Multiplex cell engineering of allogeneic anti-CD19 CAR T-cells with tuned silencing of HLA class I limits rejection by both CD8 T-cells and NK cells

Šakić A,[1] Ponsard B,[1] Mansuy M,[1] Lanz I,[1] Blancheteau V,[1] Marteyn A,[1] Genolet Y,[1] Bourrat B,[1] Turc A,[1] Cherpin O,[1] Ilmjärv S,[1] Roussel-Gervais A,[1] Alessandrini M[1]
[1] Antion Biosciences SA, Geneva, Switzerland

Introduction

Autologous chimeric antigen receptor (CAR) T-cell therapy is an effective form of treatment for hematological malignancy. This personalized approach is however challenged by high manufacturing costs and complicated logistics, limiting widespread adoption. Moreover, durable treatment response and a lack of efficacy against solid tumors depends largely on the persistence of CAR T-cells, which not only relies on cellular potency, but also the ability to avoid rejection by the patient's immune system. These challenges can be overcome with multiplex engineering of next generation CAR T-cells, such that they can be provided as an off-the-shelf solution, manufactured at scale and designed with molecular features to enhance cellular persistence. We have developed a bimodal gene construct which co-expresses a miRNA-mediated gene silencing cassette in tandem with a CAR (miCAR). This approach not only facilitates highly efficient multiplex gene silencing, but also allows for "tunable" silencing of multiple target genes.[1] Our aim was to develop allogeneic CAR T-cells with enhanced cellular persistence by tuning down the expression of HLA class I to limit rejection by both CD8 T-cells and NK cells, and thus obviating the need for co-expression of NK cell inhibitory molecules (Fig. 1).

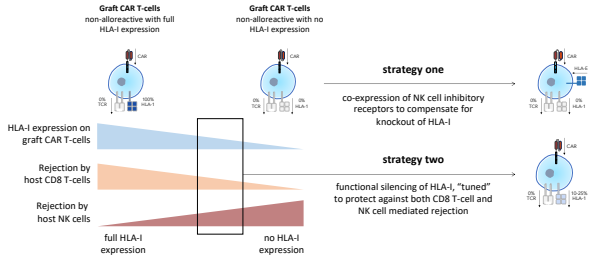

Fig 1. Strategies for engineering allogenic and hypoimmunogenic CAR T-cells.

Methods

- Primary T-cells were modified in a single vector transduction step, expanded in G-Rex cell culture plates, and purified by depletion of residual TCR-expressing cells.
- Characterization included flow cytometry immunophenotyping, functional testing against CD19-expressing tumor cells, and mixed lymphocyte reactions (MLRs).
- Preliminary *in vivo* data were generated in T-cell engrafted NSG mice.

Results

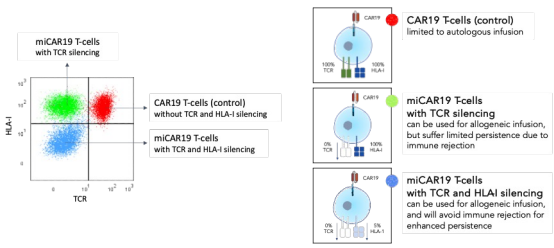

Fig 2. Highly efficient manufacturing of distinctly pure populations of multiplex engineered CAR19 T-cells. The flow cytometry dot plot represents an overlay of three uniquely manufactured CAR T-cell products, as schematically shown on the right.

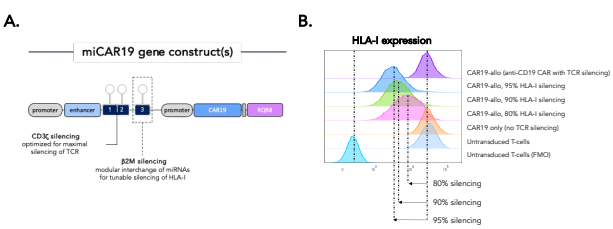

Fig 3. Engineering of allogeneic miCAR T-cells with tuned silencing of HLA-I. (A) Design of a bimodal miCAR19 gene construct with multiplexed miRNAs for high efficiency silencing of TCR[2] and tuned silencing of HLA-I; and a multi-gene addition cassette for expression of an anti-CD19 CAR (CAR19) and RQR8 safety switch molecule. (B) Histograms illustrating a tunable range of HLA-I silencing in TCR-depleted allogeneic CAR19 T-cells.

Functional characterization of miCAR19 T-cells

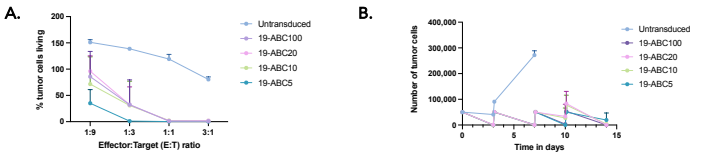

Fig 3. Allogeneic and hypoimmunogenic miCAR19 T-cells maintain efficient cytotoxicity of CD19-expressing tumor cells. (A) In short-term cytotoxicity assays, all miCAR19 T-cell populations efficiently lysed JeKo-1 target cells over a range of E:T ratios. (B) In recursive killing assays, all miCAR19 T-cells efficiently lysed target cells over four rounds of JeKo-1 tumor cell exposure, while untransduced T-cells were unable to control tumor growth. Cells abbreviated to indicate the level of HLA-ABC expression, e.g., 19-ABC10 = allogeneic miCAR19-T-cell with 10% HLA-I expression.

