P#260

Novel CLL-1-directed CAR-T cells Mediate Potent Antigen-specific Cytolytic Activity in Mouse Models of Acute Myeloid Leukemia

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INTRODUCTION

- High-risk acute myeloid leukemia (AML) patients have poor clinical outcomes.
- Chimeric antigen receptor (CAR) T cell therapy holds promise as an immunotherapeutic strategy.
- Targeting C-type lectin-like molecule-1 (CLL-1, CD371) represents an attractive approach, as CLL-1 is highly expressed on AML blasts and leukemic stem cells (1).

OBJECTIVE

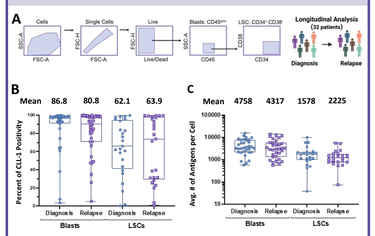
- Development, functional characterization and side-side comparison of 24 novel CLL-1-directed CAR-T cells.

METHODS

- CLL-1-directed binders were identified by phage display technology and evaluated by flow cytometric, ELISA and Octet analyses.
- Selected binders were used to generate second generation CLL-1 directed CAR constructs with a 4-1BB co-stimulatory domain.
- CLL-1 monoCARs were investigated for antigen-dependent cytotoxicity, activation and cytokine secretion, for potency at low effector to target (E:T) ratios, for long-term persistence in repeated stimulation assays, and for avidity.
- The top CLL-1 CAR candidates were screen in an *in vivo* murine xenograft model using HL60 AML cells in NSG mice.

RESULTS

Fig. 1. CLL-1 is Expressed on AML Blasts and Leukemia Stem Cells



A) Gating schema for the flow cytometric surface expression longitudinal profiling of bone marrow samples from AML patients (N=32). **B-C**. Percent positivity and number of antigens per antigen+cell in blasts (**B**) and Leukemic stem cells (LSCs) (**C**). QuantiBrite beads were used for antigen quantitation. Data shown as median ± standard error of mean.

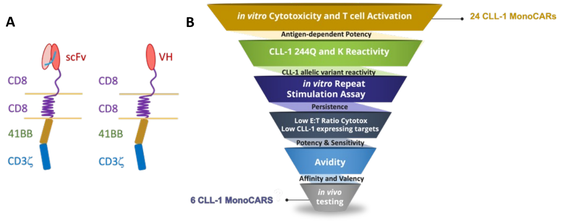
RESULTS (CONT'D)

Table 1. CLL-1-directed Binder Affinity and Kinetic Characterization

	EC50 (nM)		Affinity, KD (nM)	Aggregation (% Monomer)	Allelic variant
Binder	Multi-Point FACS (HL-60 cells)	Multi-Point ELISA CLL-1 ECD	Octet CLL-1 ECD	SEC and DLS	Q/K 244 binding
1	3.2	3.4	0.9	83.5	Both
2	59	0.9	5.1	68.4	Both
3	1.8	0.8	1.1	66	Both
4	0.2	1.1	2.6	73.7	Both
5	0.2	1.4	16.6	89	Both
6	1.6	0.6	1.7	81.7	Both
7	0.7	1.9	2.3	78.2	Both
8	2.2	1.5	2.9	88.8	Both

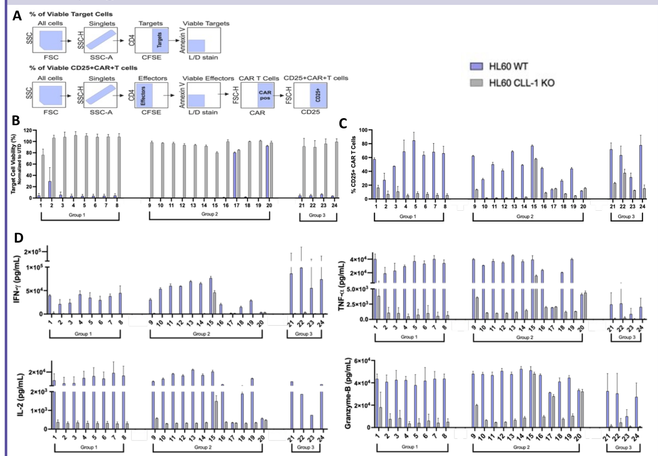
CLL-1-directed binders were identified by phage display panning against single-chain variable fragment (scFv) and heavy chain variable region (VH) libraries. Binder kinetic and affinity characterization (EC50 and KD) were performed by multipoint flow cytometric, ELISA and Octet analyses. Binder aggregation and stability screen was assessed by Size Exclusion Chromatography and Dynamic Light Scattering analyses and CLL-1 244 K/Q allelic variant binding specificity by ELISA.

