Production of chimeric antigen receptor (CAR)-T cells for preclinical testing using non-viral transposon vectors and a lymphoblastoid cell line (LCL)

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ABSTRACT

Introduction: CAR T cell immunotherapy for treatment of cancer is showing promising results in clinical trials targeting the B cell restricted CD19 antigen. Patients in clinical trials harboring B cell malignancies experienced overall response rates of 73%, with pediatric B cell acute lymphoblastic leukemia (B-ALL) patients showing the highest response rates (93%). However, there is no standard protocol for the generation of CAR-modified T cells, with different genetic modification vectors and expansion protocols being used. Viral vectors insert the transgene in the genome of the cell, providing long term CAR expression. However, their use is associated with high production costs and cumbersome quality controls, impacting the final cost of CAR-T cell therapies. Sleeping Beauty (SB) transposon system consist of plasmid-based integrative vectors that, through a cut-and-paste mechanism catalyzed by a transposase, recognize inverted terminal repeats flanking the transgene and insert it in the target cell genome. We show here that this system, combined with LCL-based T cell expansion can be used to efficiently transfect primary T lymphocytes and induce long term CAR expression throughout T cell expansion.

Objectives: To develop a protocol to expand SB-transfected CAR T cells using a LCL.

Methods: Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll and transfected using Nucleofector II electroporator. The plasmids encoding the anti-CD19 CAR and transposase (SB100X) used in the transfection were mixed based on a pre-defined optimized proportion. After transfection the cells were stimulated with the L388 LCL, a mature B cell line that expresses the target antigen (CD19) and costimulatory receptors. CAR T cells were re-stimulated up to 3 times with L388 and T cell phenotype (CAR expression, memory markers) and effector function were evaluated at the end of each cycle.

Results: Electroporation of PBMCs with transposon plasmid decreased viability and altered the frequency of memory subpopulations when compared to the mock (electroporated without plasmid) condition. However, CAR expression rescued the electroporated lymphocytes and these cells showed increased proliferation compared to mock control (28-fold vs 15-fold expansion; 19BBz vs mock). Moreover, CAR+ lymphocytes showed an increased frequency by the end of the stimulation cycle compared to d1 post electroporation, suggesting that CAR signaling enhances the activation and proliferation of these cells. NK cell depletion prior to L388 stimulation altered the composition of memory subpopulations, favoring the expansion of CD8+ CD62L- CCR7+ cells.

Discussion: The results showed that electroporation using the SB system is a simple and cost-effective method for inducing long-term CAR expression in T lymphocytes. Expansion of gene modified T cells was possible by using the L388, providing up to 3 cycles of stimulations and reaching the required cell number for preclinical testing. Experiments using B cell leukemia models in immunodeficient mice are underway to evaluate CAR T cell function *in vivo*.

