Non-Viral Engineered Human CAR-T Cells for Safe and Specific Stem Cell Transplant Conditioning

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Abstract

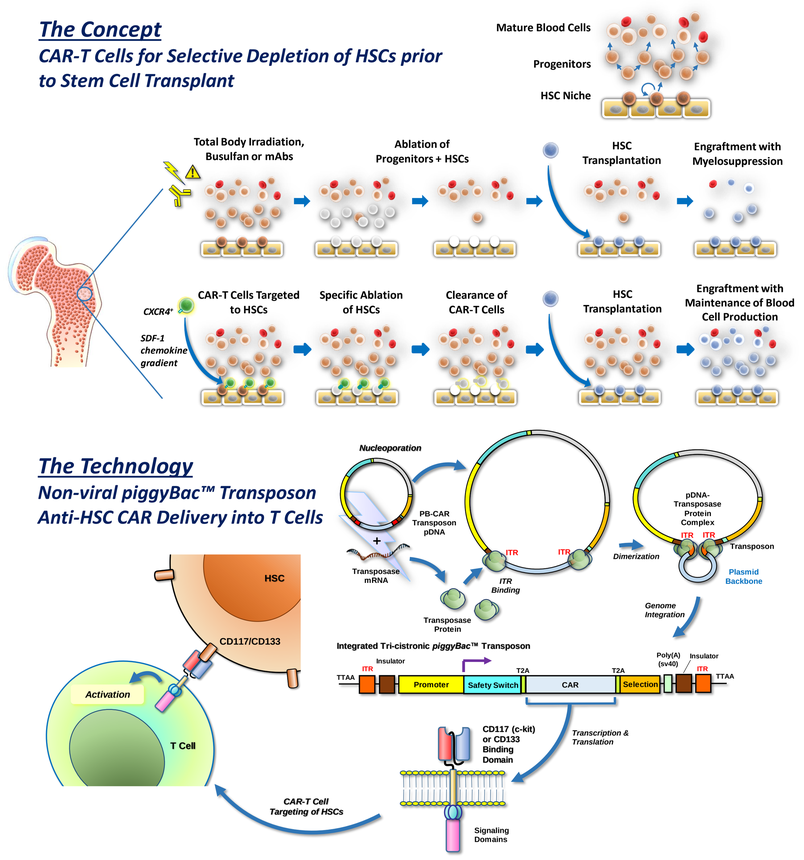
Each year in the U.S. over 5,000 patients with hematological disorders are treated with myeloablative conditioning therapy prior to reconstitution by transplanted hematopoietic stem cells (HSCs). These conditioning regimens typically consist of high doses of genotoxic radiation or busulfan, which can lead to life-threatening post-transplant complications. This has prompted the investigation of more targeted and less hazardous approaches to specifically deplete endogenous hematopoietic cells in the bone marrow (BM). To date, antibodies broadly directed against HSC antigens, such as c-kit and CD45, have been considered for transplant conditioning. However, this approach is limited by the slow rate of antibody clearance, which delays time to transplant, and wide biodistribution, which limits efficacy and increases the possibility of toxicity towards non-HSC antigen-bearing cells. Alternatively, short-lived, chimeric antigen receptor (CAR)-T cells with bone marrow-homing capability may provide more effective, selective, and safer depletion of resident HSCs. Among the CAR-T production protocols currently in development, delivery of the CAR transgene via the non-viral *piggyBac*™ (PB) transposon method has several advantages: (1) lower manufacturing costs, (2) larger cargo capacity that allows introduction of multiple genes, and (3) a predominantly stem cell memory (T_{SCM}) phenotype for bone marrow homing ability and enhanced *in vivo* potency. Here, we constructed PB vectors encoding CARs targeting either human c-kit (CD117) or prominin-1 (CD133), markers known to be antigenically expressed on HSCs. In addition to the CAR, the transposon also encodes a selection gene, for generation of entirely pure product, and an inducible safety switch that will allow rapid clearance of the reactive CAR-T cell product prior to donor HSC transplant. A panel of CAR candidates were first evaluated in vitro for their ability to specifically deplete human myeloid leukemia cells (TF-1a) expressing c-kit and CD133. Two-day co-culture of select anti-c-kit or anti-CD133 CAR-T cells with primary human or Rhesus macaque BM cells resulted in greater than 80% depletion of functional hematopoietic subsets as determined by assays quantifying the frequency of colony forming cells (CFUs) and the more primitive cobblestone area forming cells (CAFCs). The PB CAR-T manufacturing process yielded CAR-T cells with more than 60% T_{SCM} content, as determined by CD62L, CCR7, and CD45RA expression. The CAR-T cells also expressed high levels of CXCR4, a key chemokine receptor involved in selective BM trafficking. Studies evaluating the in vivo efficacy of lead anti-HSC CAR-T candidates are underway in immunodeficient NSG mice engrafted with either a human acute myeloid leukemia cell line or primary human CD34+ hematopoietic stem and progenitor cells. Our findings provide proof-of-concept that controllable and non-genotoxic CAR-T cells directed against c-kit- or CD133-bearing hematopoietic cells can be used to safely and specifically eliminate HSCs in order to allow engraftment of allogeneic or gene-corrected stem cells. This may ultimately lead to safer conditioning protocols that greatly increase the number of patients eligible for transplant and significantly reduce the damaging side-effects associated with current radiation or chemotherapy treatment.

