CRG-023, A Novel Tri-Specific CAR T Product Candidate Engineered to Prevent Antigen Escape and Sustain Durable Anti-Tumor Functionality Against B-Cell Malignancies

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Introduction

Chimeric antigen receptor (CAR) T-cell therapy has revolutionized the treatment landscape for B-cell lymphomas and leukemias. However, most patients do not achieve durable response. Mechanisms that facilitate resistance and impact survival outcomes include antigen escape, loss of CD58 co-stimulation, and CAR T-cell exhaustion. CRG-023 was designed to address these challenges.

- CRG-023 is a tri-specific CAR T-cell that targets the B-cell lineage antigens CD19, CD20, and CD22 via tri-cistronic expression of 3 distinct second-generation CARs from a single lentiviral vector.
- The CD19- and CD20-targeting CARs employ novel, human single-chain variable fragment (scFv). The CD22-targeting CAR employs the human scFv m971 (Frank MJ et al., Lancet 2024)
- Each CAR incorporates a CD3ζ signaling domain, and a distinct co-stimulatory domain derived from 4-1BB (CD22-targeting CAR), CD28 (CD19-targeting CAR), or CD2 (CD20-targeting CAR). CD2 is the costimulatory receptor required for CD58 engagement.
- Each CAR sequence and their arrangement within the tri-cistronic vector was engineered to achieve optimal CAR T-cell activity. Further codon optimization and removal of splice sites were performed to limit recombination and to ensure stable CAR expression.

Design matters: CAR optimization through engineering and screening

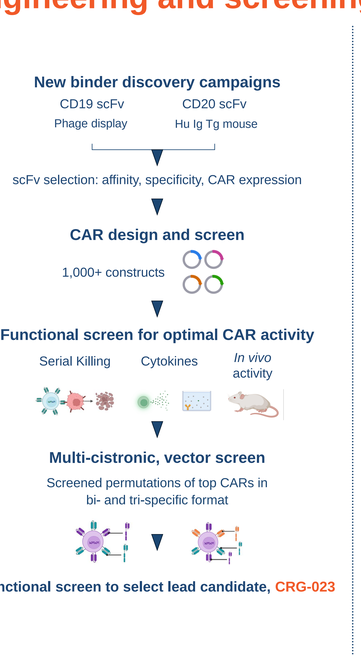


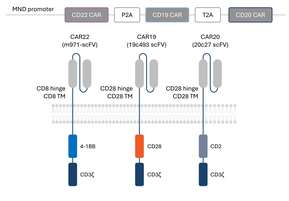
Figure 1. Overview of CRG-023 discovery process.

New CD19 and CD20 scFv binders were selected based on affinity, specificity, and ability to express in a CAR.

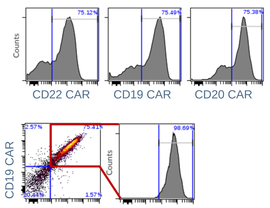
CARs underwent functional screening to identify those with superior activity, including serial killing capacity, cytokine production, and in vivo efficacy.

Selection of CRG-023 was based on engineering of top performing CARs into bi- and tri-CAR formats and screening for optimal functional activity in vitro and *in vivo* across a range of antigen expression profiles in target cells.

A. CRG-023 construct overview



B. CAR surface expression profile



C. CAR surface receptor quantification

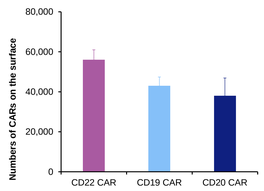


Figure 2. CRG-023 CAR T cells express the 3 CARs simultaneously

A. CRG-023 is expressed as 3 individual CARs targeting CD19, CD20 and CD22. MND promoter variant drives transgene expression.
B. Representative flow cytometry plot (N>3 repeats per donor, N>5 donors). CAR T cells were stained with CAR 19, 20 and 22 anti-Id (anti-idiotype) antibodies (gated on live cells).
C. CAR molecule quantification measured by flow cytometry using anti-Id antibodies and Bang quantum simply beads (Bang labs). N=3, mean±SD.

