# DEVELOPING A DUAL-ANTIGEN TARGETING CAR-T THERAPY DIRECTED AGAINST CD70 AND ACTIVE INTEGRIN β2 FOR ACUTE MYELOID LEUKEMIA


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## INTRODUCTION

A successful cell-based immunotherapy for acute myeloid leukemia (AML) remains elusive, hindered in part by the heterogeneity of target antigen expression on leukemic cells and the risk of on-target, off-tumor toxicity due to the expression of many leading AML targets (e.g., CD33, CD123) on normal hematopoietic stem and progenitor cells (HSPCs).

Our research group has previously generated CAR-T therapies against the active conformation of integrin β2 (aITGB2)[1] and CD70[2], two antigens expressed on AML but absent on HSPCs and other healthy tissues. Both aITGB2 and CD70 have heterogeneous expression on AML blasts, however, which may prevent complete tumor clearance and allow for antigen-negative relapse. Here, we combine these two single-antigen CAR-Ts into a dual-targeting OR-gated CAR approach that eliminates a larger proportion of AML cells while maintaining low off-tumor toxicity.

## AIMS

**Aim 1:** Demonstrate that aITGB2 and CD70 are promising target antigens for an OR-gated CAR-T therapy against AML

**Aim 2:** Generate and evaluate the best-performing dual-targeting CAR construct in *in vitro* and *in vivo* preclinical models of AML

## RESULTS

### Target Validation for AML Dual-targeting CAR-T Therapy

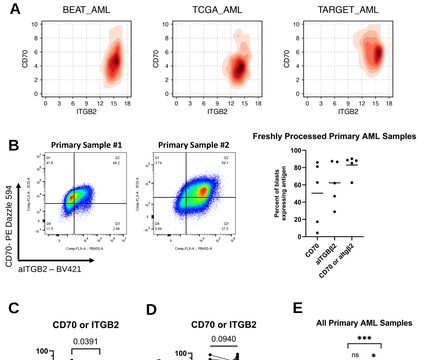



**Fig. 1:** **A)** Bulk RNA-seq analysis of 3 different AML patient sample cohorts for gene expression of *CD70* and active *ITGB2*. **B)** Representative flow cytometry plots and quantification of *CD70* and *aITGB2* expression on N = 5 freshly processed AML primary samples **C)** Quantification of decreased cell surface antigen expression following a freeze-thaw cycle of N = 4 primary AML samples **D)** Flow cytometry on N=10 primary AML samples upon thaw and after 7 days of co-culture with the HS-5 cell line and exogenous cytokines IL-3, SCF, and FLT3 Ligand. **E)** Proportion of AML blasts expressing *CD70, aITGB2,* or either antigen

### Identification of Optimal Dual-targeting CAR Constructs

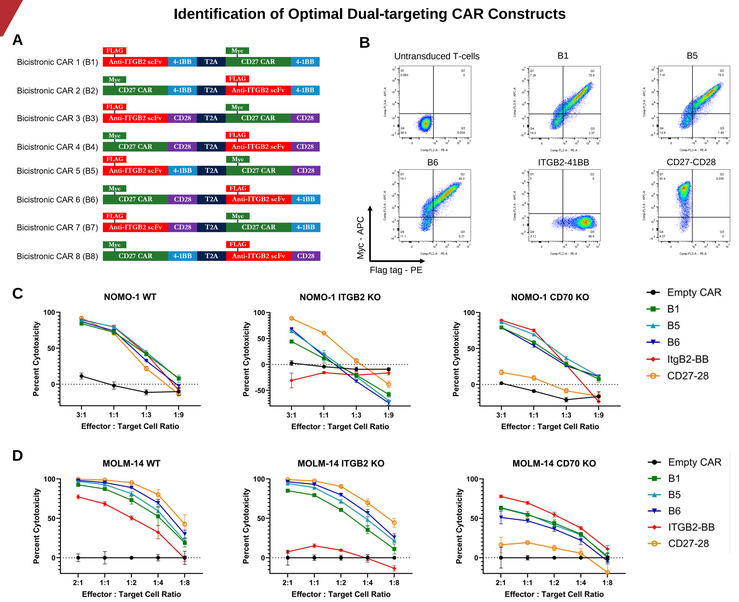



**Fig. 2:** **A)** Cartoon depiction of all dual-targeting CAR-T constructs that were cloned and tested for *in vitro* cytotoxicity. **B)** Flow cytometry to determine transduction efficiency of selected CAR-T constructs. **C-D)** Relative percent cytotoxicity of the best performing dual-targeting CAR-Ts compared to single-targeting CAR-Ts and an empty CAR negative control in two AML cell lines, **C)** NOMO-1 and **D)** MOLM-14. Percent cytotoxicity is normalized to the untransduced T-cell condition. Each point represents the average of N=3 replicates. Data shown are from two independent experiments.