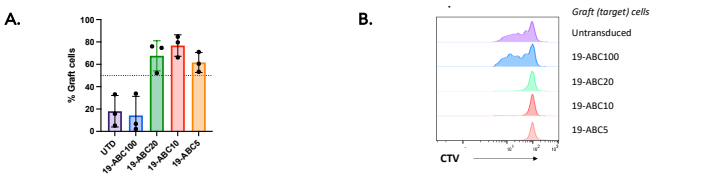

Fig 4. Engineered miCAR19 T-cells with >80% silencing of HLA-I are equally protected from allogeneic CD8 T-cell rejection. Host CD8 T-cells (effector cells, E) were primed and co-cultured with graft miCAR19 T-cells (target cells, T) at an E:T ratio of 1:1. (A) After 6 days of co-culture, untransduced T-cells and CAR19-allo T-cells with full expression of HLA-I were prone to CD8 T-cell rejection, while CAR19-allo T-cells with >80% silencing of HLA-I expression were less sensitive to CD8 T-cell cytotoxicity. (B) Flow cytometry histograms show loss of CellTrace Violet (CTV) signal and thus expansion of primed CD8 T-cells upon graft HLA-I recognition. Cells abbreviated to indicate the level of HLA-ABC expression, e.g., 19-ABC10 = allogeneic miCAR19-T-cell with 10% HLA-I expression.

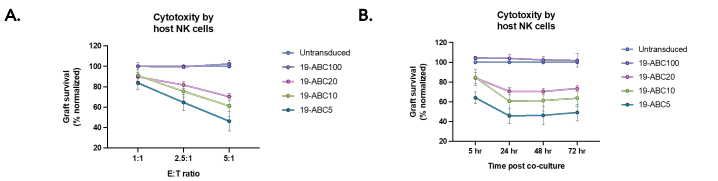

Fig 5. Tuned silencing of HLA-I protects miCAR19 T-cells from NK cell rejection according to HLA-I expression. Host NK cells (effector cells, E) were co-cultured with graft miCAR19 T-cells (target cells, T) over a range of E:T ratios. (A) Untransduced graft T-cells and miCAR19-allo T-cells with full expression of HLA-I were not lysed, while CAR19-allo T-cells with 95% silencing of HLA-I were the most sensitive to NK cell mediated cytotoxicity at all E:T ratios. Notably, graft miCAR19 T-cells with tuned HLA-I silencing of 80-90% remained largely protected from NK cell mediated cytotoxicity. (B) Host NK cells most prominently rejected graft T-cells with HLA-I silencing over the first 24 hours of co-culture (E:T ratio of 5:1). All assays were performed with n=3 NK cell donors. Cells abbreviated to indicate the level of HLA-ABC expression, e.g., 19-ABC10 = allogeneic miCAR19-T-cell with 10% HLA-I expression.

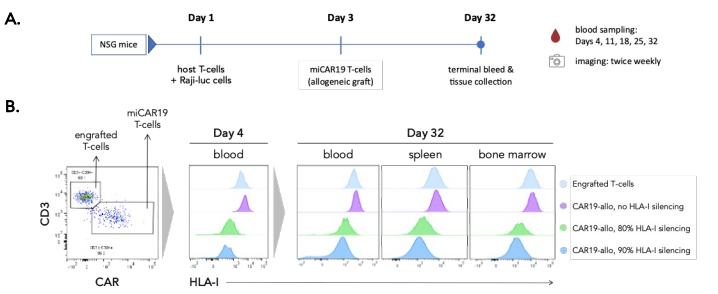

Fig 6. Sustained and "tuned" silencing of TCR/CD3 and HLA-I in an *in vivo* model of T-cell engrafted mice. (A) *In vivo* study design in NSG mice. (B) Flow cytometric tracking of miCAR19 T-cells from *in vivo* sampling. Dot plot shows definitive identification of miCAR19 T-cells based on CAR positivity and CD3 silencing. Representative histograms indicate the expression of HLA-I based on sampling from Day 4 and Day 32. Notably, "tuned" silencing of HLA-I was clearly detectable to its varying levels on Day 4 (one day post CAR T-cell infusion), which was accordingly sustained until study termination on Day 32 in all sampled tissues.

Summary and Conclusions

- We demonstrate a highly efficient means to multiplex engineer allogeneic and hypoimmunogenic CAR19 T-cells from a single gene construct
- Multiplex engineered miCAR19 T-cells are highly active against CD19-expressing tumor cells in both short-term and recursive killing assays
- Tuned down expression of HLA-I to 10-20% affords miCAR19 T-cells balanced protection from both allogeneic CD8 T-cell and NK cell mediated cytotoxicity
- Antion's multiplex gene constructs sustain CAR expression and silencing of TCR and HLA-I to optimized levels in an *in vivo* model of allogeneic T-cell engrafted mice

References: [1]Šakić et al., *Multiplex cell engineering of next generation CAR T-cells with functional silencing of six target molecules. Molecular Therapy, 31, 4S1, 2023.* Golinelli et al., Multiplex genetic engineering using microRNA (miRNA)-mediated gene silencing in mesothelin-directed CAR (M5CAR) T cells. Cytotherapy, 25, S7–S283, 2023. [2]Šakić et al., Overcoming the risk of Graft versus Host Disease by engineering non-alloreactive T-cells using a Tunable Expression Module (TEM) for high efficiency gene silencing. *Cytotherapy*, 25, S7–S283, 2023.