***CAR** = chimeric antigen receptor; **scFv** = single-chain variable fragment; **TM** = transmembrane, **P2A** = Porcine teschovirus-A (self-cleaving peptide); **T2A** = Thosea asigna virus (self-cleaving peptide).*

Sustained anti-tumor activity against tumor cells expressing CD19, CD20 and CD22 or single Ag tumor cells

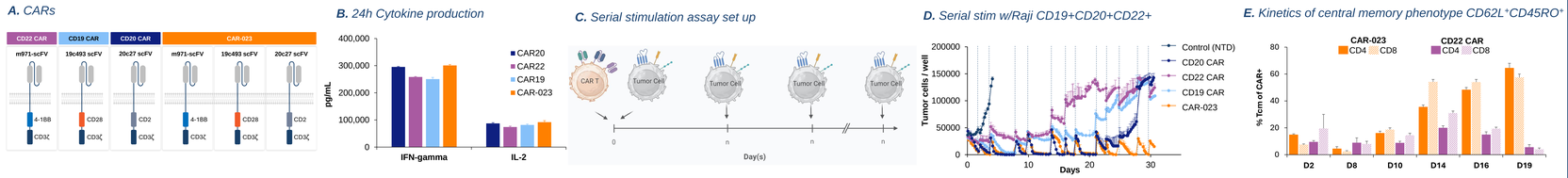


Figure 3. CAR-023 demonstrates sustained, superior cytolytic anti-tumor activity over mono-CAR T cells, with an enhanced central memory phenotype and comparable cytokine levels

A. Schematics of CAR T cells used in this study
B. Effector cytokine Production (pg/mL) using MSD assay (n=3, mean±SEM). Conditioned media was collected after 24hr co-culture with CAR T and tumor cells.
C. Tumor killing activity was continuously measured using an Incuctyte instrument for up to 30 days. CAR T cells were initially co-cultured with GFP-positive tumor cells (E:T 1:3). Additional target cells were added to the culture ~3x/week.
D. Representative serial stimulation results. Dashed lines indicate addition of new tumor cells to the culture. Y axis indicates GFP signal as a surrogate for tumor cell presence in culture (n=3, mean±SEM)
E. Flow cytometry analysis for CD62L and CD45RO expression to inform T cell differentiation state. CAR T cells were isolated at indicated timepoint from serial stimulation assay.

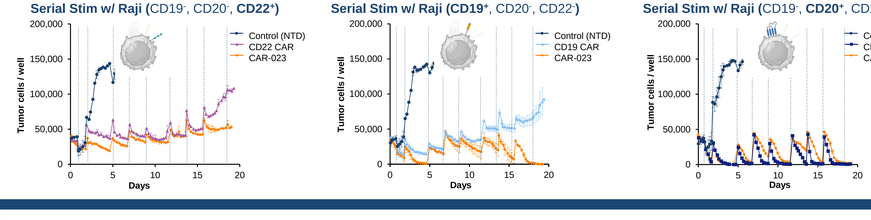


Figure 4. CAR-023 CAR T cells sustained cytolytic activity against single antigen expressing tumor cells

Three independent serial stimulation experiments were performed with three tumor cell lines engineered by genetic knockout to express one of three target antigens. CD22-specific killing (left panel), CD19-specific killing (middle panel) and CD20-specific killing (right panel) (n=3, mean±SEM).

Enhanced anti-tumor activity relative to benchmarks

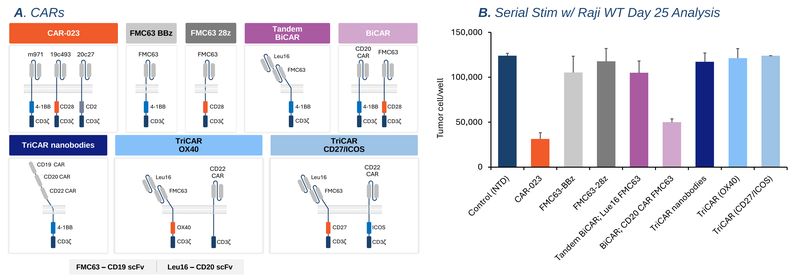


Figure 5. CAR-023 anti-tumor activity as compared to benchmark CARs

A. Schematics of benchmark CARs evaluated in serial stimulation assay, all containing MND promotor.
B. Quantification of tumor cells / well on day 25 of serial killing assay. (N=2, mean±SEM, similar results with 2 different donors).