Introduction

- Most myeloablative conditioning regimens prior to HSC transplant consist of high doses of genotoxic radiation or busulfan. The use of stem cell transplants is limited by the major complications associated with these regimens.
- Antibodies directed against antigens expressed on HSCs, such as c-kit and CD45, have been considered as alternatives.
- CAR-T cells may provide more effective, selective, and safer depletion of HSCs residing in the bone marrow.
- *PiggyBac*™ (PB) is a non-viral delivery system with a large cargo capacity that allows introduction of multiple genes, including a selection marker and a safety switch that can eliminate CAR-T cells prior to donor HSC transplant.
- PB-produced CAR-T cells exhibit a stem cell memory (T_{SCM}) phenotype for enhanced *in vivo* potency, and may better home to bone marrow than other HSC-directed pre-conditioning alternatives.

The Concept

CAR-T Cells for Selective Depletion of HSCs prior to Stem Cell Transplant



The Technology

Non-viral piggyBac™ Transposon Anti-HSC CAR Delivery into T Cells

Methods

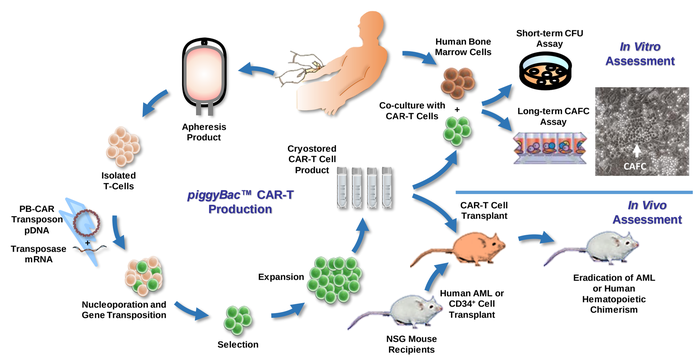


Figure 1: PB vector delivery and CAR-T manufacturing process. Pan T cells are isolated from an apheresis product and then electroporated with anti-c-kit or anti-CD133 CAR *piggyBac*™ transposon plasmid DNA and *in vitro* transcribed *piggyBac*™ transposase mRNA. The electroporated cells are then activated, selected and expanded prior to freezing. The process yields >1 x 10^9 cells with >95% CAR expression. Reactivity against hematopoietic cells was first tested *in vitro* by 2-day co-culture of the CAR-T cells with human stem and progenitor cells followed by evaluation of short-term CFUs or long-term CAFCs. Selected anti-c-kit or anti-CD133 CARs were assessed *in vivo* in NSG mice with pre-established engraftment of CD34+ cells or the $CD133^+$ TF-1a AML cell line.

Results

Phenotype of PB CAR-T Cells

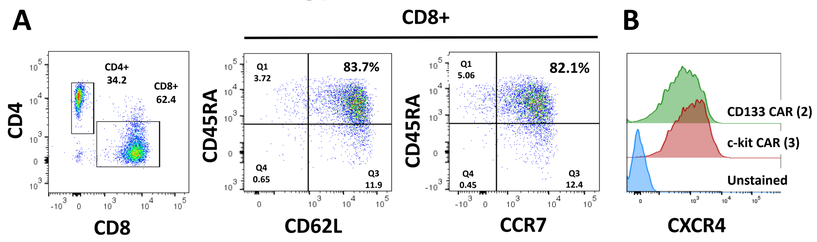


Figure 2: PB CAR-T exhibit a stem cell memory phenotype and endogenously express CXCR4. PB CAR-T cells directed against c-kit and CD133 antigens were evaluated by flow cytometry for typical T cell markers following the manufacturing process. (A) Expression of CD4, CD8 and memory markers demonstrating the stem cell memory phenotype of PB CAR-T cells; (B) PB CAR-T cells express CXCR4, a marker commonly associated with bone marrow homing.

