CD19-specific chimeric antigen receptor-modified T cells with safety switch produced under "Point-of-Care" using the *Sleeping Beauty* system for the very rapid manufacture and treatment of B-cell malignancies

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Abstract

Chimeric antigen receptor (CAR)-modified autologous T cells have shown remarkable clinical responses targeting B-cell malignancies. However, a majority of manufacturing approaches rely on expensive viral vectors and *ex vivo* activation of clinical-grade T cells for gene transfer with subsequent 2-3 week-long numeric expansion to achieve necessary dosing. We have engineered our clinically-validated *Sleeping Beauty* (SB) system for stable non-viral integration of CAR, cytokine signaling, and safety switch transgenes into T cells to eliminate need for viral vectors. We previously demonstrated that co-expression of membrane bound IL-15 (mbIL15, Proc Natl Acad Sci U S A. 2016 Nov 29;113(48):E7788-E7797) on CD19-specific CAR$^+$ T cells using SB system allows for very rapid (<2 days) manufacturing with point-of-care (P-O-C) technology (Blood 2016 128:2807). This approach to production avoids the need for activation and propagation of CAR$^+$ T cells leading to augmented persistence and anti-tumor activity in an *in vivo* model of leukemia. We now update this technology by co-expressing a safety switch, HER1t, on CD19-specific CAR$^+$ mbIL15$^+$ T cells. HER1t improves the safety profile by providing a mechanism for selective *in vivo* depletion of P-O-C-produced T cells through the administration of cetuximab, a clinically-available monoclonal antibody. We confirmed co-expression of CAR, mbIL15, and HER1t in P-O-C-manufactured T cells and demonstrated that these cells exhibited a memory-like phenotype, potent antigen-specific cytotoxicity, and produced IFNγ, TNFα, and IL-2 when co-cultured with CD19$^+$ tumor cells. Cetuximab specifically and efficiently killed mbIL15$^+$ HER1t$^+$CAR$^+$ T cells via antibody dependent cellular cytotoxicity and complement dependent cytotoxicity in a dose-dependent manner. Adoptive transfer of mbIL15$^+$HER1t$^+$CAR$^+$ T cell generated in less than 2 days under P-O-C into immunocompromised (NSG) mice bearing established CD19$^+$ leukemia (NALM-6) resulted in significant reduction in tumor burden and improvement in overall survival, compared to control mice. Blood analysis demonstrated improved expansion and persistence of mbIL15$^+$HER1t$^+$CAR$^+$ T cells compared to mbIL15^{neg}HER1t^{neg}CAR$^+$ T-cell control. In summary, we have generated T cells co-expressing mbIL15 and the HER1t safety switch in less than two days using P-O-C that demonstrated superior efficacy and persistence compared to P-O-C T cells that expressed CAR without mbIL15. These pre-clinical data demonstrate the effectiveness of very rapidly manufactured mbIL15$^+$HER1t$^+$CAR$^+$ T cells in targeting CD19$^+$ tumors and provide a rationale for clinical evaluation.

"Point-of-Care" (P-O-C) Manufacturing

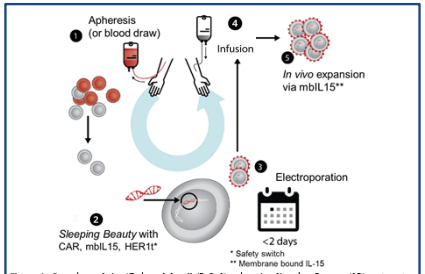


Figure 1: Overview of the "Point-of-Care" (P-O-C) using the *Sleeping Beauty* (SB) system to very rapidly generate CAR$^+$ T cells. Very rapid manufacturing process removes need for *ex vivo* culture of T cells prior to in fusion. Co-expression of mbIL15 enhances *in vivo* persistence and anti-tumor response.

Co-expression of CD19 CAR, mbIL15 And Safety Switch In T Cells

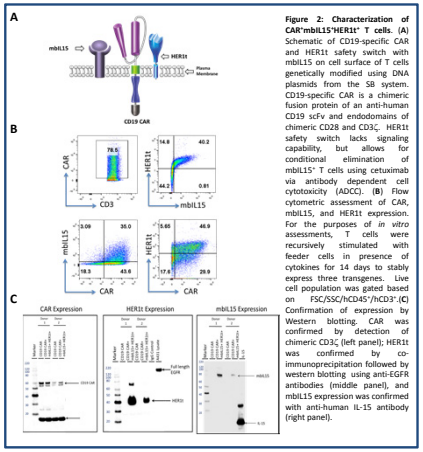
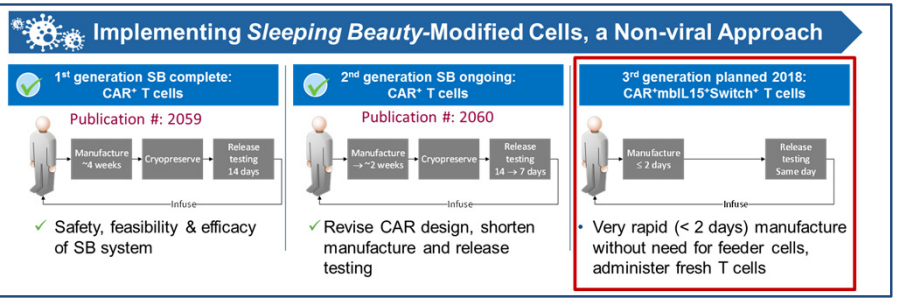


