

Original article

Feasibility of generating allogeneic BCMA-directed CAR T cells using CD45RA depletion

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Abstract

Backgrounds: Chimeric antigen receptor (CAR) T cell therapy has recently exhibited promise as cancer treatment, especially for hematologic malignancies. Currently, all of the FDA-approved CAR T therapies are autologous in nature. This presents as a limitation since generating CAR T cell using the patient's own blood leads to higher costs and a 3–4 week production time. To overcome this, allogeneic CAR T cell-therapy is being explored.

Objective: In this study, CD45RA depletion was used to generate allogeneic anti-BCMA CAR T cells. This approach aims to utilize the CD45RA-negative fraction containing memory T cells which has lower alloreactivity and reduced risk to cause graft-versus-host disease (GVHD).

Methods: The CD45RA-negative T cells were obtained through magnetic separation of CD45RA-labeled PBMCs. This process yielded the flow-through containing the unlabeled CD45RA-negative fraction which was then used to produce CAR T cells through lentiviral transduction. The generated CD45RA-negative anti-BCMA CAR T cells were compared to nondepleted anti-BCMA CAR T cells according to transduction efficiency, cell expansion, memory phenotype and cell subset. Cytotoxic function was also assessed through co-culture assays.

Results: Results show higher transduction efficiency and MFI of CD45RA-negative CAR T cells on days 4 and 11, respectively with no difference in expansion. They also feature more CD4⁺ cell subset and T^{em} memory phenotype. Results show no statistically significant difference between non-depleted and depleted anti-BCMA CAR T cells in *in vitro* co-culture assay.

Conclusion: The data demonstrate CD45RA depletion as a feasible approach to generating allogeneic anti-BCMA CAR T cells

Keywords: allogeneic, BCMA, CAR T cells, immunotherapy, multiple myeloma

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Multiple myeloma (MM) is a plasma cell neoplasm that is considered a heterogeneous disease.⁽¹⁾ It is a type of cancer with varying clinical presentations, genetic and molecular profiles, disease behaviors and treatment response from patient to patient. This contributes to patients relapsing after a period of response or becoming refractory or resistant to treatment.⁽²⁾ Treatment options for MM have expanded for the past years but it still remains incurable.⁽³⁾

Chimeric antigen receptor (CAR) T cell therapy has shown promise as treatment for hematological malignancies. Idecabtagene vicleucel (Ide-cel) and Ciltacabtagene autoleucel (Cilta-cel), the two FDA-approved BCMA-directed CAR T-cell therapies, have improved r/r MM patient outcomes.⁽⁴⁾ A major setback for this treatment strategy, even in CAR T-cell therapy in general, is that it requires personalized manufacturing as it is currently produced using autologous T cells which leads to a 3-4 week production time and increased cost.⁽⁵⁾ The development of a CAR T-cell product from an allogeneic source overcomes this logistic limitation.

One approach to develop a CAR T cell product from an allogeneic source with reduced risk of graft-versus-host disease (GVHD) is the depletion of CD45RA-expressing T cells which include naïve and effector T cells.⁽⁶⁾ Naïve T cells (T_n) have increased alloreactivity potential and effector T cells (T_e) have decreased persistence *in vivo* in comparison to CD45RA- memory T cells (T_m) which are primed to detect specific antigens and reduces the risk of GVHD.^(7, 8) This study aims to address challenges concerning CAR T cell accessibility by producing an allogeneic anti-BCMA CAR T-cell product utilizing the CD45RA depletion approach.

