# Pre-clinical efficacy of a novel anti-GPC3 *in vivo* CAR-M for hepatocellular carcinoma

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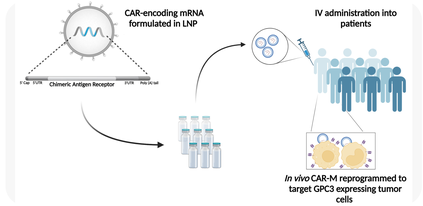
## Introduction

Chimeric antigen receptor macrophage (CAR-M) cell therapies have the potential to mediate robust anti-tumor immunity via phagocytosis, cytokine/chemokine release, activation of the tumor microenvironment (TME), T cell recruitment, and antigen presentation. Phase I data have demonstrated that autologous *ex vivo* CAR-M are well-tolerated, induce reprogramming of the solid TME, promote epitope spreading, and mediate anti-tumor activity [1].

Here, we have developed a novel off-the-shelf approach to directly reprogram endogenous myeloid cells *in vivo* by systemically delivering lipid nanoparticles (LNP) encapsulating mRNA encoding a CAR targeting glypican-3 (GPC3). GPC3 is a tumor-associated surface antigen that is overexpressed in hepatocellular carcinoma (HCC) with minimal expression on normal tissues. The CAR architecture was optimized to maximize antigen-dependent myeloid activation.

## Objectives

### mRNA/LNP for *In Vivo* Anti-GPC3 CAR-M Engineering




**Goal:** Develop an off-the-shelf *in vivo* strategy for reprogramming myeloid cells to elicit antigen-specific anti-tumor activity.

1. Identify an LNP encapsulated mRNA encoding anti-GPC3 CAR that induces GPC3 antigen-driven macrophage activation and anti-tumor activity.
2. Demonstrate pre-clinical anti-tumor efficacy *in vivo* by systemically delivering LNP encapsulated mRNA encoding CAR targeting GPC3.

## Methods

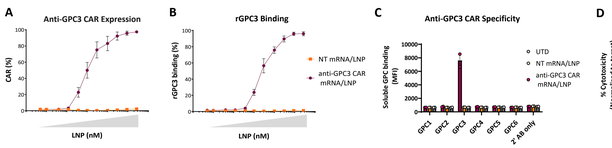
For *in vitro* assessment of anti-GPC3 CAR-M phenotype and function, primary human macrophages were transfected with anti-GPC3 CAR mRNA/LNP. Anti-GPC3 CAR expression, M1/M2 phenotype, rGPC3 protein binding, target cell killing, and cytokine production were assessed on days 1 or 7 post-transfection using flow cytometry, the Incucyte® Live Cell Analysis System, and multiplex Meso Scale Discovery (MSD) assay. Untreated macrophages (UTD) and macrophages transfected with non-translating (NT) mRNA were included as control groups.

Soluble GPC3 protein in serum from 10 healthy donors and 70 HCC patients was measured with ELISA. The effect of soluble rGPC3 on the anti-GPC3 CAR-M binding to target cells and killing activity was measured using flow cytometry and the Incucyte® Live Cell Analysis System, respectively.

The efficacy and tolerability of anti-GPC3 CAR mRNA/LNP were evaluated *in vivo* in CD34⁺ HSC humanized NSG-S and C57BL/6 syngeneic GPC3+ solid tumor mouse models.

### mRNA/LNP transfection generated target-specific and highly functional anti-GPC3 CAR-M *in vitro*

Engineering human macrophages with anti-GPC3 CAR mRNA/LNP led to titratable CAR expression and rGPC3 binding on day 1 (**A** and **B**). Anti-GPC3 CAR expressing human macrophages are highly specific to GPC3 antigen (**C**). Anti-GPC3 CAR-M exhibits cytotoxicity against HEPG2 target cells that positively correlates with CAR expression (Spearman r:0.8531, p-value: 0.0008) (**D**).