Figure 2: Characterization of CAR$^+$mbIL15$^+$HER1t$^+$ T cells. (**A**) Schematic of CD19-specific CAR and HER1t safety switch with mbIL15 on cell surface of T cells genetically modified using DNA plasmids from the SB system. CD19-specific CAR is a chimeric fusion protein of an anti-human CD19 scFv and endodomains of chimeric CD28 and CD3ζ. HER1t safety switch lacks signaling capability, but allows for conditional elimination of mbIL15$^+$ T cells using cetuximab via antibody dependent cell cytotoxicity (ADCC). (**B**) Flow cytometric assessment of CAR, mbIL15, and HER1t expression. For the purposes of *in vitro* assessments, T cells were recursively stimulated with feeder cells in presence of cytokines for 14 days to stably express three transgenes. Live cell population was gated based on FSC/SSC/hCD45$^+$/hCD3$^+$.(**C**) Confirmation of expression by Western blotting. CAR was confirmed by detection of chimeric CD3ζ (left panel); HER1t was confirmed by co-immunoprecipitation followed by western blotting using anti-EGFR antibodies (middle panel), and mbIL15 expression was confirmed with anti-human IL-15 antibody (right panel).

Implementing *Sleeping Beauty*-Modified Cells, a Non-viral Approach



Antigen Specific Cytotoxicity And Cytokine Production By CD19 CAR$^+$ T Cells

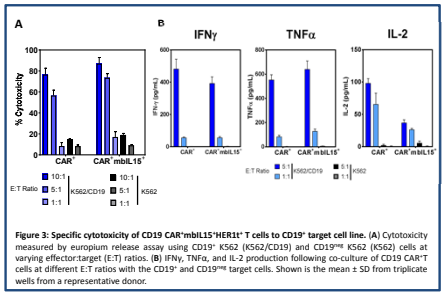


Figure 3: Specific cytotoxicity of CD19 CAR$^+$mbIL15$^+$HER1t$^+$ T cells to CD19$^+$ target cell line. (**A**) Cytotoxicity measured by europium release assay using CD19$^+$ K562 (K562/CD19) and CD19^{neg} K562 (K562) cells at varying effector:target (E:T) ratios. (**B**) IFNγ, TNFα, and IL-2 production following co-culture of CD19 CAR$^+$T cells at different E:T ratios with the CD19$^+$ and CD19^{neg} target cells. Shown is the mean ± SD from triplicate wells from a representative donor.

Conditional Elimination of HER1t Expressing CD19 CAR$^+$ T Cells

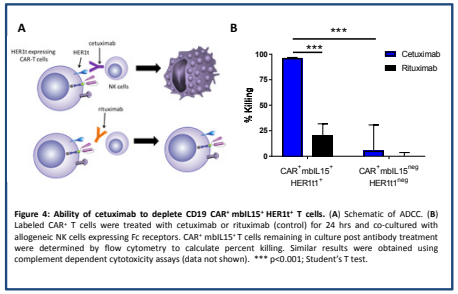


Figure 4: Ability of cetuximab to deplete CD19 CAR$^+$ mbIL15$^+$ HER1t$^+$ T cells. (**A**) Schematic of ADCC. (**B**) Labeled CAR$^+$ T cells were treated with cetuximab or rituximab (control) for 24 hrs and co-cultured with allogeneic NK cells expressing Fc receptors. CAR$^+$ mbIL15$^+$T cells remaining in culture post antibody treatment were determined by flow cytometry to calculate percent killing. Similar results were obtained using complement dependent cytotoxicity assays (data not shown). *** p<0.001; Student's T test.

"Point-of-Care" CD19 CAR$^+$ T Cells Eliminate Leukemia And Improve Survival In Mice

