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Generation and characterization of anti-CD19 CAR-T cells overexpressing the protein PHF19

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INTRODUCTION

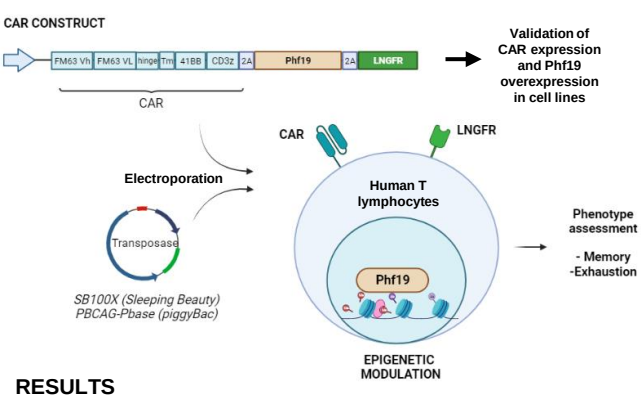
- CAR-T cells are T lymphocytes genetically modified to express a synthetic receptor that can recognize membrane antigens in tumor cells or other cell types. CAR-T cell therapy has had enormous success in the last decade agains B-cell malignancies through targeting of the common B cell antigen CD19. Nonetheless, there are some major challenges still to be addressed in the development of CAR-T cells, such as the high cost of production and the low long-term persistence of T cells *in vivo*, often linked to the acquisition of an exhausted phenotype.

- Transposon systems for gene delivery like Sleeping Beauty (SB) and piggyBac (PB) can greatly reduce time and cost of production compared to traditional viral vectors, as they are easier to propagate and apply in standard biosafety level 2 infrastructure. In addition, the epigenetic modulation of T cells is a promising approach for modulating memory and exhaustion phenotype: recent work showed that Phf19, an accessory protein of the Polycomb Repressor Complex 2 (PRC2) can be harnessed to modulate T cell phenotype by downregulating exhaustion-associated transcription factors and avoiding terminal differentiation

OBJECTIVE

This work aimed to address CAR-T cell phenotype modulation trough overexpressing the protein Phf19 on CAR-T cells generated with transposon systems Sleeping Beauty and piggyBac.

METHODS



RESULTS

1. Validation of Phf19 overexpression on 293T cells

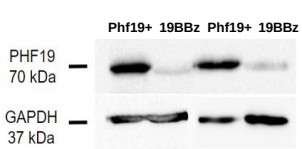


Figure 1. Western blotting analysis of Phf19 expression. HEK293T cells were transfected with control CAR 19BBz or 19BBz-Phf19+. Cells were sorted and lysed for protein extraction. Bands show the same pair of samples in duplicate.

2. Sleeping beauty system fails to provide stable CAR-Phf19+ transgene expression in human peripheral mononuclear cells (PBMCs)

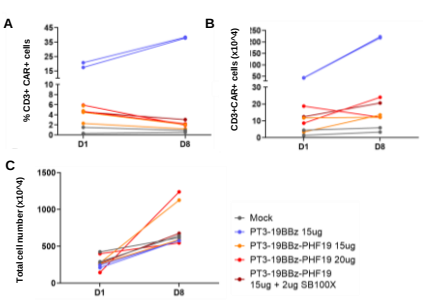


Figure 2. CAR-T generation with the Sleeping Beauty system in primary human cells.

Human PBMCs from healthy donors were isolated and transfected with transposons carrying the control CAR 19BBz or 19BBz-Phf19+. Cells were then activated with anti-CD3/CD28 beads and cultivated in complete media with IL-2.

19BBz-Phf19+ CAR was tested in 3 different conditions - 15ug, 20ug or 15ug + 2ug of the transposase plasmid (SB100X). CAR expression **(A)**, CAR+ cell number **(B)** and total cell number **(C)** were assessed at day 1 (D1) and after 8 days of expansion (D8).
N = 2 independent donors

3. Piggybac system is more efficient in generating stable expression of the transgene in human PBMCs

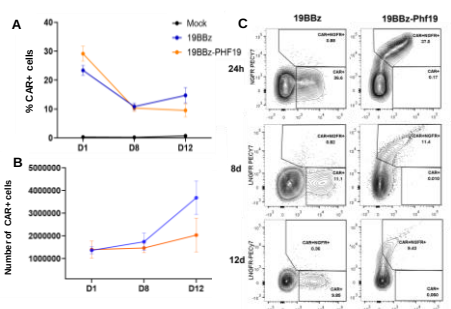


Figure 3. CAR-T generation with the piggyBac (PB) system in primary human cells.

Human PBMCs from healthy donors were isolated and transfected with piggyBac transposons carrying the control CAR 19BBz or 19BBz-Phf19+, along with the transposase plasmid (20ug). Cells were then activated with anti-CD3/CD28 beads and cultivated in complete media with IL-2 for 12 days.