Fig. 2. Experimental Study Design



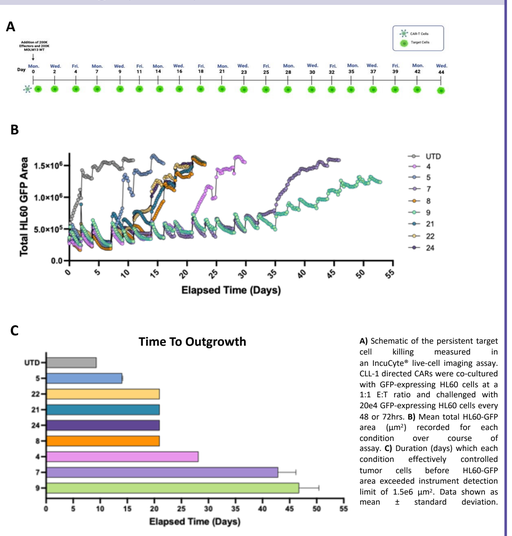
A) Schematic of the CLL-1-directed 4-1BB CAR structures. Binding domain comprised of a single chain variable fragment (scFv) or variable domain of heavy chain antibody (VH).
B) Experimental design. Co-culture experiments were conducted for 24 and 48 hours with CLL-1-expressing WT and CLL-1 knockout (KO) HL60 target cells. CAR candidates showing robust antigen-dependent activity were further evaluated for CLL-1244Q and K binding affinity, for potency by bioluminescent-based assays at low E:T ratios, for long-term persistence in repeated stimulation assays, and for avidity measurements in acoustic force microscopy assays. The top CLL-1 CAR candidates were further studied in an *in vivo* murine xenograft model using HL60 AML cells in NSG mice.

Fig. 3. CLL-1-directed CARs Mediate Potent CLL-1-specific Cytolytic Activity Against AML Cells in Vitro



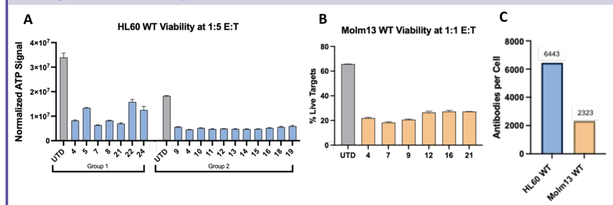
CLL1-directed CAR T cells were co-cultured with CFSE labeled HL60 WT and CLL-1KO cell lines as AML target cells at 1:1 ratio for 24 (n=3) and 48 hours (n=2). **A)** Gating schema for the flow cytometric analysis of specific target cytolysis and CAR+T cell activation. **B)** Percentage of viable target cells was determined by the absence of Annexin V and fixable viability dye reactivity by flow cytometry at 48 hours. **C)** Antigen-specific activation of CAR+ T cells was defined by CD25 expression by flow cytometry at 48h. **D)** CLL-1 CARs exhibit antigen-specific cytokine secretion. The supernatants of 24h co-cultures were analyzed for cytokine secretion using a Luminex 17-Plex kit. Data shown as mean ± standard deviation.

Fig. 4. CLL-1-directed CARs Display Persistent in vitro Killing Upon Repeated Stimulation



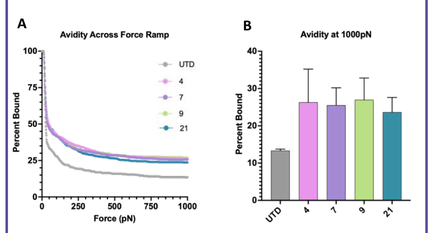
A) Schematic of the persistent target cell killing measured in an IncuCyte® live-cell imaging assay. CLL-1 directed CARs were co-cultured with GFP-expressing HL60 cells at a 1:1 E:T ratio and challenged with 20e4 GFP-expressing HL60 cells every 48 or 72hrs. **B)** Mean total HL60-GFP area (μm^2) recorded for each condition over course of assay. **C)** Duration (days) which each condition effectively controlled tumor cells before HL60-GFP area exceeded instrument detection limit of 1.5e6 μm^2. Data shown as mean ± standard deviation.

