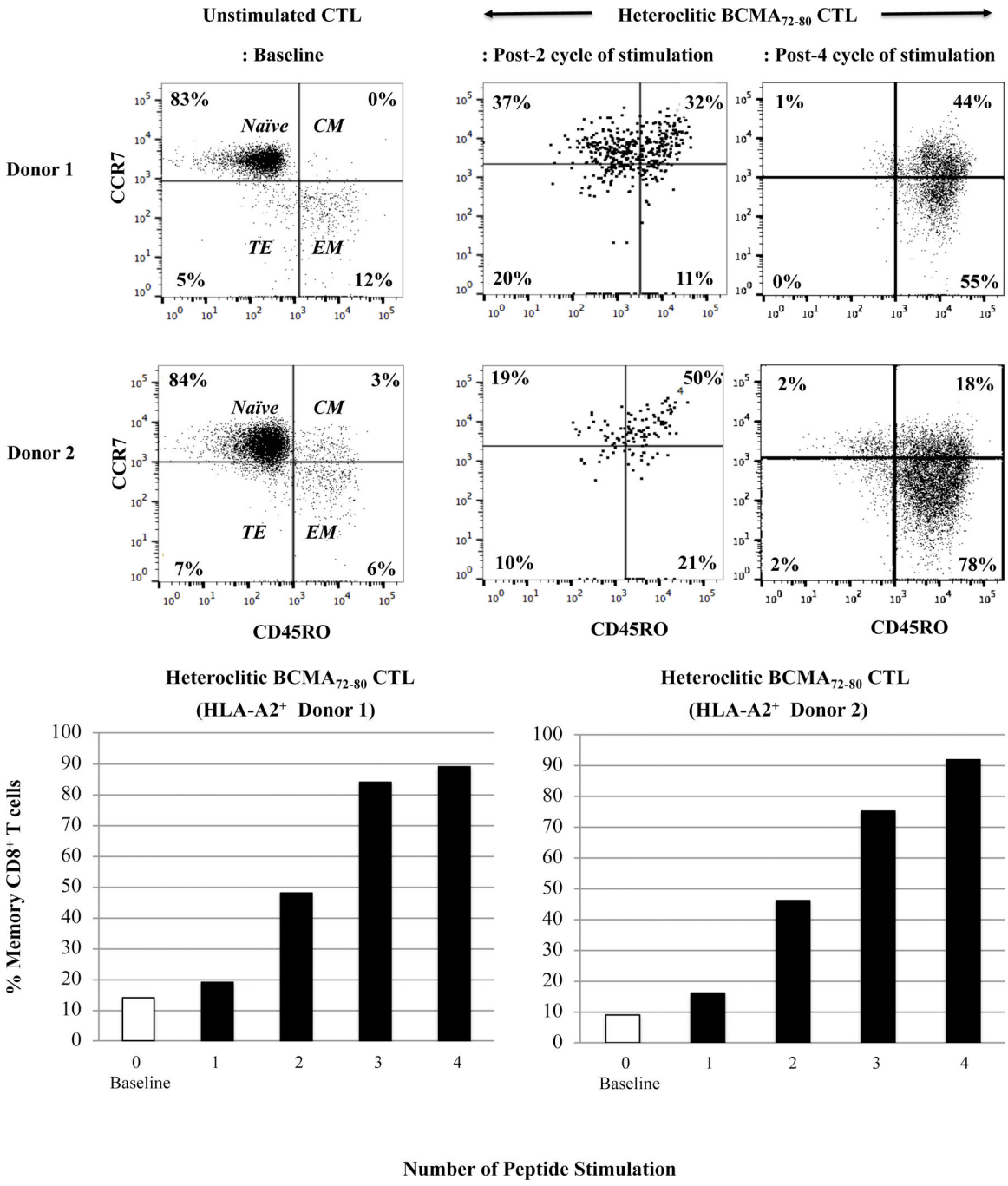


Figure 5. Key antigen expression and functional activities of specific Tetramer$^+$ hBCMA$_{72–80}$ CTL against MM cells.