### *In Vivo* Validation of Dual-Targeting Efficacy in AML Antigen Heterogeneity
### Model: mixed CD70/ITGB2 KO THP-1 xenograft

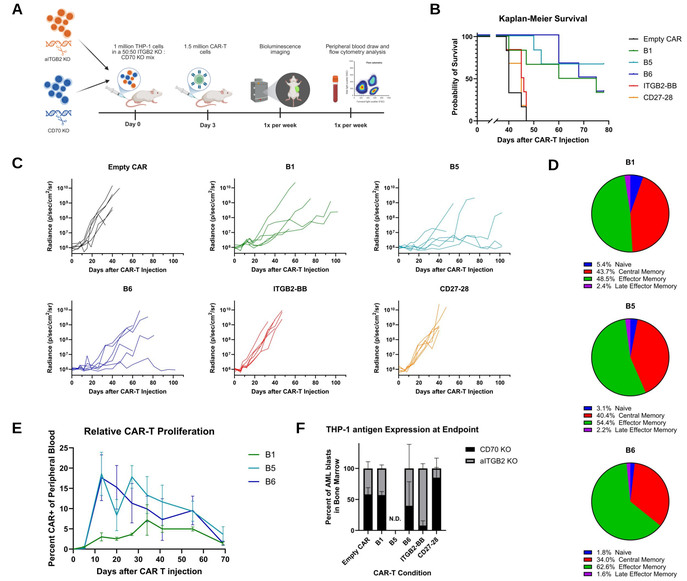
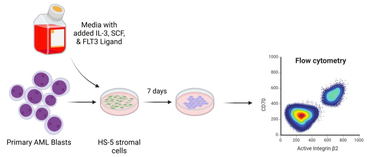



**Fig. 3:** **A)** Cartoon timeline of heterogenous THP-1 xenograft experiment, N=6 mice per treatment group. **B)** Kaplan-Meier Survival curve of mice by treatment group. **C)** Quantification of bioluminescence signal per mouse in each treatment group. **D)** T-cell memory/effector phenotype proportions of each bicistronic CAR on day 20 of the *in vivo* study, as assessed by flow cytometry of CD62L and CD45RA expression on CAR-T cells isolated from peripheral blood. N=3 mice evaluated per treatment group. **E)** Proliferation of CAR-Ts in each treatment group, as measured by flow cytometry, as a percent of all live singlet-gated events from weekly peripheral blood draws. N=3 mice evaluated at each time point. **F)** Relative proportion of each KO THP-1 line found in the bone marrow at clinical endpoint, as assessed in N=3 mice per group by flow cytometry

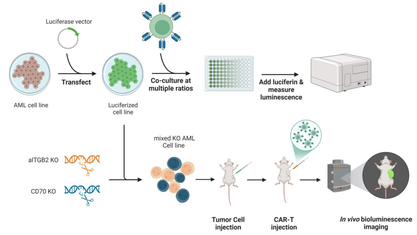
## METHODS

### Aim




To measure the co-expression of CD70 and aITGB2 on AML, a co-culture strategy with N=10 primary AML patient samples, HS-5 bone marrow stromal cells, and exogenous cytokines (25 ng/ml IL-3, SCF, and FLT3 Ligand) was used to simulate the endogenous tumor microenvironment and re-establish cell surface homeostasis after the shedding of surface proteins following a freeze-thaw cycle. Flow cytometry for antigen expression was performed every 2-3 days during the 7-day co-culture period.

### Aim 2:




To generate CAR-Ts targeting both CD70 or aITGB2, several permutations of our previously validated anti-aITGB2 scFv CAR and anti-CD70 natural ligand-based CAR were designed with all 8 possible combinations of 4-1BB or CD28 costimulatory domains and CAR expression order in a bicistronic plasmid design. To measure each construct's anti-tumor efficacy, lentivirally transduced CAR-T cells were co-incubated with the luciferized AML cell lines NOMO-1 and MOLM-14 at multiple effector-to-target cell (E:T) ratios to measure average dose-dependent killing. CRISPR knockout versions of these cell lines for both target antigens were developed to validate the efficacy of both CARs in each construct. To measure the *in vivo* efficacy of these bicistronic CARs, CAR-T constructs were tested in an AML antigen heterogeneity model, in which NSG mice were injected with a population of 1 million THP-1 AML cells, consisting of a 50%:50% mixture of CD70 KO or ITGB2 KO THP-1 cells. Four days later, N=6 mice per treatment group were injected with 1.5 million CAR-Ts, followed by weekly bioluminescence imaging and biweekly blood draws to evaluate CAR-T expansion in the peripheral blood.

## CONCLUSIONS

1. A dual-targeting OR-gated approach against the AML antigens CD70 and active ITGB2 greatly increases the proportion of AML blasts that can be targeted.
2. Among our screened dual-targeting CAR constructs, binders 1, 5, and 6 show the greatest promise *in vitro* in AML cell line models.
3. Our dual-targeting CARs reduce tumor burden and greatly extend lifespan in a mouse model of AML antigen heterogeneity.
4. Future safety studies are underway to confirm a lack of hematopoietic cell toxicity for our lead bicistronic CAR designs

## REFERENCES


1. Mandal, K *et al.* Structural surfaceomics reveals an AML-specific conformation of integrin $\beta_2$ as a CAR T cellular therapy target. *Nat Cancer 2023; 4*: 1592–1609
2. Kasap, C *et al.* Targeting high risk multiple myeloma genotypes with optimized anti-CD70 CAR-T cells. *Blood.* [EPub before print] (2025).


## ACKNOWLEDGEMENTS


We thank all members of the Wiita & Eyquem labs for their experimental guidance and support. This project was supported by funding from the National Institutes of Health (NIH NIGMS T32GM141323) and the UCSF Catalyst Award program.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