CAR-T and nanoCAR-T for multiple myeloma: Does smaller mean smarter ?

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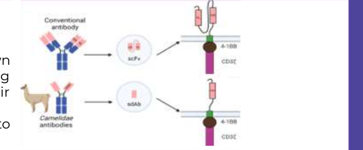
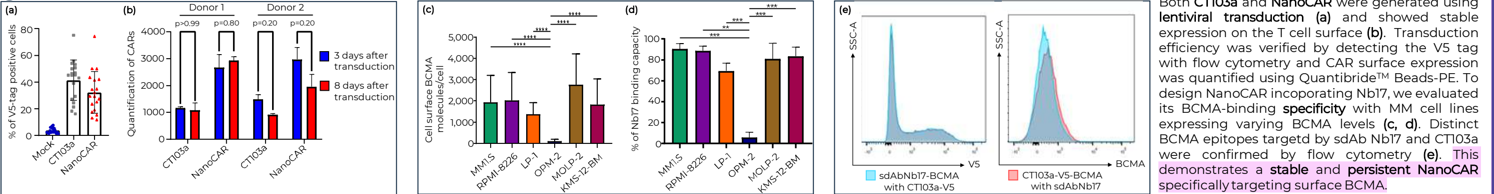
Introduction & Objective

- **Multiple myeloma** (MM) is an incurable hematologic malignancy of plasma cells. **Chimeric antigen receptor T lymphocyte** (CAR-T) immunotherapy has shown remarkable results in relapse patients. **Single-domain antibody** (sdAb) offers an excellent alternative to **scFv** due to the advantage of having a small and stable folding structure, therefore avoiding tonic signaling. Despite the development of numerous nanoCAR-Ts, there is limited data demonstrating the direct differences and their mechanisms of action.
- This **project** aims to characterize and compare the activity of a **nanoCAR-T** containing the sdAb Nb17 to the **ScFv CAR-T CT103a**, an FDA-approved CAR-T, to try to elucidate differences of mechanisms between them.



Methods & Results

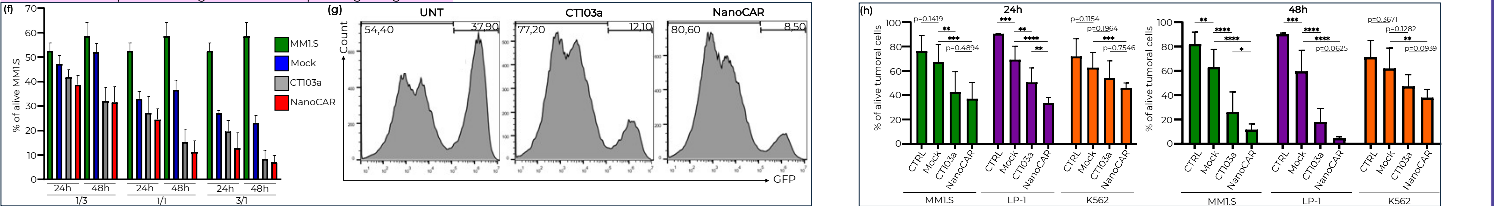
I Construction of a NanoCAR sequence containing sdAb Nb17



Both **CT103a** and **NanoCAR** were generated using **lentiviral transduction (a)** and showed stable expression on the T cell surface **(b)**. Transduction efficiency was verified by detecting the V5 tag with flow cytometry and CAR surface expression was quantified using Quantibride™ Beads-PE. To design NanoCAR incoporating Nb17, we evaluated its BCMA-binding **specificity** with MM cell lines expressing varying BCMA levels **(c, d)**. Distinct BCMA epitopes targetd by sdAb Nb17 and CT103a were confirmed by flow cytometry **(e)**. This demonstrates a **stable** and **persistent NanoCAR** specifically targeting surface BCMA.

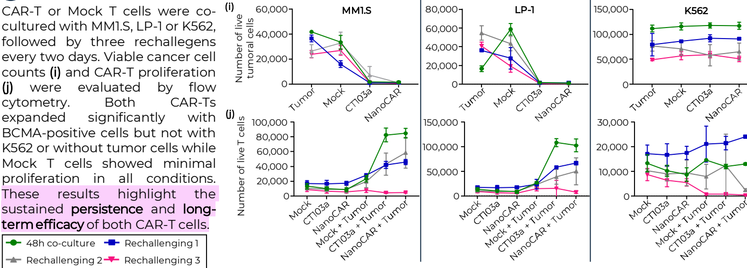
II In vitro efficacy of CT103a and NanoCAR in killing BCMA-positive MM cell lines

The cytotoxic capacity of NanoCAR, CT103a and Mock T cells was evaluated by flow cytometry after co-culture with **MM1.S** for 24 to 48 hours at **effector-to-target** (E/T) **ratios** of 1/3, 1/1 and 3/1. Killing efficiency improved with higher ratios and longer incubation **(f, g)**. A 1/1 ratio killing assay confirmed specific targeting of BCMA-positive cells (MM1.S, LP-1) while sparing BCMA-negative K562 cells **(h)**. These results demonstrate the efficacy and selectivity of CT103a and NanoCAR as therapeutic strategies for BCMA-expressing malignancies.