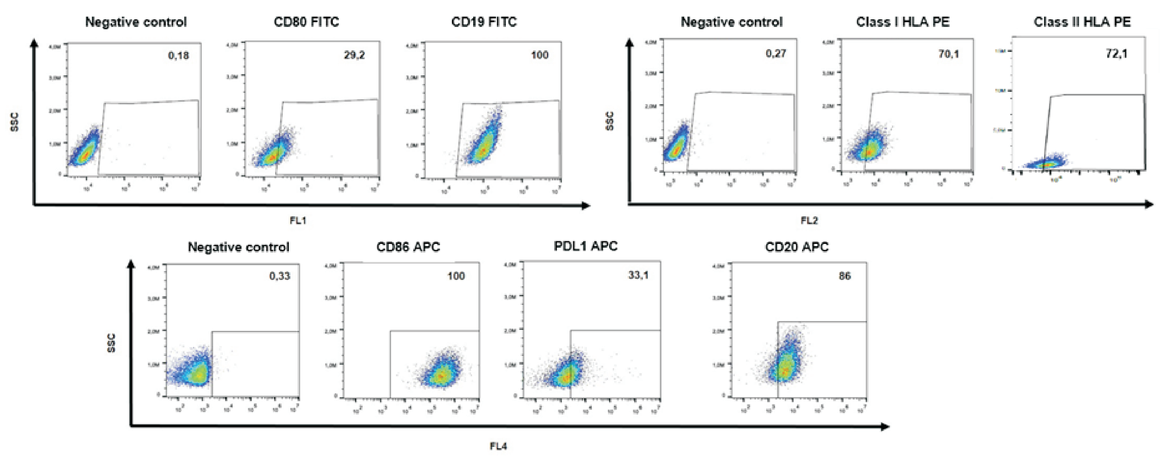

Figure 1: Phenotype of LAZ 388 (L388) cells used for the T cell expansion protocol. This cell line expresses high levels of HLA and costimulatory (CD86, CD80) molecules. Moreover, it has high levels of CD19, the target molecule for 19BBz CAR. Numbers in plots represents the percentage of gated (positive) cells.

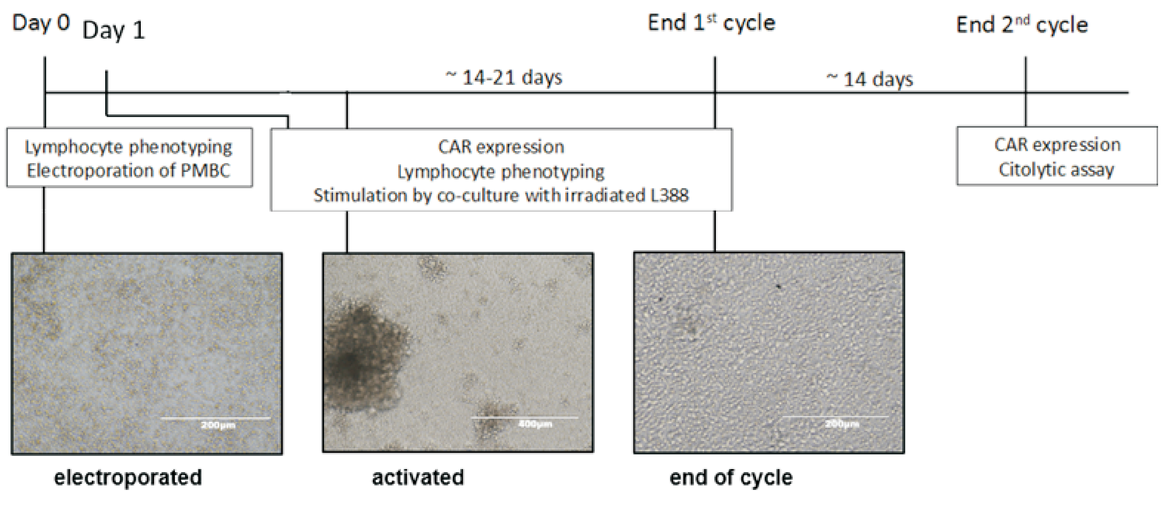

Figure 2: Schematic view of CAR+ T cells expansion protocol. At day 0, PBMCs obtained from healthy blood donors were immunophenotyped by flow cytometry and electroporated with plasmids encoding CAR and SB100X transposase. , Cells from the same donor maintained in culture or subjected to electroporation in the absence of DNA (mock) were used as experimental controls. At the following day, CAR expression was assessed by flow cytometry and irradiated LAZ 388 (L388) cells were added to the culture at 5:1 ratio (PBMC:L388). Bars represent 400 m at the middle picture and 200 m at the right and left panels.