In Vitro Activity of CAR-T Cells Against an AML Cell Line

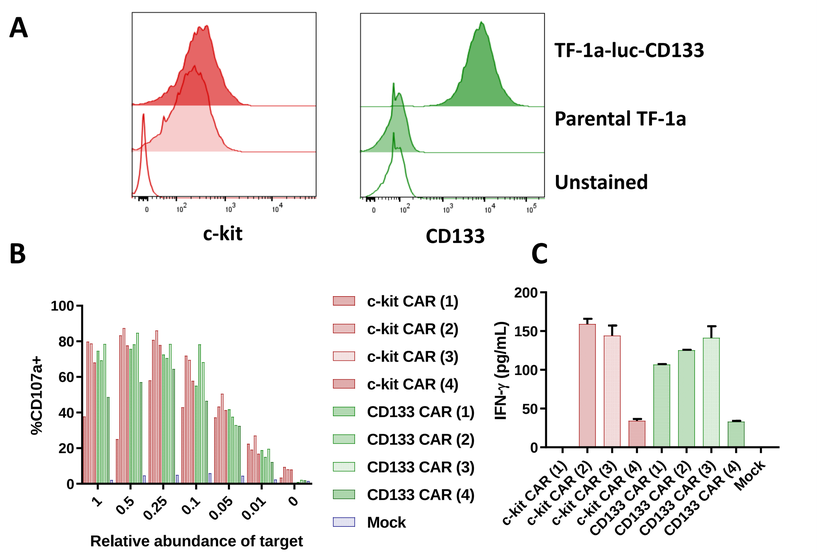


Figure 3: CAR-T cells degranulate and secrete IFN-γ in response to target expressing TF-1a-luc-CD133 cells. CAR-T cells directed against c-kit and CD133 antigens were evaluated for specific activity against target expressing cells *in vitro*. (A) The TF-1a AML cell line endogenously expresses c-kit but not CD133. PB was used to introduce an expression cassette containing CD133 and CBG99 luciferase, creating TF-1a-luc-CD133 cells, which express both c-kit and CD133. (B) TF-1a-luc-CD133 cells were spiked into non-target expressing cells (Raji) at varying amounts and incubated with CAR-T cells. After 4 hours of co-culture the fraction of CD107a expressing CAR-T cells was quantified by flow cytometry. (C) After 48 hours co-culture of CAR-T cells with TF-1a-luc-CD133 cells at an effector: target ratio of 2.5:1, the level of IFN-γ in the supernatant was quantified by ELISA.

Depletion of Hematopoietic Progenitors by CAR-T

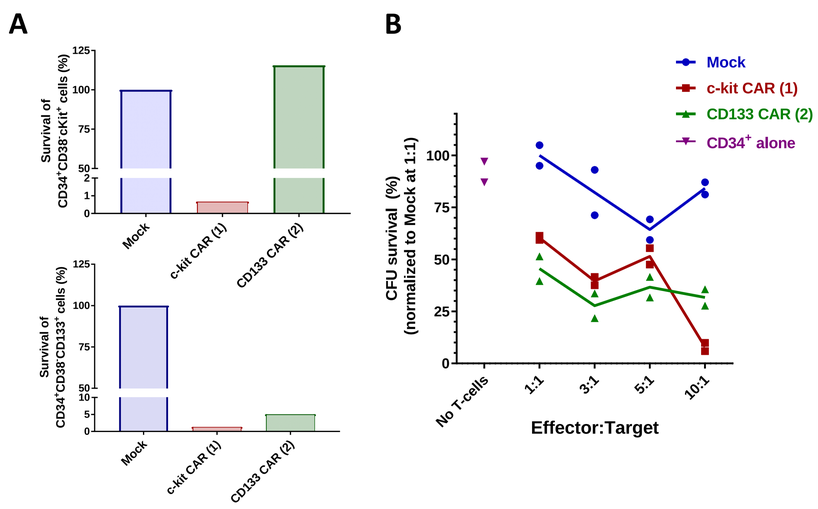


Figure 3: Anti-c-kit and anti-CD133 CAR-T cells effectively target the $CD34^+CD38^-$ progenitor population and colony forming cells in peripheral blood $CD34^+$ cells. $CD34^+$ cells isolated from human mobilized peripheral blood were incubated with anti-c-kit or anti-CD133 CAR-T cells for 48h followed by FACS phenotyping of remaining cells and CFU survival assay. (A) The anti-c-kit CAR-T depleted >95% of c-kit^+ and $CD133^+$ cells from the primitive $CD34^+CD38^-$ population, while the anti-CD133 CAR-T depleted >90% of $CD133^+$ cells from this population. (B) Both the anti-c-kit and anti-CD133 CAR-T cells reduced colony formation at all effector:target ratios tested.

