# PiggyBac™-Produced CAR-T Cells Exhibit Stem-Cell Memory Phenotype

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## ABSTRACT

Immunotherapy using chimeric-antigen receptor (CAR)-T cells is emerging as an exciting therapeutic approach for cancer therapies. Autologous CAR-modified T cells targeting a tumor-associated antigen (Ag) can result in robust tumor killing, in some cases resulting in complete remission of CD19⁺ hematological malignancies. Unlike traditional biologics and chemotherapeutics, CAR-T cells possess the capacity to rapidly reproduce upon Ag recognition, thereby potentially obviating the need for repeat treatments. To achieve this, CAR-T cells must not only drive tumor destruction initially, but must also persist in the patient as a stable population of viable memory T cells to prevent potential cancer relapses. Many CART therapies have achieved the first part of this plan by displaying impressive initial efficacy, but have failed in the second part due to a poor durability of response. Thus, intensive efforts have been focused on the development of CAR molecules that do not cause T cell exhaustion through Ag-independent (tonic) signaling, as well as of a CAR-T product containing early memory cells, especially stem cell memory ($T_{SCM}$). It is hypothesized that a stem cell-like CAR-T would exhibit the greatest capacity for self-renewal and multipotent capacity to derive central memory ($T_{CM}$), effector memory ($T_{EM}$) and effector T cells ($T_E$), thereby producing better tumor eradication and long-term CAR-T engraftment.

We developed a novel Centyrin™-based CAR, referred to as a CARTyrin, that is specific for human B cell maturation antigen (BCMA). Centyrins™ are alternative scaffold molecules based on human consensus tenascin FN3 domain, are smaller than scFv molecules, and can be selected for monomeric properties that favor stability and decrease the likelihood of tonic signaling in CAR molecules. We produced a plasmid DNA transposon encoding the CARTyrin that was flanked by two cis-regulatory insulator elements to help stabilize CARTyrin expression by blocking improper gene activation or silencing. The piggyBac™ (PB) Transposon System was used for stable integration of anti-BCMA CARTyrin into resting pan T cells. To achieve this, the transposon was co-delivered along with an mRNA transposase enzyme, called Super piggyBac™ (SPB), in a single electroporation reaction. Delivery of piggyBac™ transposon into untouched, resting primary human pan T cells resulted in 20-30% of cells with stable integration and expression of PB-delivered genes. Surprisingly, we observed that a majority of these cells were positive for expression of CD62L and CD45RA, markers commonly associated with $T_{SCM}$ cells. To see if this phenotype was retained upon CAR-T cell stimulation and expansion, we activated the cells via stimulation of CD3 and CD28, and later show that > 60% of CARTyrin+ T cells exhibited a stem-cell memory phenotype. Furthermore, these cells were fully capable of expressing potent anti-tumor effector function.

To determine whether or not the PB system directly contributed to enhancing the expression of stem-like markers, we compared the phenotype of CAR-T cells generated either by PB transposition or lentiviral (LV) transduction. To do this, we constructed a new vector by subcloning the CARTyrin transgene into a common LV construct for production of virus. Following introduction of the CARTyrin to untouched resting T cells either by PB-transposition or LV-transduction, we expanded the CARTyrin⁺ cells and then allowed them to return to a resting state. A variety of phenotypic and functional characteristics were measured, including kinetic analysis of memory and exhaustion-associated markers, secondary proliferation in response to homeostatic cytokine or tumor-associated Ag, cytokine production, and lytic capability in response to BCMA⁺ tumor cells. Unlike the PB-transposed CARTyrin⁺ T cells, we found that the LV-transduced CARTyrin⁺ T cells did not exhibit an augmented memory phenotype. In addition, PB-transposed cells exhibited a comparable or greater capability for secondary proliferation and killing of BCMA⁺ tumor cells. The difference in final phenotype between PB-transposed and LV-transduced cells may be explained in part by the initial modification event. In sorted T cell subsets, PB shows a preference for transposing naïve T cells, while LV shows an inability to transduce naive T cells.

Together, these data demonstrate that CAR-T cells produced by PB transposition are predominantly $T_{SCM}$ cells, a highly desirable product phenotype in the CAR-T field. Furthermore, these CARTyrin⁺ T cells exhibit strong anti-tumor activity and may give rise to cells that persist longer *in vivo* (a hypothesis that is supported by data shown on the poster referenced below) due to the use of a Centyrin™-based CAR, which may be less prone to tonic signaling and functional exhaustion.