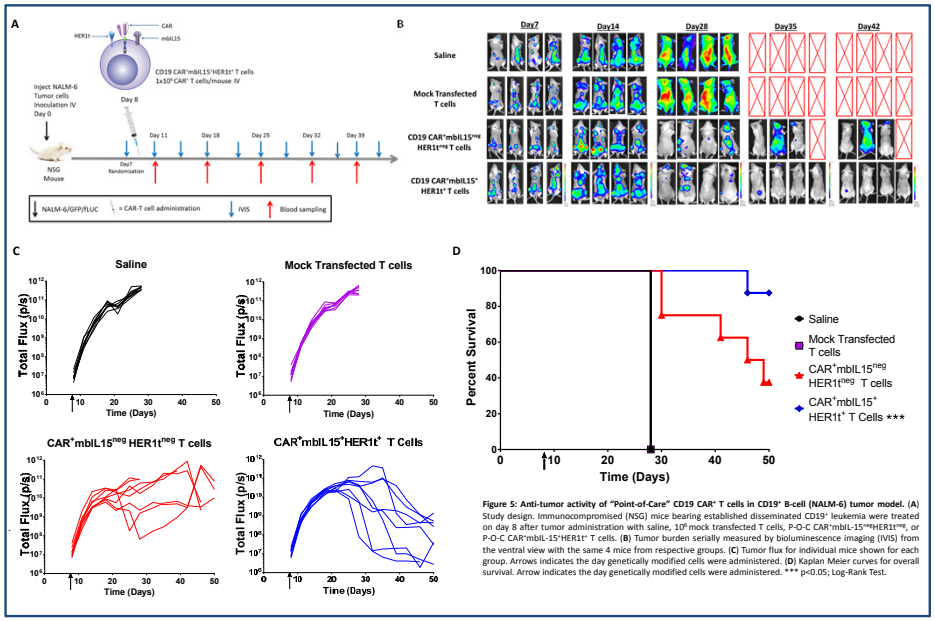


Figure 5: Anti-tumor activity of "Point-of-Care" CD19 CAR$^+$ T cells in CD19$^+$ B-cell (NALM-6) tumor model. (**A**) Study design. Immunocompromised (NSG) mice bearing established disseminated CD19$^+$ leukemia were treated on day 8 after tumor administration with saline, 10^6 mock transfected T cells, P-O-C CAR$^+$mbIL-15^{neg}HER1t^{neg}, or P-O-C CAR$^+$mbIL-15$^+$HER1t$^+$ T cells. (**B**) Tumor burden serially measured by bioluminescence imaging (IVIS) from the ventral view with the same 4 mice from respective groups. (**C**) Tumor flux for individual mice shown for each group. Arrows indicates the day genetically modified cells were administered. (**D**) Kaplan Meier curves for overall survival. Arrow indicates the day genetically modified cells were administered. *** p<0.05; Log-Rank Test.

P-O-C CD19 CAR$^+$ T Cells Expand *In Vivo* And Persist At High Frequencies

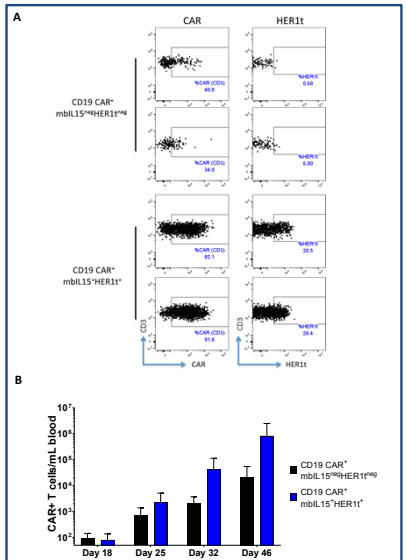


Figure 6: *In vivo* expansion and persistence of "Point-of-Care" CD19 CAR$^+$ T cells in tumor bearing mice. (**A**) Representative flow cytometric data shown from whole blood evaluating expression of CAR and HER1t on day 32. Cell populations are gated based upon FSC/SSC/hCD45$^+$/CD3$^+$. (**B**) Quantification of the CAR$^+$ T cells detected in whole blood. Shown is the mean ± SEM. n = 4 to 11mice/group.

Summary

- T cells expressing CD19-specific CAR, mbIL15 and safety switch were generated via very rapid manufacturing process ("Point-of-Care") using the *Sleeping Beauty* system.
- "Point-of-Care" CAR$^+$ T cells reduces time to treatment by eliminating the need for *ex vivo* (i) activation and (ii) propagation of T cells.
- Safety switch provides a mechanism for conditional elimination post infusion.
- Co-expression of mbIL15 enhances *in vivo* expansion and persistence of "Point-of-Care" CAR$^+$ T cells and improves anti-tumor response.
- "Point-of-Care" CD19 CAR$^+$ T cells eliminated CD19$^+$ tumor and improved survival in mice.
- These data support clinical evaluation of CAR$^+$ T cells co-expressing mbIL15 and safety switch rapidly manufactured using the *Sleeping Beauty* system.

Conflict of Interest Disclosures:
Chan: *Intrexon Corporation*: Employment. **Gallagher:** *Intrexon Corporation*: Employment. **Cheng:** *Intrexon Corporation*: Employment. **Carvajal-Borda:** *Intrexon Corporation*: Employment. **Plummer:** *Intrexon Corporation*: Employment. **Govekung:** *Intrexon Corporation*: Employment. **Barrett:** *ZIOPHARM Oncology*: Employment. **Khare:** *Ziopharm Oncology*: Employment. **Cooper:** *Mitteny Biotec*: Honoraria; *Ampliphi Biosciences*: Equity Ownership; *Procter & Gamble*: Equity Ownership; *Organovo Holdings*: Equity Ownership; *Targazyme, Inc.*: Equity Ownership; *Argos Therapeutics*: Equity Ownership; *Intrexon Corporation*: Patents, Equity Ownership; *Immatics*: Equity Ownership, Patents & Royalties; *Sangamo Biosciences*: Patents & Royalties; *ZIOPHARM Oncology*: Employment, Equity Ownership, Research Funding; **Shah:** *Intrexon Corporation*: Employment.