Depletion of Primitive Hematopoietic Cells in Long-Term Cultures by both Anti-c-kit and Anti-CD133 CAR-T

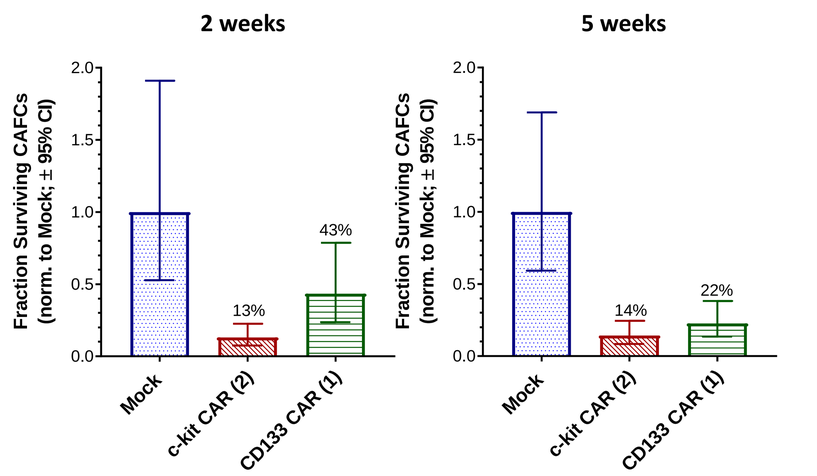
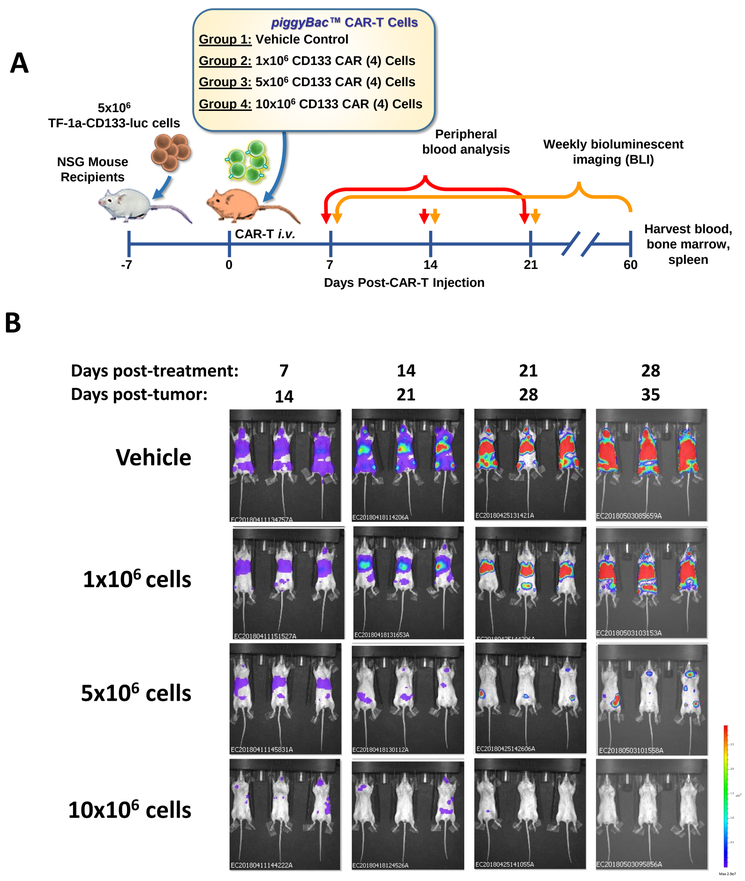


Figure 4: Activity of anti-c-kit and anti-CD133 CAR-T cells against long-term cobblestone area forming cells (CAFCs): Following co-culture of the CAR-T cells with human mobilized peripheral blood $CD34^+$ cells for 48h (effector:target cell ratio of 3:1), serial dilutions were plated on MS-5 stromal cells for the generation of CAFCs over 2 months. At 5 weeks post-plating, both CARs significantly reduced the absolute number of CAFCs, suggesting these CAR-T cells successfully target very primitive cells.