Materials and methods

Isolation, depletion and activation of peripheral blood mononuclear cells (PBMCs)

PBMCs were collected from healthy donors, isolated following the Ficoll-Paque technique and labeled with CD45RA MicroBeads (Miltenyi Biotec Germany). CD45RA depletion was done through magnetic separation using an MS Column (Miltenyi Biotec USA) following manufacturer's instructions. The flow-through of this process containing unlabeled CD45RA-negative cells were collected to generate CAR T cells. Nondepleted and CD45RA-negative T cells were cultured in TexMACS medium (Miltenyi

Biotec, Germany) with 5% fetal bovine serum (FBS) and supplemented with 10 ng/mL of human IL7 (Miltenyi Biotec, Germany) and 5 ng/mL of human IL15 (Miltenyi Biotec, Germany). Cells were then seeded to a 24-well treated plate with a density of 1×10^6 cells per well and activated using T-Cell TransAct™ (Miltenyi Biotec, Germany). Incubation for 24 hours at 37°C in a 5% CO₂ incubator then followed.

Generation of nondepleted and CD45RA-negative anti-BCMA CAR T-cells

Lentivirus was produced through the transfection of the BCMA CAR transgene and packaging plasmids RSV, VSVG and pMDLg to 293T cells with GeneJuice transfection reagent (Merck). The BCMA construct had an anti-BCMA single-chain variable fragment (scFv), CD28 transmembrane, 41BB costimulatory and CD3 zeta activation domain. The activated T-cells were transduced with the lentivirus following a multiplicity of infection (MOI) of 3 and were transferred to their respective RetroNectin-coated well. The generated CAR T cells were maintained by replacing media with TexMACS medium (Miltenyi Biotec, Germany) with 5% FBS supplemented with 10 ng/mL IL7 (Miltenyi Biotec, Germany) and 5 ng/mL of human IL15 (Miltenyi Biotec, Germany) every 3-4 days.

Characterization of generated CAR T-cells was done using BD Accuri™ C6 Plus flow cytometer (BD Biosciences, USA) and FlowJo version 10 (Treestar, USA). The expression of BCMA on the BCMA CAR T cells was detected using PE anti-human BCMA (Acro Biosystems). Memory phenotype was determined according to the expression of specific surface markers wherein naïve (T_n) were CD3⁺CD45RA⁺CCR7⁺CD62L⁺, effector (T_e) were CD3⁺CD45RA⁺CCR7⁻CD62L⁻, central memory (T_{cm}) were CD3⁺CD45RA⁻CCR7⁺CD62L⁺ and effector memory (T_{em}) were CD3⁺CD45RA⁻CCR7⁻CD62L⁻. Cell subset was differentiated as CD4 (CD3⁺CD8⁻) and CD4 (CD3⁺CD8⁺).

Short term and sequential co-culture assay

Short term co-culture was done following 2:1, 1:1 and 1:2 effector:target (E:T) ratios with a fixed target concentration of 100,000 cells per 100ul. Co-cultured cells were collected and analyzed after 24 hours. Cytotoxic activity was assessed through flow cytometry and the percentage of inhibition was calculated using the formula:

$$\% \text{ inhibition} = 100 - \left(\frac{\text{Number of target cells after co-culture}}{\text{Number of target cells alone}} \times 100 \right)$$

Sequential co-culture was conducted following a normalized E : T ratio of 1:1 and with target fixed at 1×10^6 /well in a 24-well G-rex plate (Wilson Wolf, Saint Paul, Minnesota, United States). Each well contained 1ml normalized CAR T cell, 1ml of target (1×10^6 cells) and 6ml of TexMACS (Miltenyi Biotec, Germany) with 5% FBS. Every four days, 6 ml of media was discarded and T cells were collected for flow cytometry analysis. After, 1 ml of target (1×10^6 cells) and 5ml of media were added to each well. The same method of analyzing cytotoxic activity with short term co-culture was employed.

Data analysis and statistics

GraphPad Prism version 8.4.0 was used for statistical analysis and visualization. Statistical significance between two groups was determined using t-test while analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was employed for comparison between more than two groups. A *P*-value of less than 0.05 ($P < 0.05$) was considered significant. Data are shown as mean $\pm$ SEM.