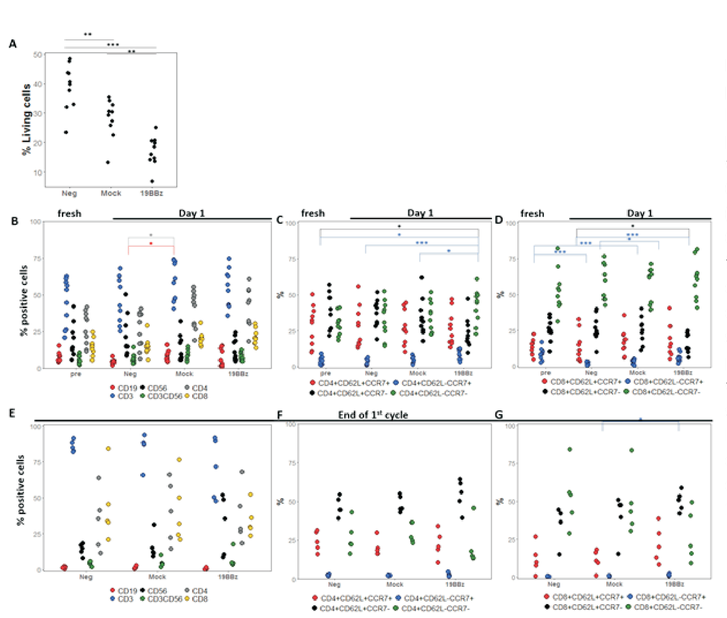

Figure 3: The effect of electroporation and DNA plasmid transfection on human primary cells. A) Evaluation of cell viability after electroporation in the presence or absence of plasmid. At day 1 in culture cells were evaluated by flow cytometry for **(B)** B, T, CD4, CD8, NK, NKT cell contribution to PBMC composition **(C)** CD4+CD62L+CCR7- cell proportions and **(D)** CD8+CD62L+CCR7- cell populations. At the end of the first cycle of expansion, cells were evaluated by flow cytometry to determine **(E)** B, T, CD4, CD8, NK, NKT cells in culture **(F)** CD4+CD2L+/-CCR7+/- subpopulations or **(G)** CD8+CD62L-CCR7+ cells populations. * p-value < 0.05. ** p-value < 0.01. *** p-value < 0.001. Star colors represent the population statistically significant difference. pre: fresh human PBMC. Neg: cultured not-electroporated cells. Mock: cultured electroporated cells without plasmid. 19BBζ: cultured electroporated cells that received 20µg of 19BBζ CAR and 1µg of SB100X plasmids.