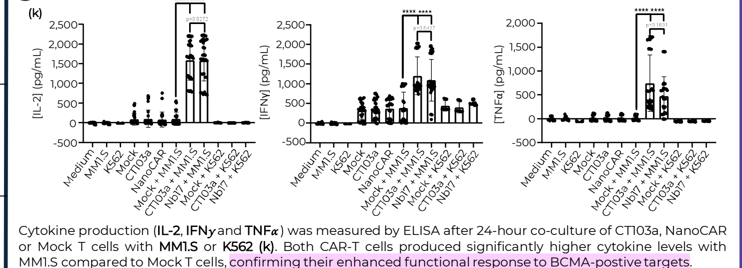


III Persistence of CAR-T killing ability following repeated antigen challenges

CAR-T or Mock T cells were co-cultured with MM1.S, LP-1 or K562, followed by three rechallenges every two days. Viable cancer cell counts **(i)** and CAR-T proliferation **(j)** were evaluated by flow cytometry. Both CAR-Ts expanded significantly with BCMA-positive cells but not with K562 or without tumor cells while Mock T cells showed minimal proliferation in all conditions. These results highlight the sustained **persistence** and **long-term efficacy** of both CAR-T cells.



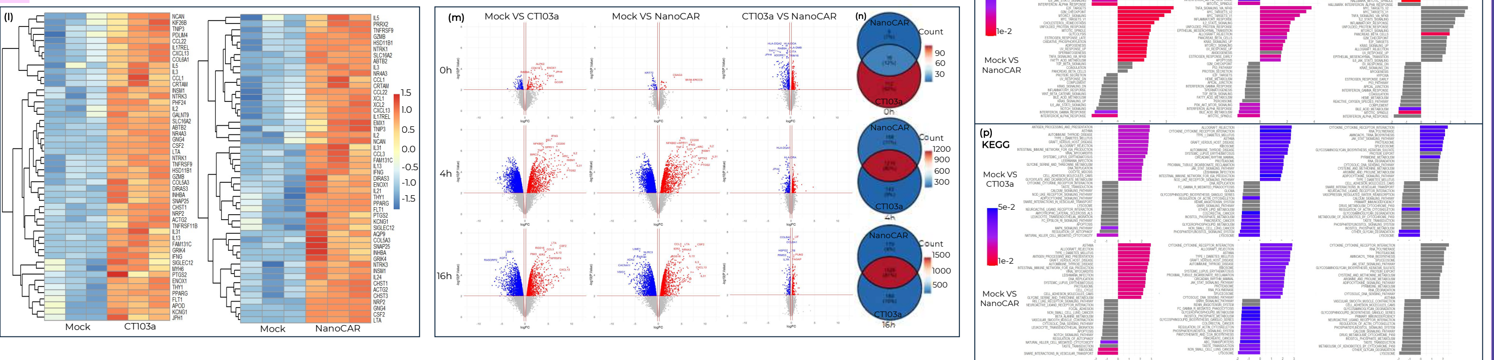
IV CT103a and NanoCAR produce cytokines when co-cultured with MM



Cytokine production (**IL-2**, **IFNγ** and **TNFα**) was measured by ELISA after 24-hour co-culture of CT103a, NanoCAR or Mock T cells with **MM1.S** or **K562 (k)**. Both CAR-T cells produced significantly higher cytokine levels with MM1.S compared to Mock T cells, confirming their enhanced functional response to BCMA-postive targets.

V CT103a and NanoCAR expressed similar set of genes or pathways when co-cultured with MM1.S

Bulk RNA seq compared transcriptional profiles of CT103a, NanoCAR and Mock T cells at **0h**, **4h** and **16h** post-incubation with MM1.S. Mock T cells served as a negative control and CT103a as the comparator. **Heatmaps** of the **top 50 genes** at 16h **(l)** showed upregulation of activation, proliferation and effector function genes in both CAR-T cells. **Volcano plots** highlighted **minimal differences** between CT103a and NanoCAR with many upregulated and downregulated genes shared **(m, n)**. **GSEA** (**Hallmark** and **KEGG**) at 4h revealed enriched cytokine production, cytokine interactions and proliferation pathways in both CARs compared to Mock **(o, p)**. However, these differerences disappeared by 16h due to basal cytokine production by pre-activated Mock T cells. Despite **similar transcriptional profiles**, both CT103a and NanoCAR showed tumor-specific activation mechanisms distinct from Mock T cells.



Conclusion(s)

To conclude, we first designed a persistant and stable **NanoCAR** containing the **sdAb Nb17**. The specificity of **CT103a** and the NanoCAR CAR-T cells for BCMA-positive targets while sparing BCMA-negative cells, such as the K562 leukemia cell line, confirmed the potential of CT103a and NanoCAR as effective and selective therapeutic strategies for targeting **BCMA-expressing malignancies**. Morevoer, both CT103a and the NanoCAR demonstrated sustained **persistence** and **long-term efficacy**, suggesting their potential for prolonged tumor control. In addition, the **cytokine production** highlighted the robust cytotoxic potental and functional activity of CT103a and NanoCAR, confirming their functional readiness to engage and kill target. Also, according to overall bulk RNA seq data, while CT103a and NanoCAR demonstrated similar transcriptional profiles, their distinct gene signatures relative to Mock revealed shared mechanisms of tumor-specific activation. Thus, our nanoCART demonstrates excellent anticancer efficacy as CT103a *in vitro*. Further investigation *in vivo* in a mice model should finally validated *in vitro* results.

Acknowledgements

Members of the Laboratory of Hematology

Viral Vector platform (GIGA) – Flow Cytometry platform (GIGA) – RNA seq platform (GIGA)

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