[1] Abdou Y, Dees EC, Mortimer JE. A phase-1, first-in-human (FIH) study of autologous macrophages engineered to express an anti-HER2 chimeric antigen receptor (CAR) in participants (pts) with HER2-overexpressing solid tumors. J. Clin. Oncol. 2023; 41: 16 suppl.

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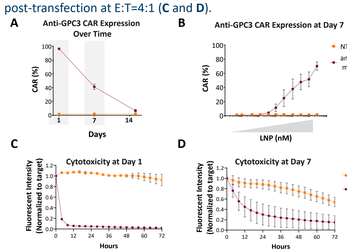
Disclosure: Zhen Zhang, Christine Lukacs, Lin Guey and Simone Mori are employees of Moderna, Inc. and may hold stock/stock options in the company.

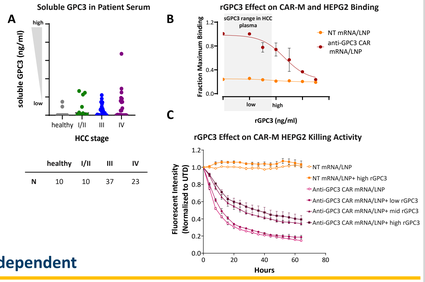
### Kinetics of CAR expression and anti-GPC3 CAR-M tumor cell killing

Kinetics and dose-dependent expression of anti-GPC3 CAR after transfection with 9 nM (**A** ) or increasing concentration of LNP *in vitro* (**B**). Potent anti-GPC3 CAR-M killing against HEPG2 cells on day 1 and day 7 post-transfection at E:T=4:1 (**C** and **D**).



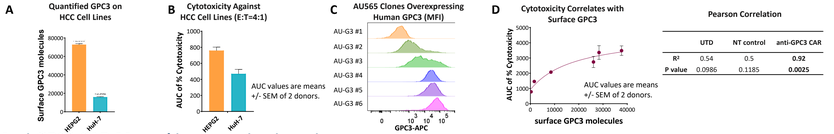

### rGPC3 does not inhibit CAR-M activity at physiologically relevant concentrations

Quantification of serum GPC3 in HCC patients (**A**). High soluble [GPC3] reduces anti-GPC3 CAR-M/HEPG2 interaction (**B**) or anti-GPC3 CAR-M cytotoxicity at E:T=4:1 (**C**).




### Cytotoxic activity of anti-GPC3 CAR-M is antigen density-dependent

Anti-GPC3 CAR-M cytotoxic activity was evaluated *in vitro* using HCC cell lines with endogenous GPC3 expression (**A** and **B**) and AU565 cells overexpressing different levels of surface human GPC3 (**C**). Cytotoxicity, represented as area under the curve (AUC), positively correlates with target GPC3 expression E:T=1:1 (**D**).

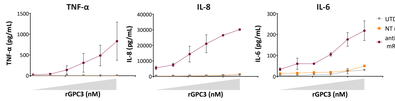



Pearson Correlation

| | UTD | NT control | anti-GPC3 CAR |
|---|---|---|---|
| $R^2$ | 0.54 | 0.5 | **0.92** |
| P value | 0.0986 | 0.1185 | **0.0025** |

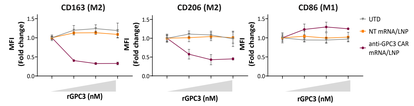
### Anti-GPC3 CAR-M cytokine secretion is antigen dependent

rGPC3 stimulation leads to dose-dependent pro-inflammatory cytokine secretion by anti-GPC3 CAR-M.



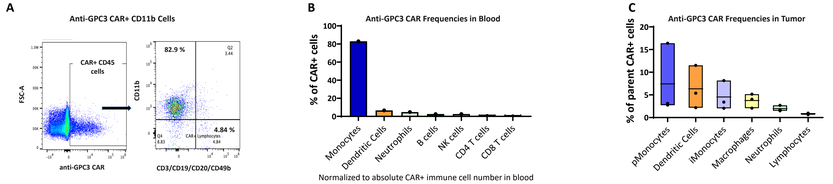

### Ag stimulation polarizes CAR-M to an M1-phenotype

rGPC3 stimulation increases M1 (CD86) and decreases M2 (CD163, CD206) surface markers on anti-GPC3 CAR-M.