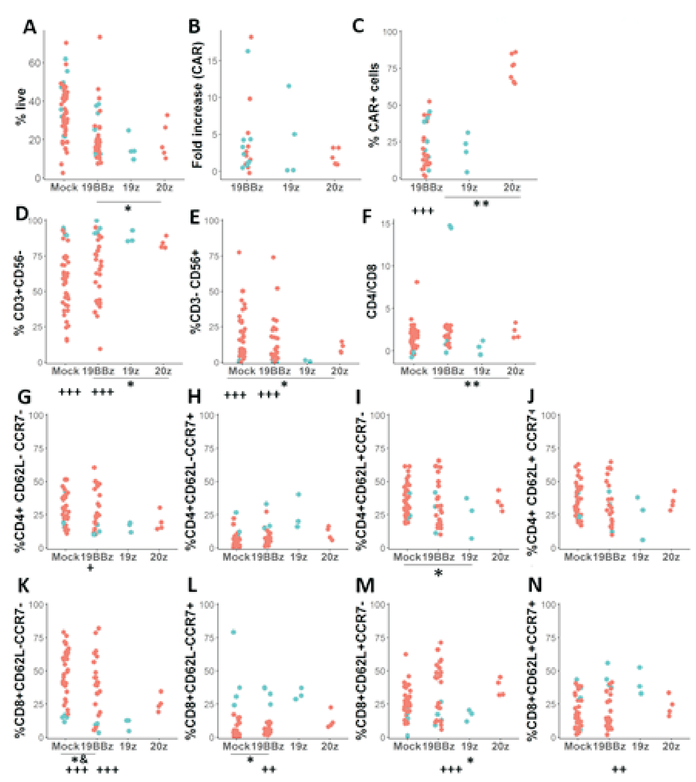

Figure 4: The impact of different CARs and NK cell depletion on lymphocyte populations after the first cycle of expansion. A) The percentages of viable cells in Mock, 19BBζ, 19ζ and 20ζ conditions at day 1. **B)** The percentage of CAR+ in 19BBζ, 19ζ and 20ζ NK depleted conditions at the end of the first cycle. **C)** Fold increase in the number of CAR+ cells for the three CARs expression along the culture upon activation by L388 cells. **D)** Percentage of CD3+CD56- cells after the first cycle of expansion. **E)** Percentage of CD3-CD56+ cells after the first cycle of expansion. **F)** CD4/CD8 cell ratio in 19BBζ, 19ζ and 20ζ CAR expression conditions **G)** CD4+D62L-CCR7-, **H)** CD4+CD62L-CCR7+, **I)** CD4+CD62L+CCR7-, **J)** CD4+CD62L+CCR7+, **K)** CD8+CD62L-CCR7-, **L)** CD8+CD62L-CCR7+, **M)** CD8+CD62L-CCR7- and **N)** CD8+CD62L-CCR7- cell population proportion in cultures with (blue dots) or without (red dots) NK depletion and 19BBζ, 19ζ and 20ζ CARs presence. + marks represent statistical difference between NK depleted or not depleted cultures in the same condition (Mock or CAR expressing cultures). * represents significant statistical difference (p<0.05), ** p<0.01, *** p<0.001. All * corresponds to the comparison between CAR condition groups. Comparison was made considering NK depletion *per group*. For this analysis was applied one-way ANOVA statistical test with Tukey post-test. + p<0.05, ++ p<0.01, +++ p<0.001. All "+" corresponds to Student's t-test comparing NK depletion or not within the same CAR group.

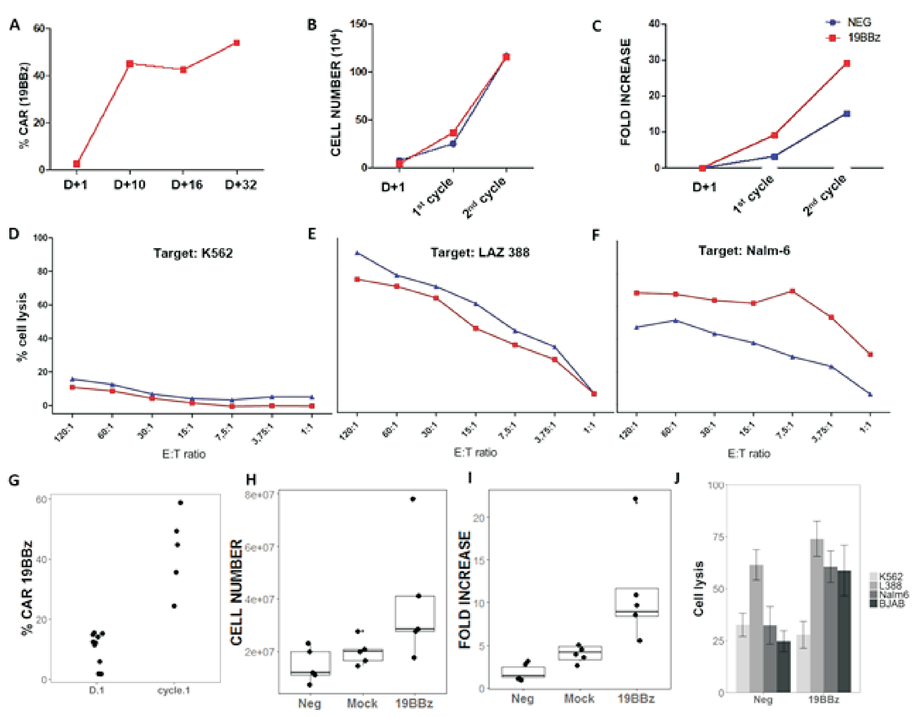

Figure 5: 19BBζ CAR+ cells proliferate and kill target cells *in vitro*. A-C) Percentage (A), absolute cell numbers (B) and fold increase compared to d+1 (C) of CAR+ T cells during cell expansion after stimulation with L388. **D-F)** Target cell lysis measured by chrome release assay. **D)** K562, **E)** L388, and **F)** Nalm-6 cell lines were used as target. This result is representative of five independent donors. **G)** Percentage of 19BBζ CAR+ cells at day 1 and at the end of the first cycle for 5 donors. **H)** Number of cells of five donors at the end of the first cycle of expansion. **I)** Fold expansion of the cells put in culture at day 0 for five donors. Number of input viable cells was used to calculate expansion rates. **J)** Cell lysis measured by chrome release assay from 5 different donors with K562, L388, Nalm-6 and BJAB cell lines as targets at 3:1 (E:T) ratio.

Projeto Gráfico: Setor de Edição e Informação Técnico-Científica / INCA

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