Ethical statement

This study obtained ethics approval from the Institutional Review Board, Faculty of Medicine, Chulalongkorn University (No. 0377/69).

Results

CD45RA depletion enables effective separation of CD45RA-negative fraction

Following PBMC isolation, CD45RA depletion was done through magnetic separation. After depletion, $99.33 \pm 0.53\%$ of the cells were $CD3^+CD45RA^-$ which shows an effective depletion of CD45RA-positive T cells (**Figure 1**).

Characterization of nondepleted and CD45RA-negative CAR T-cells

To assess the feasibility of producing CAR T-cells from CD45RA-negative cells, the characteristics of the generated CD45RA-negative anti-BCMA CAR T-cell were compared to the nondepleted CAR T-cells.

CD45RA-negative anti-BCMA CAR had a significant increase in transduction efficiency on day 11 at $70.83 \pm 3.00\%$ and mean fluorescence intensity (MFI) on day 4 at 5418 ± 150 (**Figure 2a**). No difference was observed in terms of cell expansion between nondepleted and CD45RA-negative CARs on days 4, 7, and 11 (**Figure 2b**). The depleted anti-BCMA CAR T-cells features more $CD4^+$ cell subset ($83.43 \pm 3.84\%$) and T^m memory phenotype ($48.03 \pm 4.49\%$) than the nondepleted CAR T cells which had an almost equal composition of $CD4^+$ and $CD8^+$ subset ($51.10 \pm 8.17\%$; $48.90 \pm 8.17\%$) and majority of T^n memory phenotype ($58.37 \pm 21.86\%$) (**Figure 2c–d**).

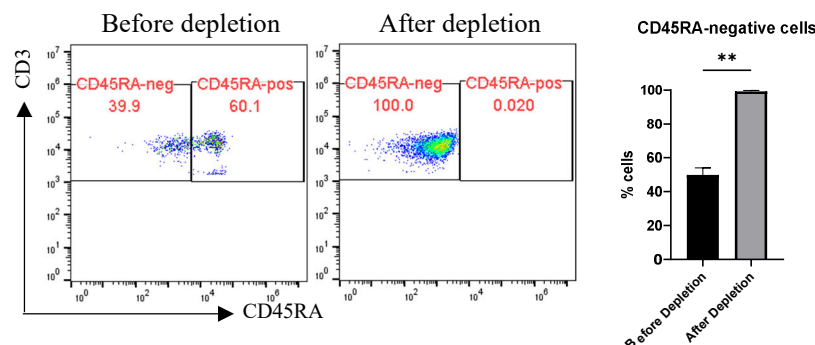


Figure 1. Flow cytometry analysis results (left) of CD45RA-positive and-negative population before and after CD45RA depletion and bar graph showing the percentage of CD45RA-negative cells (right).