Reactivity against a Human AML Cell Line *In Vivo*



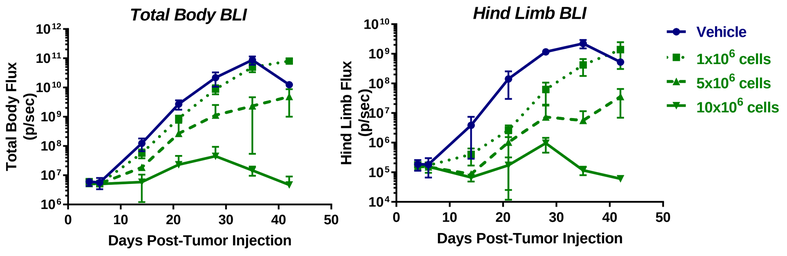


Figure 5: Anti-CD133 CAR-T cells target and kill AML cells *in vivo*. (A) NSG mice were inoculated with 5e6 TF-1a-luc-CD133 cells followed by *i.v.* delivery of 1e6, 5e6, or 10e6 anti-CD133 CAR-T cells 7 days post-tumor injection. (B and C) The 10e6 dose completely eliminated tumor in all animals. The 5e6 dose slowed tumor growth across all tissues, while the lowest dose specifically slowed tumor growth in the bone marrow. This study is ongoing.

Reactivity against Human Stem Cell Grafts *In Vivo*

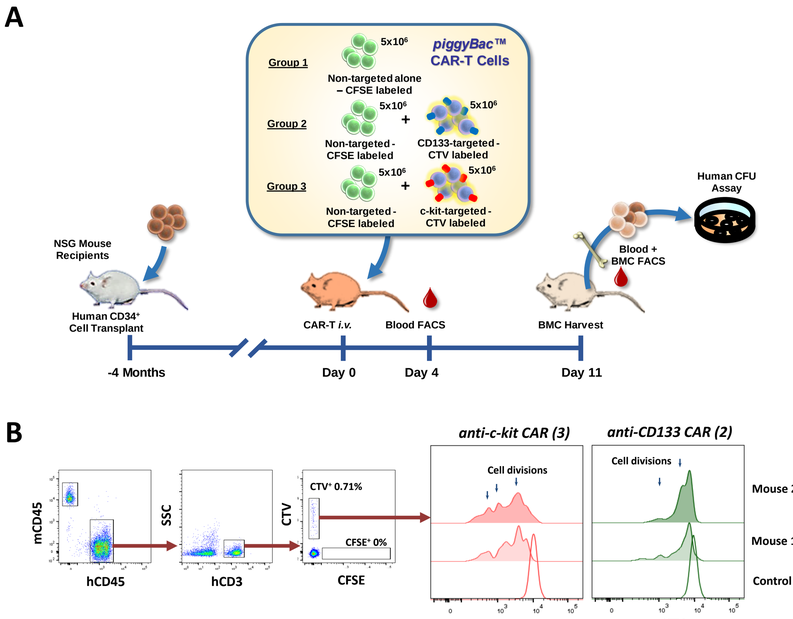


Figure 6: Anti-c-kit and anti-CD133 CAR-T cells proliferate in response to antigens on transplanted human cord blood-derived CD34+ cells *in vivo*. (A) Anti-c-kit or anti-CD133 CAR-T cells were labelled with CTV, mixed with CFSE-labelled non-target-directed CAR-T cells (internal control for partial HLA matched donor CD34+ and CAR-T cells), and injected into humanized NSG mice. (B) Four days post-injection, CTV-labelled cells were detected in the peripheral blood of all four mice. No CFSE-labelled cells were detected. Loss of CTV intensity compared to control labelled cells, which were incubated in the absence of any target cells, indicates proliferation of the CAR-T cells *in vivo*. This study is on-going.

Conclusions

- **PB CAR-T cells exhibit a stem cell memory (T_{SCM}) phenotype and robust *in vivo* activity. T_{SCM} cells naturally express high levels of CXCR4, which may be important for homing of the CAR-T cells to bone marrow**
- **PB CAR-T cells targeted against c-kit or CD133 efficiently and selectively deplete primitive human hematopoietic cells *in vitro***
- **Anti-CD133 PB CAR-T cells can completely eliminate CD133-bearing AML cells in a xenogeneic mouse model**
- **Anti-c-kit and anti-CD133 PB CAR-T cells proliferate *in vivo* in response to antigens presented by engrafted human cells in NSG mice**
- **These data support the use of PB CAR-T cells to target endogenous HSCs in the bone marrow as a minimal, non-genotoxic HSC transplant regimen**