Heteroclitic hBCMA$_{72–80}$ CTL were evaluated for phenotype and anti-MM activities related to Tetramer-positivity. **Fig. 5A.** Representative flow analyses of key activation (CD38) and costimulatory (CD40L, 41BB, OX40, GITR) molecules expression in hBCMA$_{72–80}$ peptide specific Tetramer-positive or Tetramer-negative CTL subset. **Fig. 5B.** Flow cytometry analyses of functional CD107a degranulation by Tetramer-positive or Tetramer-negative cells in hBCMA$_{72–80}$ CTL (Donor A, Donor B, Donor C; N=3) against HLA-A2$^+$ U266 MM cells. **Fig. 5C.** Flow cytometry analyses of Tetramer-positivity within the functional CD107a$^+$ hBCMA$_{72–80}$ CTL (Donor A, Donor B, Donor C; N=3) in response to HLA-A2$^+$ U266 MM cells.



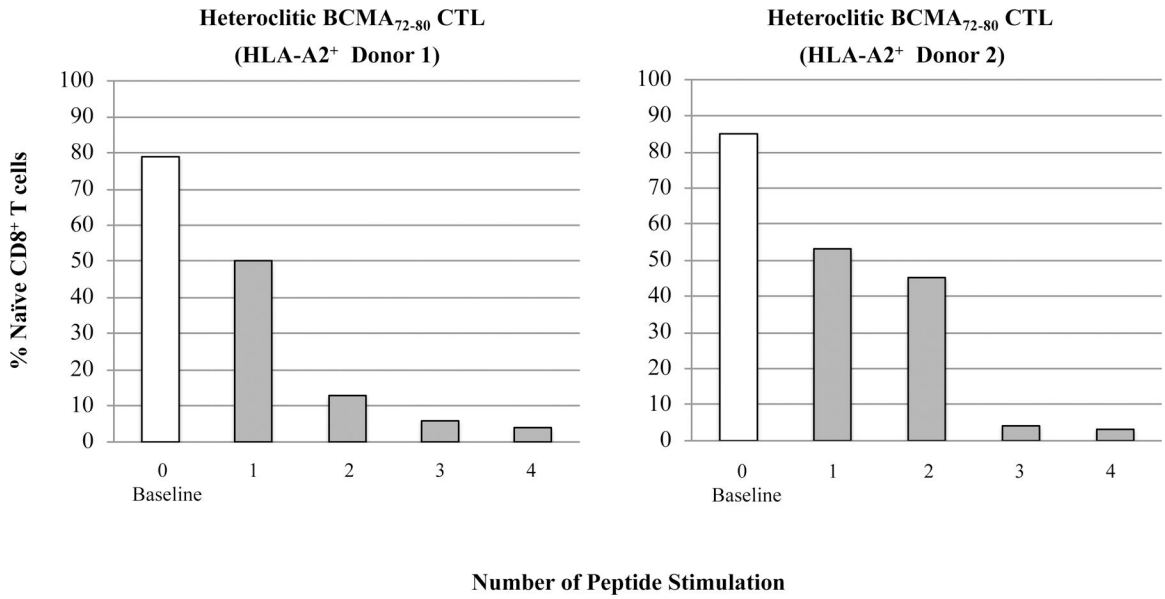
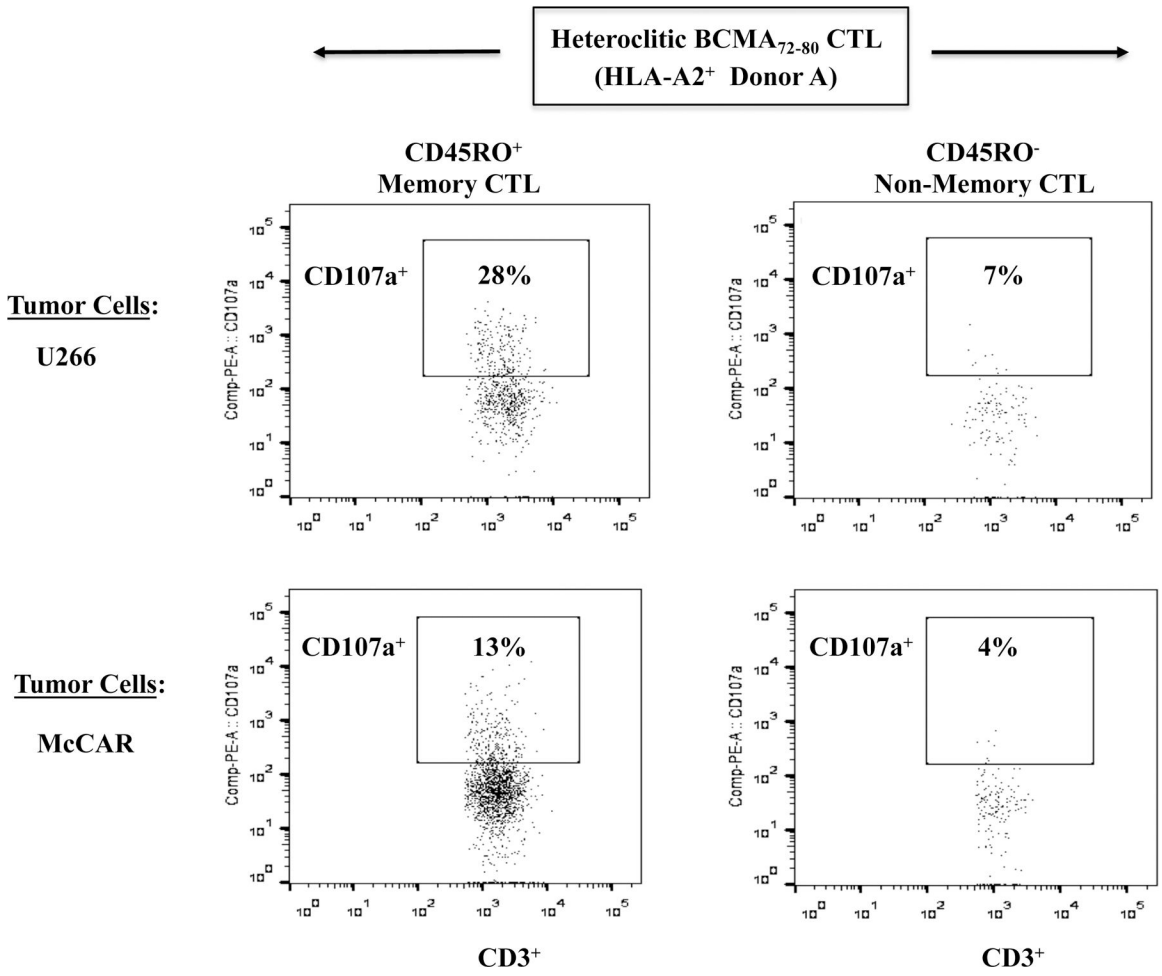
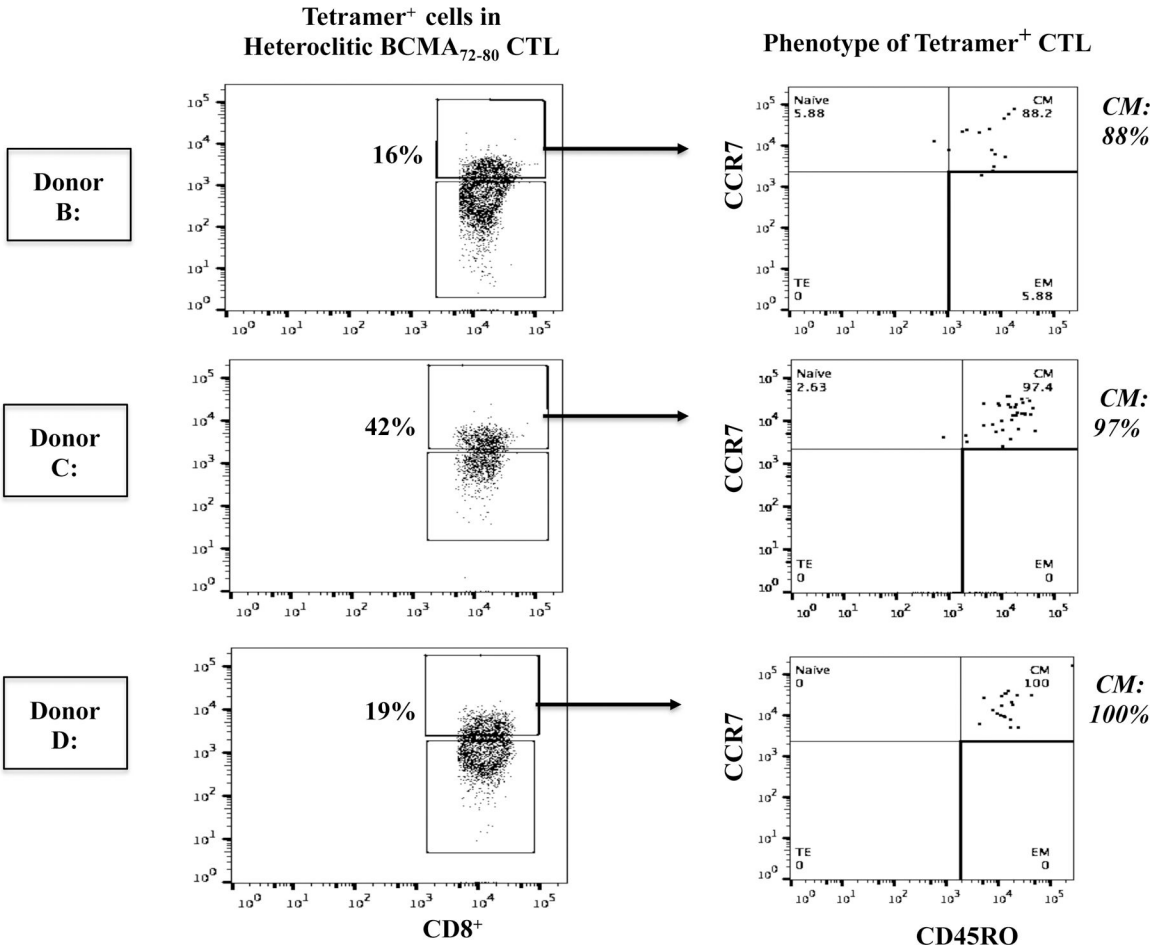


Figure 6. Phenotypic characterization of hBCMA$_{72–80}$ specific memory CTL development upon stimulation with heteroclitic hBCMA$_{72–80}$ peptide.

The Naïve:Memory phenotype of heteroclitic hBCMA$_{72–80}$ CTL (Donor 1, Donor 2) was analyzed at baseline (no peptide stimulation) or one week after each cycle of peptide stimulation. **Fig. 6A.** Flow cytometry analyses of hBCMA$_{72–80}$ CTL differentiation from naïve into memory CD8$^+$ T cells at baseline, 2 cycles and 4 cycles of BCMA$_{72–80}$ peptide stimulation. **Fig. 6B and Fig. 6C.** Summary of changes in memory (CD45RO$^+$CCR7$^+$ and CD45RO$^+$CCR7$^-$) and naïve (CD45ROCCR7$^+$) CD8$^+$ T cells frequency after each cycle of peptide stimulation.