Fig. 5. CLL-1 directed CARs Display Potent Killing and High Antigen Sensitivity



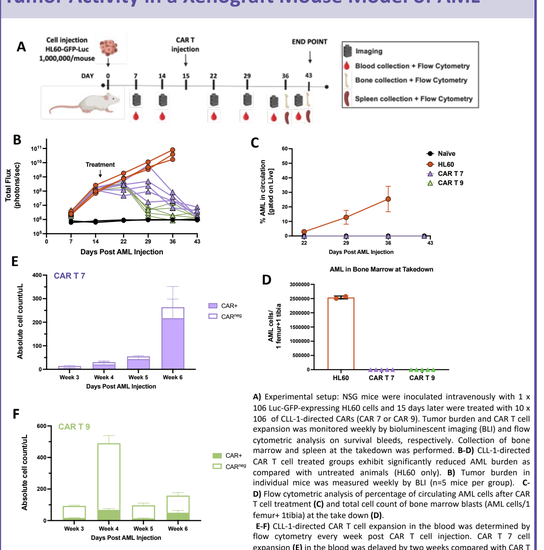
A) CLL-1-directed CAR candidates were cocultured with HL60 WT cells for 24hr at E:T ratio of 1:5. Target cell killing potency was assessed by CellTiterGlo assay. Data shown as mean ± standard deviation.
B) Molm13 WT cells expressing indicated low density of surface CLL-1 molecules (**C**) were labelled with CFSE and then cocultured with CLL-1 directed monoCAR cells for 24 hrs at a 1:1 E:T ratio. Target cell killing was assessed by flow cytometric analysis.(**C**). QuantiBrite beads were used for antigen quantitation. Data shown as mean ± standard deviation.

Fig. 6. Top Candidates Display Similar Avidity



Cell binding avidity between CLL-1 directed CAR T cells and HL60 WT targets was assessed via acoustic force microfluidic microscopy (z-Movi analysis). HL60 WT cells were seeded on z-Movi® microfluidic chips and CLL-1-directed CARs were serially flushed in and allowed to bind to the target cells prior to the application of an acoustic force ramp. **A)** The percentage of bound T cells was calculated across entirety of force ramp. **B)** The percentage of bound T cells at the end of force ramp (1000pN). Data represent mean ± standard deviation from (n=3) replicates.

Fig. 7. Top CLL-1 directed monoCARs exhibit Robust Anti-Tumor Activity in a Xenograft Mouse Model of AML



A) Experimental setup: NSG mice were inoculated intravenously with 1 x 106 Luc-GFP-expressing HL60 cells and 15 days later were treated with 10 x 106 of CLL-1-directed CARs (CAR 7 or CAR 9). Tumor burden and CAR T cell expansion was monitored weekly by bioluminescent imaging (BLI) and flow cytometric analysis on survival bleeds, respectively. Collection of bone marrow and spleen at the takedown was performed. **B-D)** CLL-1-directed CAR T cell treated groups exhibit significantly reduced AML burden as compared with untreated animals (HL60 only). **B)** Tumor burden in individual mice was measured weekly by BLI (n=5 mice per group). **C-D)** Flow cytometric analysis of percentage of circulating AML cells after CAR T cell treatment (**C**) and total cell count of bone marrow blasts (AML cells/1 femur+ 1tibia) at the take down (**D**).
E-F) CLL-1-directed CAR T cell expansion in the blood was determined by flow cytometry every week post CAR T cell injection. CAR T 7 cell expansion (**E**) in the blood was delayed by two weeks compared with CAR T 9 (**F**), which was reflective of a slower tumor control activity (**B**).

CONCLUSION

- CLL-1 is expressed on a high percentage of AML blasts and LSCs at both diagnosis and relapse timepoints.
- Novel CLL-1-directed CAR T cells displayed potent and CLL-1-specific cytolytic activity, activation and Th1 cytokine secretion, and long-term persistence *in vitro*.
- Top CLL-1-directed CAR T cell candidates mediated robust and rapid anti-tumor activity in a xenograft mouse model of AML.

References

1. Ma, H., Padmanabhan, I.S., Parmar, S. et al. Targeting CLL-1 for acute myeloid leukemia therapy. J Hematol Oncol. 2019; 12(1):41

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