# Zinc Finger Repressors Against Immune Checkpoint Molecules To Improve Anti-Tumor Activity of Gene-Modified T Cells

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### Introduction

The adoptive transfer of gene-modified T cells has recently emerged as a revolutionary new pillar in cancer therapy. Despite its remarkable success, many barriers still limit a more broadened therapeutic efficacy in solid tumors and hematological malignancies. Among these, T cell exhaustion and an immunosuppressive tumor microenvironment (TME) represent critical constraints to maintaining effector functions and persistence and achieving durable clinical potency. To overcome these significant challenges, innovative strategies and approaches are necessary to engineer more potent gene-modified T cells with improved anti-tumor activity.

We have developed a platform capable of efficient and durable epigenetic cell engineering using ZF-Repressors (ZF-Rs). These consist of zinc finger proteins (ZFPs) fused to different KRAB repressors, capable of silencing gene expression without the need of DNA double-strand breaks. Their compact size allows multiple ZF-Rs to be combined in a single lentiviral (LV) construct to achieve concomitant multi-gene epigenetic silencing in a single transduction event.



Using this technology, primary human T cells were engineered with a bidirectional LV construct encoding one or two ZF-Rs, either with or without an anti-CD19-CAR, to knock down cell surface proteins described as negative regulators of the anti-tumor activity. These include immune checkpoints, successfully targeted by immunotherapy, and factors playing a key role in the TME and the TCR signaling. Overall, our study proposes an innovative cell engineering approach to generate gene-modified T cells with an improved tumor-eradicating potential.







Desensitize human T cell from TME suppressive factors. Cancer cells take advantage of normal cellular homeostatic mechanisms to evade immune surveillance. Indeed, immune checkpoint pathways including PD-1, LAG3, TIM3, CTLA4 and TIGIT are often activated to inhibit anti-tumor immune response. Also, cancer cells secrete soluble factors, such as TGF $\beta$ , that induce a pro-tumoral state of immune tolerance by deregulating the immune balance towards immunosuppression. Drugs blocking some of these pathways have demonstrated durable clinical activities in a subset of cancer patients. Sangamo's strategy aims at desensitizing T cells against these TME inhibitory signals by knocking-down immune checkpoint and other receptors described to dampen T cell response against tumors.

#### Results



Efficient and durable MHCI and CD5 repression upon LV-mediated delivery of ZF-Rs in CD3+ cells. (A) Schematic representation of CD5 gene mRNA knockdown versus the CD5 ZF-R binding sites (triangles); darker colors indicate stronger repression. Selected CD5 ZF-R is highlighted in blue. (B) Panel of LV particles delivering up to two ZFRs were generated to assess repression efficiency in CD3+ cells. (C) CD5 (left) and B2M (right) repression efficiency were quantified by measuring the percentage of NGFR+/MHCI- and NGFR+/CD5- CD3+cells by flow cytometry. (D) Durability of B2M and CD5 repression was assessed by monitoring NGFR+/MHCI- and NGFR+/CD5- CD3+ cells injected in NXG mice for 10 weeks. (E) FACS plots showing concomitant MHCI and CD5 repression in transduced CD3+ cells before injection (left) and 10 weeks post-injection in blood (middle) and spleen (right).

#### Optimization of ZF-Rs specificity by designing phosphate-contact variants (PCVs)



interaction and increase specificity



Specificity optimization of PDI- and TIM3-ZF-Rs. (A) Schematic of the interaction between zinc finger domain and genomic DNA<sup>1,2</sup>. (B) The percentage of PDI- and TIM3-ZF-R-expressing T cells decreases rapidly over time using non-optimized ZF-Rs (left). In contrast, the percentage of PDI- and TIM3-ZF-R PCV-expressing T cells is stable following introduction of PCV mutations reducing non-specific interactions of ZF-Rs with the DNA phosphate contact backbone (right). (C) PDI (left) and TIM3 (right) repression efficiency were quantified by measuring the percentage of PDI + and TIM3 + transduced cells by flow cytometry.

## Generation of fully functional ZF-R-expressing CAR-T cells



In vitro characterization of ZF-R expressing CAR-T cells. (A) Panel of LV constructs delivering CD19-CAR and up to two ZF-Rs targeting PD1 and TIGIT. (B) Transduction efficiency of the indicated LVs was quantified as the percentage of CAR+ cells. (C) Durability of PDI and TIGIT repression was assessed in vitro by monitoring CAR+/PDI+ (left) and CAR+/TIGIT+ cells (right) from day 5 to day 14. Of note, cells were re-activated at day 7 with anti-CD3/CD28 beads. (D) CAR-T cell activation was determined by measuring the percentage of CAR+/CD69+ cells following overnight incubation with the listed activating agents. (E) Killing activity of CAR-T cells was evaluated by measuring the cell death of Raji (left) and K562-CD19 (right) at different effector (Eff.)-target ratio upon overnight incubation. (F) IFN- $\gamma$  and TNF- $\alpha$  were measured following overnight incubation of CAR-T cells with PDLI-expressing Nalm6 cells.

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# CTR-0 20 40 60 80 NGFR+ cells (%)

Generation of TILs expressing ZF-Rs targeting immune checkpoints. (A) Schematic representation of the workflow leading to the generation of ZF-R-expressing TILs. Briefly, TILs were isolated from biopsies resected from colorectal liver metastatic (CRLM) patients following mincing, enzymatic and mechanic dissociation and ficoll separation. Next, isolated TILs are transduced and expanded in vitro for at least 2 weeks. (B) Panel of LV constructs delivering  $\Delta$ NGFR and up to two ZF-Rs targeting PDI, LAG3 and TIGIT. (C) Transduction efficiency of the indicated LVs was quantified as the percentage of NGFR+ cells. (D) PD1 (left), LAG3 (middle) and TIGIT (right) repression efficiency were quantified by measuring the percentage of NGFR+/PD1+, NGFR+/LAG3+ and NGFR+/TIGIT+TILs by flow cytometry, respectively. (E) Preliminary data showing the killing activity of modified-TILs incubated for 48 hours with SNU-C5 cells in presence of anti-CD3/CD28 beads (bead:TIL ratio=1:10, left) or the bispecific EpCAM/CD3 engager Solitomab (Ing/mL, right). Cell lysis was evaluated by measuring lactate dehydrogenase (LDH) release.

#### Conclusion

- As a proof of concept, co-repression of constitutively and highly expressed B2M and CD5 genes with ZF-Rs resulted in efficient and long term MHCI and CD5 protein knockdown in vivo.
- Introduction of PCV mutations to abrogate some interactions between ZF-R and the DNA phosphate backbone strongly improved the specificity of ZF-Rs without compromising on-target repression efficiency.
- PDI and TIGIT ZF-Rs did not affect in vitro functionality of gene-modified CAR-T cells, warranting further evaluation of their anti-tumor activity in animal models.
- TILs from CRLM patients could be efficiently engineered with ZF-Rs targeting immune checkpoints.
- Multiplexed, epigenetic cell engineering using ZF-transcriptional regulators offers an innovative approach to the development of highly customized T cell therapies with improved therapeutic potential.

#### References

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#### Disclosures

DM, MD, YZ, ST, CD, CJ, GS, IM, AM, JF, AR, MdIR and DF are Sangamo Therapeutics employees. Sangamo Therapeutics owns patents covering the technology described in this poster. PS, MF and IKN received funding from Sangamo Therapeutics for the experiments.

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• A bidirectional LV architecture was designed to deliver multiple ZF-Rs in T cells.

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