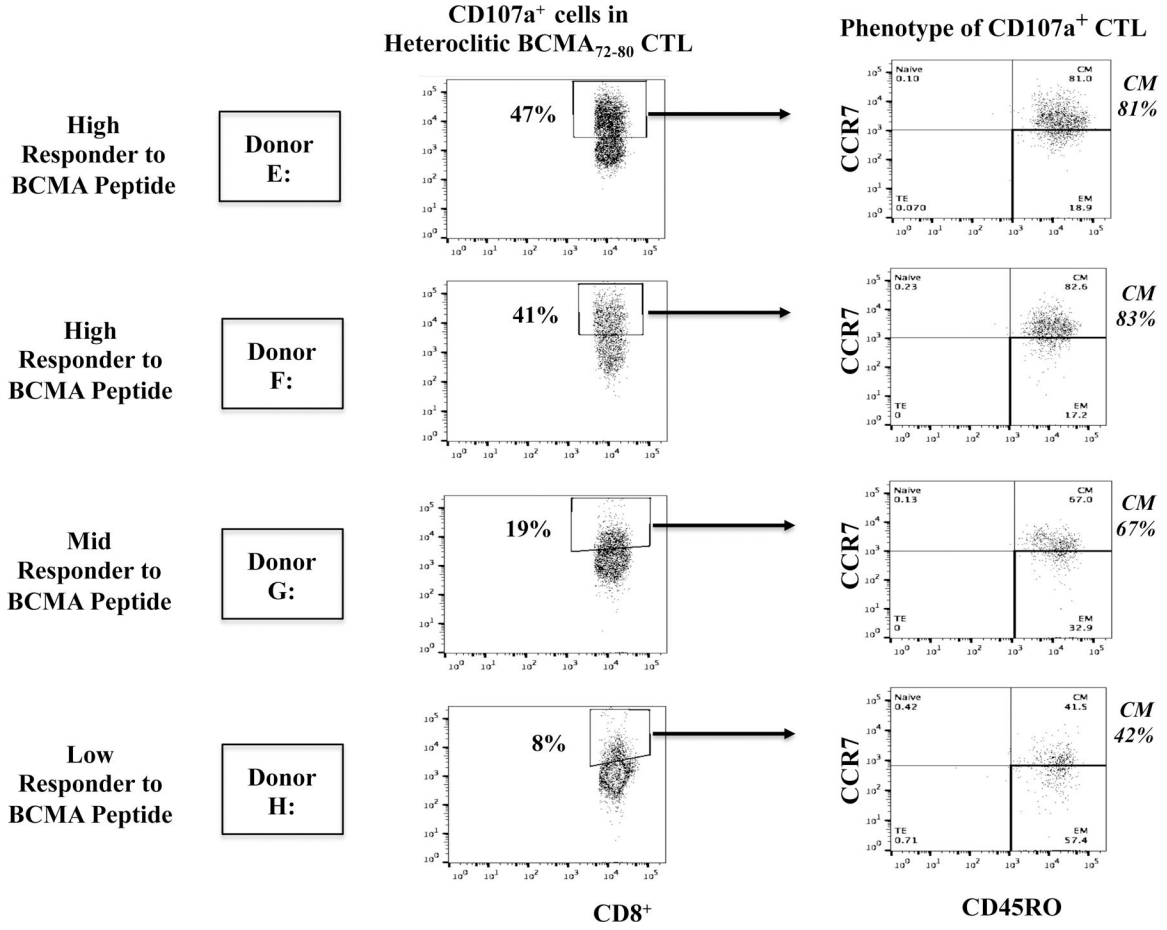
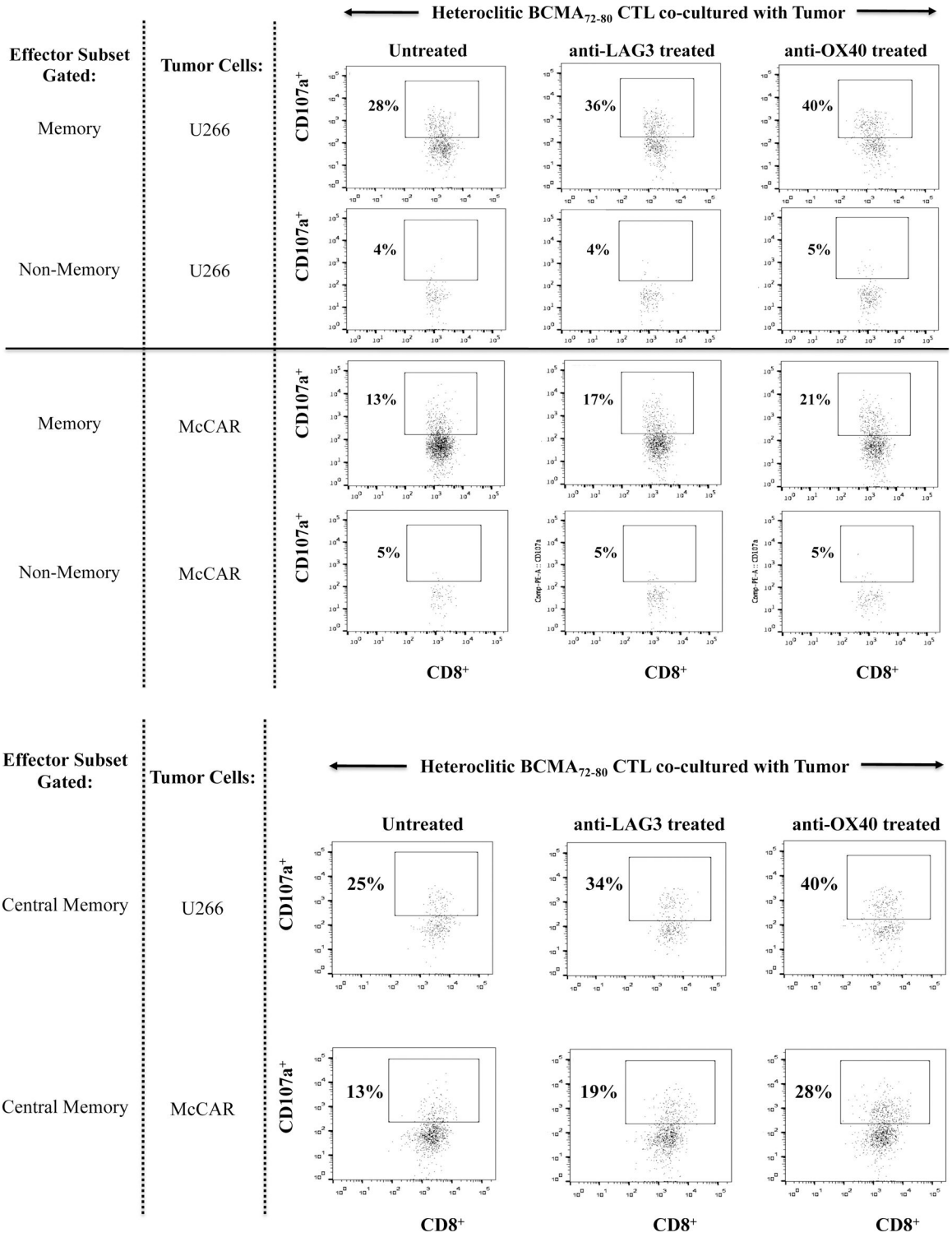


Figure 7. High anti-MM activities by heteroclitic $BCMA_{72–80}$ peptide-specific central memory $CD8^+$ T cells.

The Naïve:Memory cell subsets within $hBCMA_{72–80}$ CTL were evaluated for their functional immune responses against HLA-$A2^+$ MM cells (U266, McCAR). **Fig. 7A.** The CD107a degranulation by $CD45RO^+$ memory cells or $CD45RO^-$ non-memory cells in $hBCMA_{72–80}$ CTL (Donor A) against U266 [BCMA high expression] or McCAR [BCMA low expression] MM cells. **Fig. 7B.** The Naïve:Memory phenotype of $Tetramer^+$ CTL in $hBCMA_{72–80}$ CTL (Donor B, Donor C, Donor D). **Fig. 7C.** The frequency of heteroclitic $BCMA_{72–80}$ peptide-specific central memory CTL and level of anti-tumor activities against U266 cells by High (Donors E and F), Mid (Donor G) and Low (Donor (H) responders.