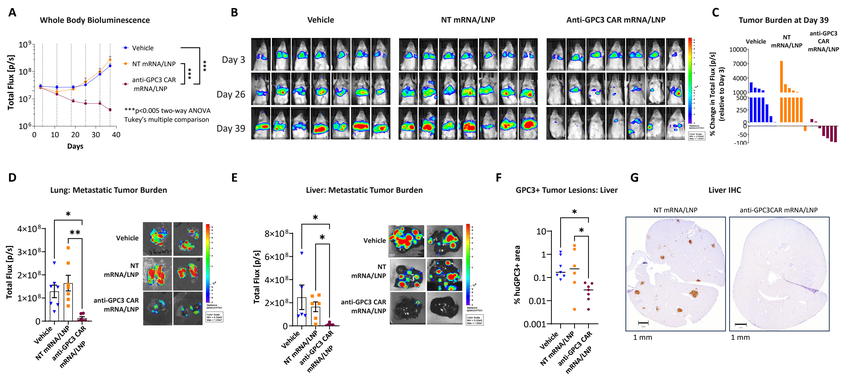

### Myeloid cells are the primary CAR+ immune cells *in vivo*

CD45+ immune cells were isolated from blood (**A** and **B**) or subcutaneous MC38_GPC3 tumors (**C**) of C57BL/6 mice. Flow analysis demonstrated that anti-GPC3 CAR expression is primarily expressed in monocytes in the blood (**A** and **B**) and myeloid cells in the tumor (**C**). pMonocytes = patrolling monocytes; iMonocytes = inflammatory monocytes.



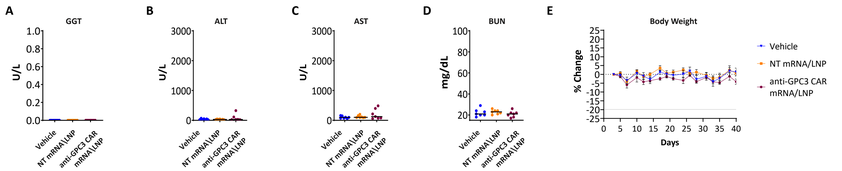

### *In vivo* anti-GPC3-CAR mRNA/LNP treatment leads to significant control of disseminated tumor growth

Weekly dosing of anti-GPC3 CAR mRNA/LNP starting day 4 in i.v. PANC-1_GPC3 engrafted CD34⁺ HSC humanized NSG-S mice inhibits tumor growth (**A, B** and **C**) and reduces tumor burden in lung (**D**) and in liver (**E, F** and **G**) compared to NT mRNA/LNP.




### *In vivo* anti-GPC3-CAR mRNA/LNP treatment does not impact liver and kidney function or body weight

Systemic intravenous delivery (six weekly doses) of anti-GPC3 CAR mRNA/LNP does not increase the levels of serum biomarkers for liver and kidney function ( **A**, **B**, **C** and **D**). No adverse events or changes in body weight are observed in any groups (**E**)




## Conclusions

Anti-GPC3 CAR-M have a high affinity for human GPC3 without cross-reactivity to other members of the GPC protein family.

LNP transfected human macrophages express anti-GPC3 CAR for more than 7 days *in vitro* and exhibit Ag-specific and dose-dependent tumor cell cytotoxicity, inflammatory cytokine release, and polarization toward a pro-inflammatory phenotype.

Physiological concentrations of soluble GPC3 in HCC patients does not interfere with anti-GPC3 CAR-M cytotoxicity.

Systemic administration of anti-GPC3 CAR mRNA/LNP induces robust anti-tumor activity in humanized metastatic solid tumor models without toxicity.

Direct *in vivo* reprogramming of endogenous myeloid cells with CARs using mRNA/LNP technology is a promising off-the-shelf therapy for patients with advanced solid tumors, including those with advanced hepatocellular carcinoma.