A) CAR+ cells percentage and total CAR+ cell number **(B)** were assessed at days 1, 8 and 12. **C)** Representative cytometry plots of CAR and LNGFR reporter staining.

4. Phf19 overexpressing CAR-T cells develop more central-memory like phenotype when compared to control CAR-T cells

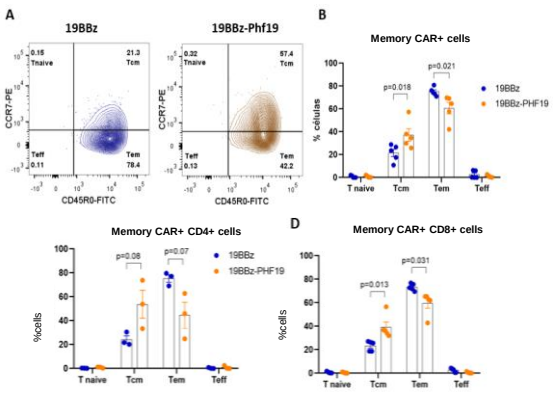


Figure 4. Memory phenotype of CAR-T cells overexpressing Phf19. A) Representative data. **B)** Memory phenotype of total CAR+ population. **C)** Phenotype inside CAR+CD4+ cells and **D)** CAR+ CD8+ cells. Tcm – central memory, Tem-effector memory, Teff- effector T cells. Populations were determined based on CD45R0 and CCR7 expression and gating strategy was according to FMO controls. Graphs show mean +- SEM. Paired Student's t test. N=3-5 independent blood donors.

5. Phf19 overexpressing CAR-T cells show higher expression of PD-1 and higher percentage of PD-1+TIM-3+ cells, specially within CD4+ cells.

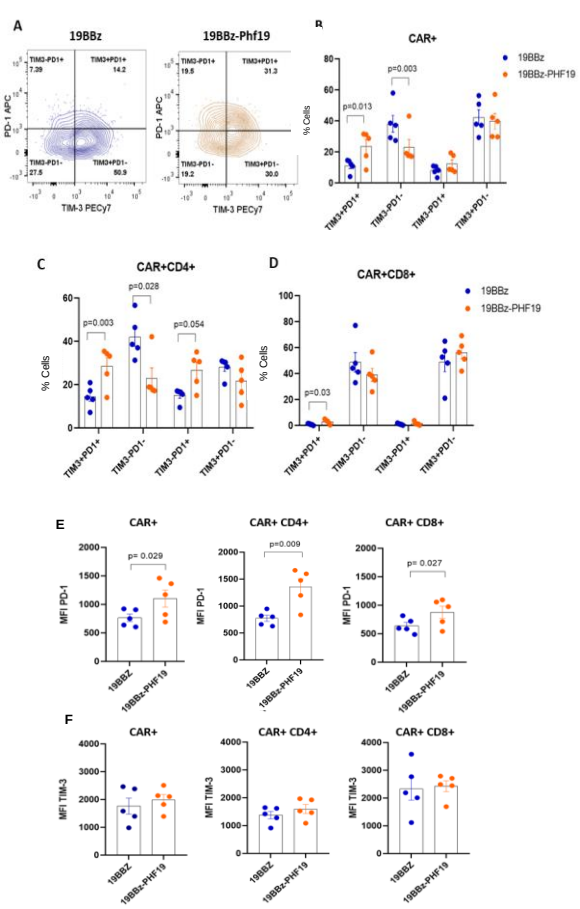


Figure 5. Exhaustion phenotype of CAR-T cells overexpressing Phf19. A) Representative data. **B)** Exhaustion phenotype of total CAR+ population. **C)** Phenotype inside CAR+CD4+ cells and **D)** CAR+ CD8+ cells. Populations were determined based on expression of the inhibitory receptors PD-1 and TIM-3. Graphs show mean +- SEM. Paired Student's t test. N=5 independent blood donors. **E)** Mean fluorescence intensity (MFI) of PD-1 and **(F)** TIM-3.

CONCLUSIONS AND PERSPECTIVES

Our results suggest an ambiguous role for Phf19 in T lymphocytes that seems to differ between CD8+ and CD4+ cells and paves the way for assessment of the effector function of these cells *in vivo*, as well as transcriptional and epigenetic profiles characterization. Of notice, we believe that Phf19 is involved in the induction of CCR7 expression, a chemokine receptor expressed by central memory T cells, but also of PD-1 in CD4 T cells, a finding that we intend to further explore. In sum, we hope this work will help develop new strategies to generate cost-effective CAR-T cells modulated to improve memory and exhaustion phenotypes.

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