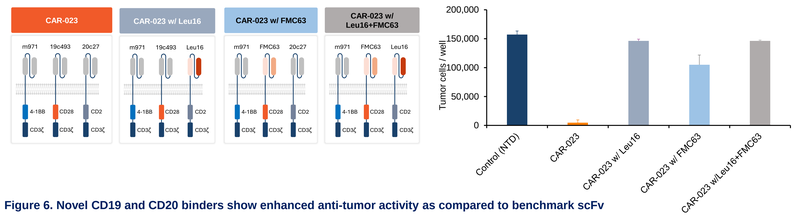
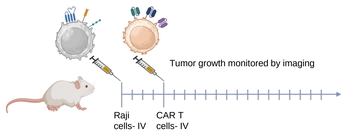


Figure 6. Novel CD19 and CD20 binders show enhanced anti-tumor activity as compared to benchmark scFv

A. Schematics of TriCARs expressing different scFv.
B. Quantification of tumor cell/ well on day 34 of serial killing assay against GFP-positive Raji WT target cells. CAR T cells were co-cultured with GFP-expressing target cells as described in (Figure 3A) (N=2, mean±SEM similar results with 2 different donors).

A. Dose-response using Raji expressing all 3 target antigens



B. Tumor burden over time

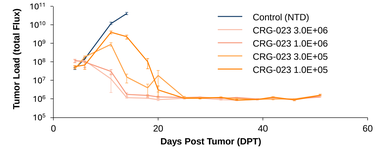


Figure 7. Tumor clearance was observed at the lowest CRG-023 CAR T dose

A. 1E+6 Raji WT cells were implanted to NCG mice (n=10/group). Four days later, 3E+6, 1E+6, 3E+5 or 1E+5 CART cells were injected into each group.
B. IVIS imaging was conducted to measure flux data (mean±SEM; one representative of 3 donors) indicative of tumor burden. NTD = Not transduced

A. Model using a mixture of single antigen KO Raji cells

B. Model using a mixture of Raji WT and Raji CD19 KO cells

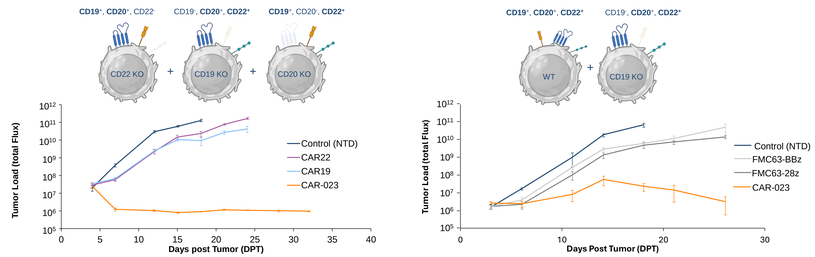


Figure 8. CAR-023 T cells maintain anti-tumor activity in tumor loss models as compared to mono-CAR T cells

A. 0.25E+6 1:1:1 mix of CD19-KO, CD20-KO, and CD22-KO Raji cells, were implanted into NCG mice (n=4 per group). Four days later, 5E+6 CAR-T cells were administered to each group. Tumor burden was monitored 2x/week using an IVIS imaging system (mean ± SEM).
B. 2.25E+5 Raji WT + 0.25E+5 Raji CD19KO cells were implanted to NCG mice (n=5/group). Four days later, 3E+6 MND-FMC63-28z, MND-FMC63-BBz or CAR23 CAR-T cells were injected into each group. Tumor burden was monitored 2x/week using an IVIS imaging system (mean±SEM).

Conclusions

Significant engineering and screening were undertaken to develop CRG-023, a highly active tri-specific CAR T-cell product candidate with differentiated pre-clinical activity.

- CRG-023 is a tri-specific, tri-cistronic CAR T cell product candidate engineered to target CD19, CD20, and CD22
- New binders for CD19, CD20 and a CD2 co-stimulatory domain were some of the design features added to enhance CAR-mediated activity
- CAR-023 had durable anti-tumor activity *in vitro* when tumor cells expressed either three target antigens or just one
- CAR-023 outperformed benchmark controls
- Durable anti-lymphoma activity was observed *in vivo*, even at the lowest dose levels assessed and in antigen loss models
- Mechanistic insights inform preclinical data to support the planned IND

References

CD22-directed CAR T-cell therapy for large B-cell lymphomas progressing after CD19-directed CAR T-cell therapy: a dose-finding phase 1 study
Frank, Matthew J., Baird, John H. et al.
The Lancet, Volume 404, Issue 10450, 353 - 363

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