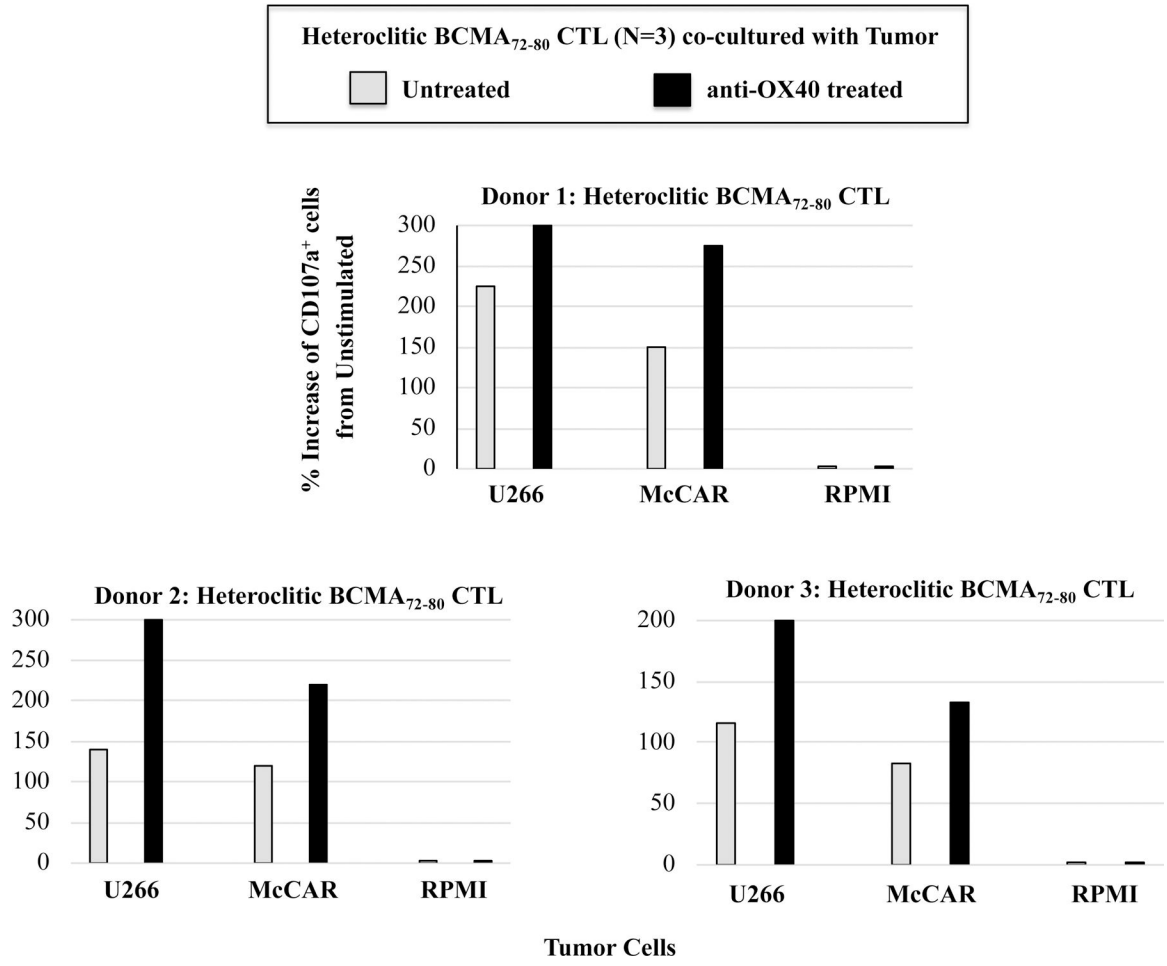


Figure 8. Enhanced immune function of heteroclitic $BCMA_{72–80}$ peptide-specific memory CTL in combination with anti-LAG3 or anti-OX40.

Anti-myeloma activities were investigated in treatment of $hBCMA_{72–80}$ CTL with anti-LAG3 or anti-OX40. **Fig. 8A.** The CD107a degranulation by $CD45RO^+$ memory cells or $CD45RO^-$ non-memory cells in $hBCMA_{72–80}$ CTL, untreated or treated, against U266 or McCAR cells. **Fig. 8B.** The CD107a degranulation by central memory ($CD45RO^+CCR7^+$) $CD3^+CD8^+$ T cells in $hBCMA_{72–80}$ CTL, untreated or treated, against U266 or McCAR cells. **Fig. 8C.** The percent increase of $CD107a^+$ cells induced by $hBCMA_{72–80}$ CTL generated from three different $HLA\text{-}A2^+$ donors (N=3), untreated or treated with anti-OX40, against U266, McCAR or RPMI cells.