## INTRODUCTION

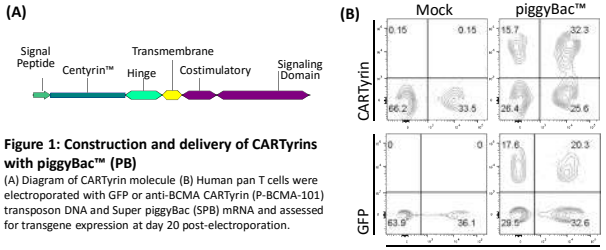


**Figure 1: Construction and delivery of CARTyrins with piggyBac™ (PB)**
(A) Diagram of CARTyrin molecule (B) Human pan T cells were electroporated with GFP or anti-BCMA CARTyrin (P-BCMA-101) transposon DNA and Super piggyBac (SPB) mRNA and assessed for transgene expression at day 20 post-electroporation.

**I. Development of Novel Non-Immunoglobulin Centryin™-Based CARs (CARTyrins) Targeting Human BCMA** Session 703 Poster III
**II. A Novel Bcma-Specific, Centyrin™-Based CAR-T Product for the Treatment of Multiple Myeloma** Session 653 Poster I

## METHODS & RESULTS

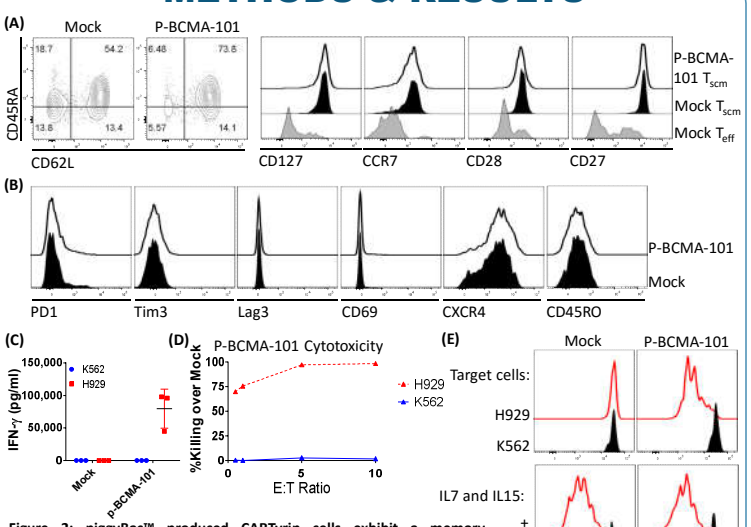


**Figure 2: piggyBac™ produced CARTyrin cells exhibit a memory phenotype and anti-tumor efficacy**
Human pan T cells were transposed with P-BCMA-101, expanded through αCD3/CD28 bead stimulation, positively selected by drug resistance, and examined at day 20 post-transposition. (A) Mock transposed and P-BCMA-101 transposed CD8⁺ T cells were examined for CD62L and CD45RA expression to distinguish $CD62L^+CD45RA^+$ $T_{scm}$, $CD62L^+CD45RA^-$ $T_{cm}$, $CD62L^-CD45RA^-$ $T_{em}$, and $CD62L^-CD45RA^+$ $T_{eff}$. The $T_{scm}$ from P-BCMA-101 and mock transposed were examined for important memory molecules and compared to $T_{eff}$ from mock transposed cells. (B) P-BCMA-101 and mock transposed CD8⁺ T cells were examined for activation, exhaustion, and homing molecules. (C) P-BCMA-101 cells secrete high levels of IFN-γ when cultured with BCMA⁺ H929 cells (10:1 E:T). (D) P-BCMA-101 cells exhibit potent BCMA-specific cytotoxicity in a 24 hour flow-based killing assay. (E) P-BCMA-101 cells proliferate vigorously in response to BCMA antigen (1:2 E:T) and IL7/IL15 treatment *in vitro*. For all experiments, n>3.