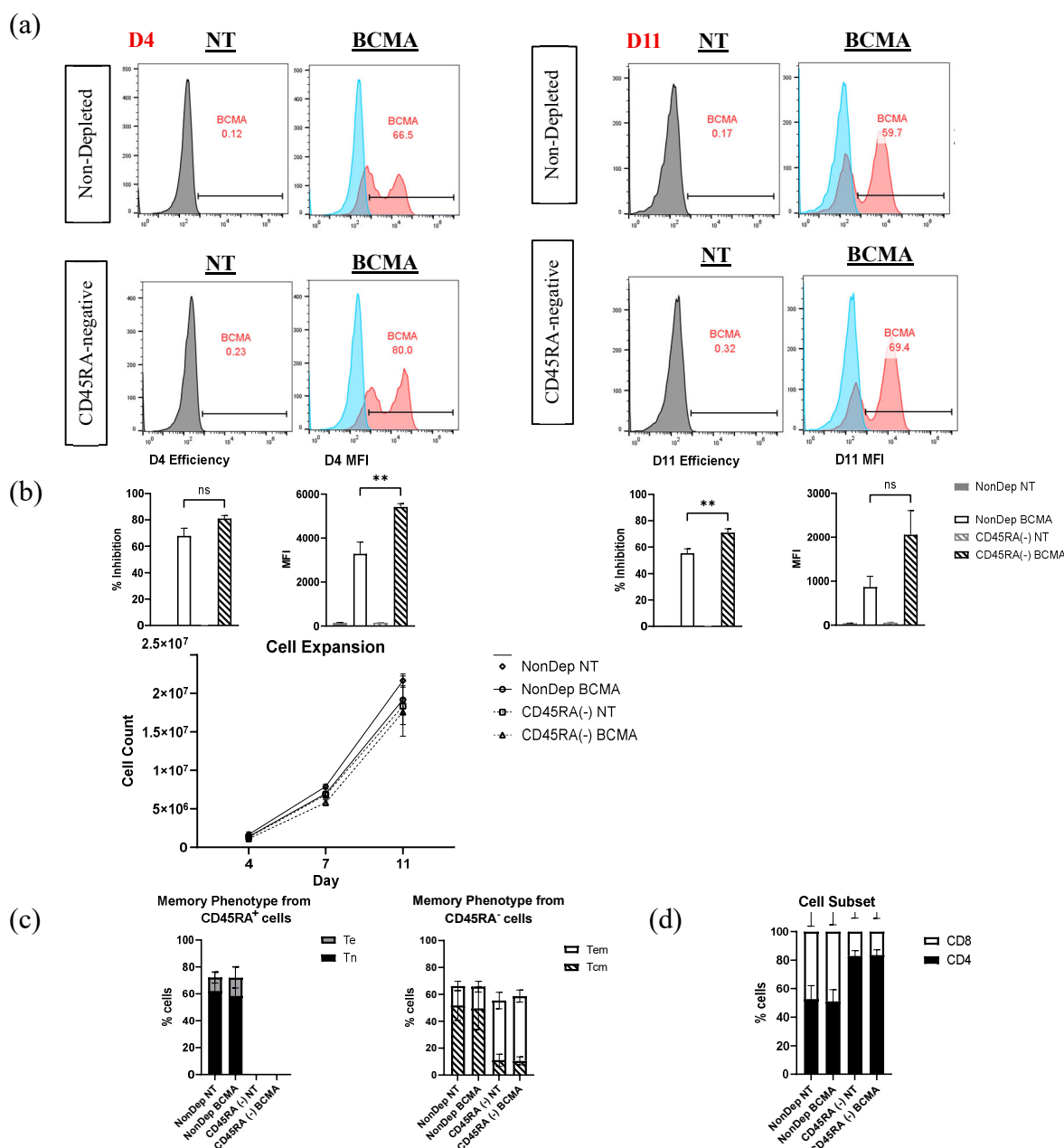


Figure 2. Characteristics of generated CAR T-cells. (a) Transduction efficiency and MFI on days 4 and 11 with representative flow cytometry histogram (b) Cell proliferation on days 4, 7 and 11 (c) Memory phenotype profiles of CARs: effector (T_e), naïve (T_n), effector memory (T_{em}) and central memory (T_{cm}) (d) Proportion of CD4 and CD8 subsets.

CD45RA-negative and nondepleted CAR T-cells have comparable in vitro anti-cancer activity

Short term and sequential co-culture assays were conducted to discover how the CD45RA-negative CAR T cells differ from nondepleted CARs in terms of function. In short term co-culture, CARs were tested against the following BCMA-positive cell lines: Nalm6-BCMA-GFP, RPMI-GFP and MM1R-GFP. The nondepleted anti-BCMA CARs showed the highest inhibition percentage in the 2:1 ratio with an average of 98.92% against Nalm6-BCMA-GFP, 86.83% with RPMI-GFP and 92.23% with MM1R-GFP. Similarly, the CD45RA-negative anti-BCMA CARs showed the same trend with the mean

percentage of inhibition against Nalm6-BCMA-GFP at 97.43%, RPMI-GFP at 77.26% and MM1R-GFP at 90.01% (**Figure 3a**). Both CARs also exhibit the lowest cytotoxic activity in the 1:2 ratio which suggests that they function against their targets in a dose dependent manner.