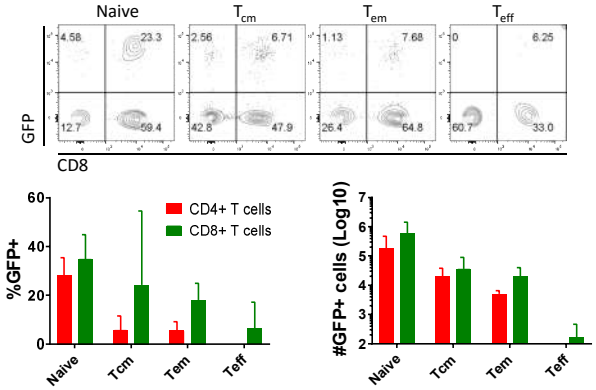


**Figure 3: piggyBac™ preferentially transposes naïve T cells**
Human pan T cells were sorted into naïve, $T_{cm}$, $T_{em}$, and $T_{eff}$ subsets (as defined in Figure 2). The sorted subsets were transposed with PB-GFP, stimulated with αCD3/CD28 beads at day 2 post-transposition, expanded, and examined on day 19 post-transposition. Flow cytometry for CD8 and GFP is shown in the top panel. The quantifications of the frequency of transposition and the final number of transposed T cells are shown in the two graphs. n=3 donors.

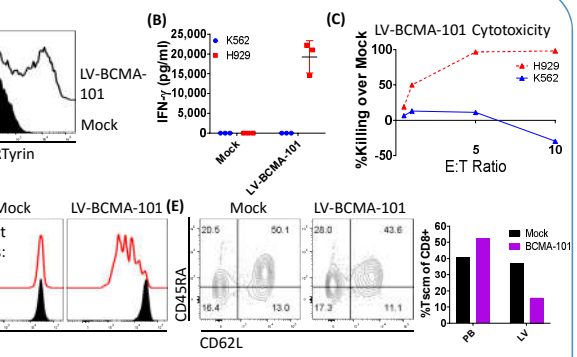


**Figure 4: Lentivirus-produced CARTyrin cells exhibit anti-tumor efficacy, but do not have an enhanced memory-like phenotype**
Human pan T cells were stimulated with αCD3/CD28 beads, transduced at day 2 post-stimulation with lentivirus encoding BCMA-101 (LV-BCMA-101, MOI 5), expanded, and examined at day 19 post-stimulation. (A) LV-BCMA-101 transduced cells express high levels of CARTyrin over mock cells. LV-BCMA-101 cells respond to BCMA⁺ H929 target cells by (B) producing high levels of IFN-γ (10:1 E:T), (C) killing target cells, and (D) proliferating (1:2 E:T). (E) LV-BCMA-101 cells do not show any propensity to differentiate into $T_{scm}$. This is especially revealed when $T_{scm}$ are thoroughly identified as CD45RA+CD62L+CCR7+CD127+CD27+CD28+ CD8+ T cells (far right graph). These data are representative of 2+ independent experiments and donors.

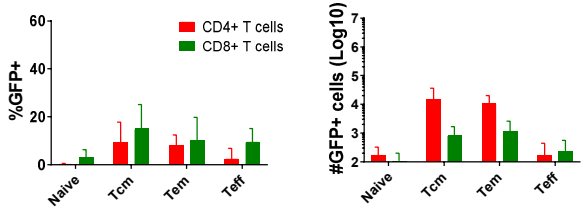


**Figure 5: Lentiviral vectors preferentially transduce non-naïve T cells**
Human pan T cells were sorted into naïve, $T_{cm}$, $T_{em}$, and $T_{eff}$ subsets (as defined in Figure 2). The sorted subsets were stimulated with αCD3/CD28 beads, transduced with lentivirus encoding GFP (MOI 5), expanded, and examined on day 19 post-stimulation. $CD4^+$ and $CD8^+$ $GFP^+$ T cells were identified by flow cytometry as in Figure 3. The quantification of the frequency of $GFP^+$ cells and the final numbers of $GFP^+$ cells are shown in the graphs above. n=3 donors.

## CONCLUSIONS

- P-BCMA-101 cells exhibit potent anti-tumor activity in the form of cytotoxicity, proliferation, and cytokine production
- Importantly, P-BCMA-101 T cells have a high frequency of $T_{scm}$ cells, possibly indicating more potential for a durable functionality *in vivo*.
- This propensity to differentiate into $T_{scm}$ is explained, in part, by the tendency for piggyBac™ to transpose naïve cells over other cell types.
- In stark contrast to PB-transposed cells (>60% $T_{scm}$), lentivirus-transduced cells have a lower frequency of $T_{scm}$ cells (<20%).
- This may also be partially explained by lentivirus preferentially transducing non-naïve T cell subsets over naïve T cells.