Sequential co-culture was done to evaluate CAR function after multiple antigen stimulation. This assay was conducted against Nalm6-BCMA-GFP and MM1R as targets. No significant difference was observed between CD45RA-negative and nondepleted anti-BCMA CARs across both targets in terms of killing activity in all rounds of co-culture (**Figure 3b–c**).

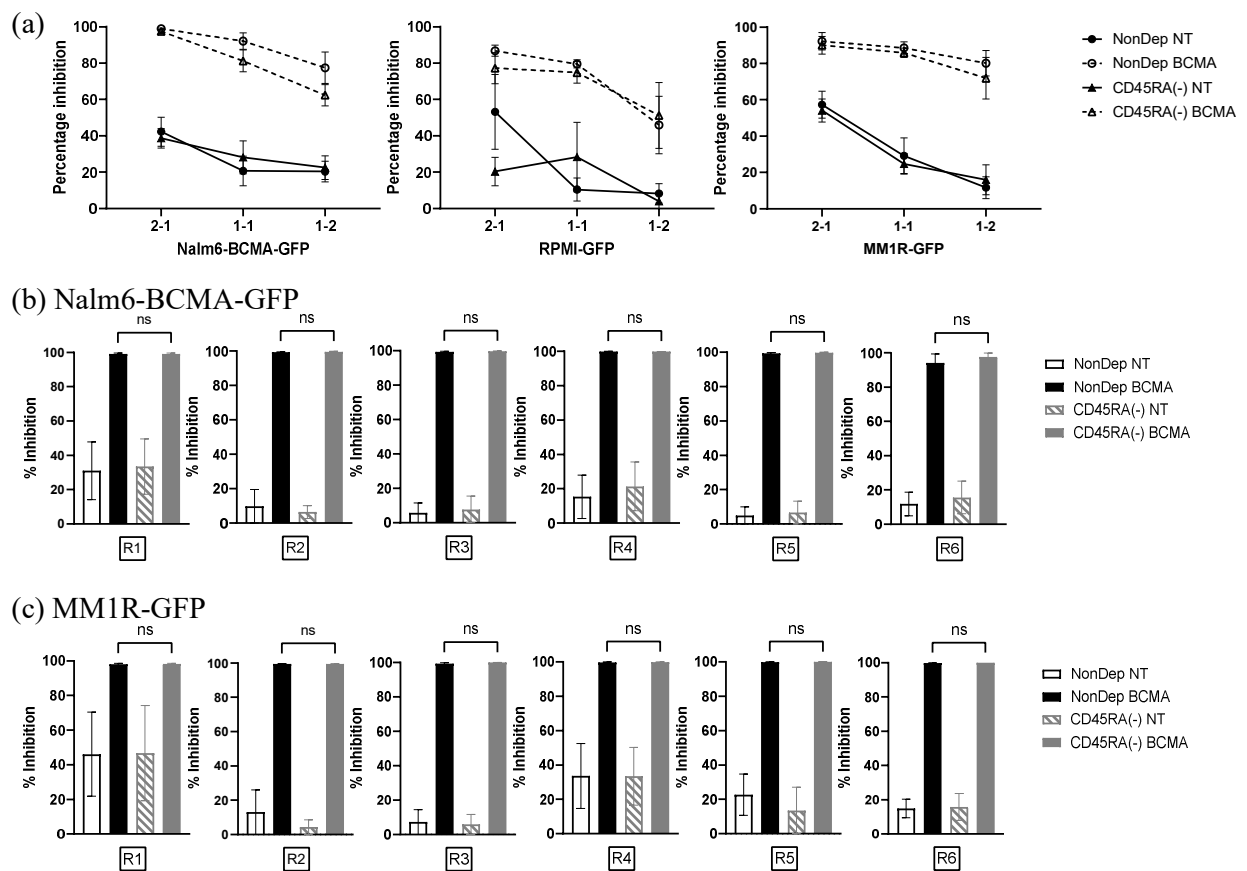


Figure 3. CAR T cell function (a) Short term coculture against BCMA-expressing targets in 2:1, 1:1 and 1:2 E:T ratios. (b) Sequential coculture against Nalm6-BCMA-GFP. (c) Sequential coculture against MM1R GFP.

Discussion

In order to generate an allogeneic anti-BCMA CAR T cell, CD45RA depletion was utilized. This investigation revealed that the magnetic separation of the CD45RA-positive and -negative fraction is an effective means of removing the CD45RA-positive T cells that contain the all reactive T^h phenotype. This aligns with a preclinical study using the same approach to generate CD19-directed CAR T-cells.⁽⁹⁾ Currently, this method is applied as donor lymphocyte infusion (DLI) for patients post hematopoietic cell transplantation (HCT).⁽¹⁰⁻¹²⁾

In this study, the CD45RA-negative T cells were used to generate anti-BCMA CAR T cells. As a preliminary step, they were characterized according to transduction efficiency, cell expansion, memory phenotype and cell subset profiles to see if the depletion step affected these key CAR T cell features. The CD45RA-negative anti-BCMA CAR T-cells showed higher transduction efficiency and similar expansion than the nondepleted CAR T cells. There was also an observed change in the phenotypic and cell subset profiles wherein it exhibited a predominantly T^m memory phenotype and CD4⁺ subset than the nondepleted CARs which featured more of T^h

memory phenotype an almost 1:1 CD4:CD8 composition. These profiles are consistent with previous studies which employed CD45RA depletion to produce CAR T cells directed against CD19.^(8,9,13)

Previous investigations on CD45RA-negative CAR T cells with CD19 specificity reported its anti-leukemic activity in vitro against CD19⁺ *MLL*-rearranged cell lines.⁽⁸⁾ To support this further, CD45RA-negative cells expressing NKG2D ligand (NKG2DL) CAR showed high cytotoxicity to NKG2D osteosarcoma cell lines.⁽¹⁴⁾ This aligns with the results of this study which revealed the CD45RA-negative anti-BCMA CAR T-cells had high anti-cancer activity in both short term and sequential coculture and has comparable results to its nondepleted counterpart. The abovementioned studies also conducted in vivo research through mice models both with results showing extended overall survival without GVHD.^(8,14)

Currently, a Phase I clinical trial called MEMCAR19: Allogeneic CAR T-Cell Therapy for Relapsed/Refractory CD19-Positive Leukemia (NCT04881240) is ongoing at St. Jude Children's Research Hospital, USA. These collectively highlight the promise of this approach for producing allogeneic CAR T-cells.

Conclusion

The results of this study show that it is feasible to generate allogeneic anti-BCMA CAR T-cells from CD45RA-negative cells. Despite the shift to a T^{em} memory phenotype and CD4⁺ subset profile of the CD45RA-negative CAR T-cells, they still exhibit comparable *in vitro* anti-cancer activity in short term and sequential co-culture assays.

This study focused on assessing the feasibility of generating anti-BCMA CAR T-cells from CD45RA-negative cells as an approach to develop allogeneic CAR T-cells. Further validation on the alloreactivity and GVHD risk of CD45RA-negative CAR T-cells through *in vitro* tests such as mixed lymphocyte reaction (MLR) and *in vivo* mouse models should be conducted.

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Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data sharing statement

The data sets generated or analyzed during the present study are available for non-commercial purposes from the corresponding author upon reasonable request.

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