Design and characterization of novel nanobody-conjugated lipid nanoparticles for targeted *in vivo* CAR-T therapy

Miina Ojansivu[1,2,3], Yanjie Huang[1,2,3], Oliver Hayes[1,2,3], Ruben De Coen[4], Sabah Kasmi[4], Stefaan De Koker[4], Samir El Andaloussi[1,2,3]

(1) Biomolecular and Cellular Medicine, Department of Laboratory Medicine, Karolinska Institute, Stockholm, Sweden, (2) Karolinska ATMP Center, Karolinska Institute, Stockholm, Sweden, (3) Department of Cellular Therapy and Allogeneic Stem Cell Transplantation (CAST), Karolinska University Hospital, Stockholm, Sweden, (4) eTheRNA Immunotherapies, Gent, Belgium

INTRODUCTION

Lipid nanoparticles (LNPs) have emerged as a promising platform for *in vivo* CAR-T therapy by enabling direct delivery of CAR-encoding nucleic acids to T-cells inside the patient, bypassing the need for complex and expensive *ex vivo* cell manufacturing [1]. However, effective LNP targeting remains challenging due to the LNP tendency to accumulate in the liver. Here we aimed to identify LNP compositions allowing for increased T-cell delivery and further boosted the specificity by decorating with anti-CD8 nanobodies. Moreover, we aimed to evaluate the effect of nanobody density on LNP targeting and delivery. The outline of the project is depicted in **Fig. 1**.

OUTLINE

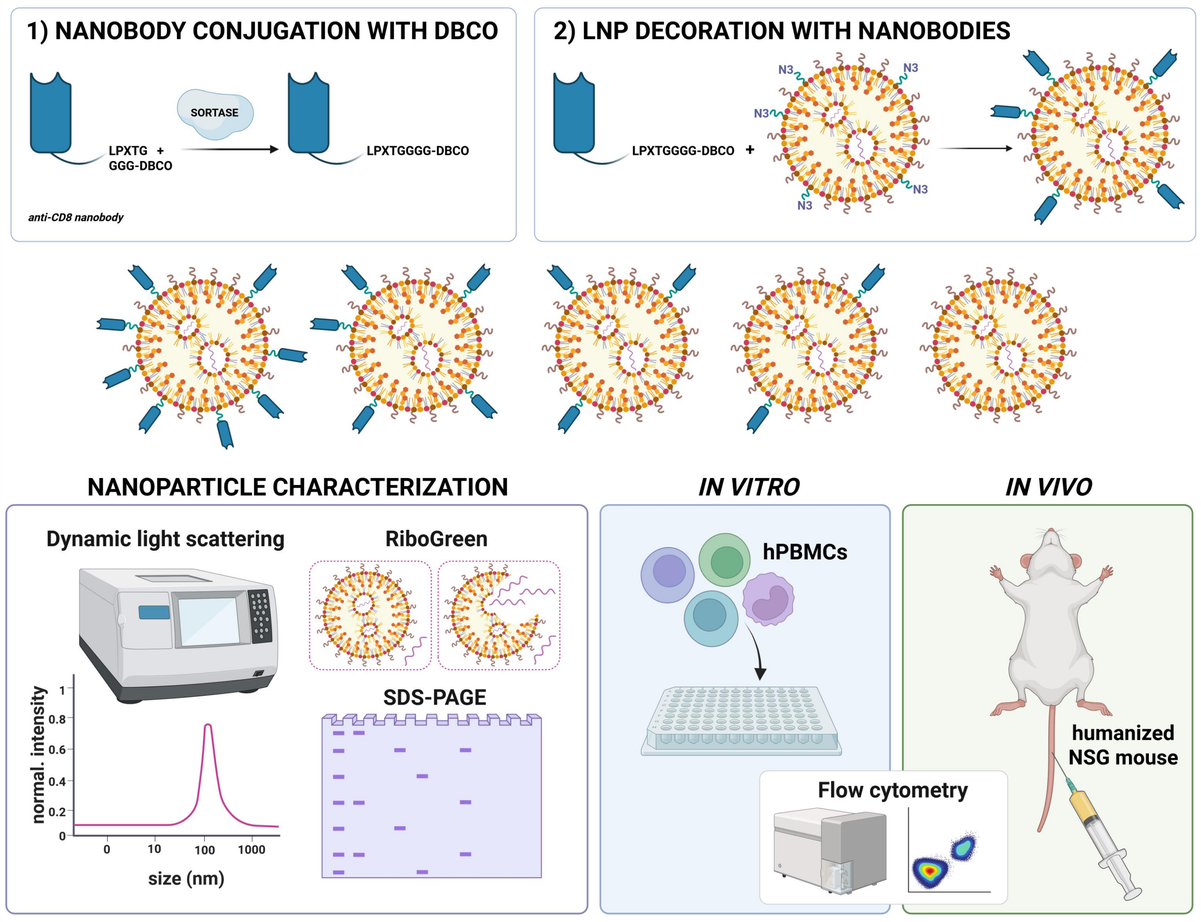

Figure 1. *Outline of the project. LNP = lipid nanoparticle, hPBMC = human peripheral blood mononuclear cell. Image created in BioRender.*

METHODS

Lipid nanoparticles with two novel ionizable lipids, ETS-29 and ETS-14, were formulated with a microfluidic mixing method. Anti-CD8 nanobody containing a sortase tag was conjugated to DBCO in a sortase-mediated reaction, and then covalently clicked onto the surface of the LNPs containing a range of different mol-% (0-0.7) of DSPE-PEG2000-azide handles. Unreacted nanobody was removed using Capto Core 700 beads. Size and polydispersity of the LNPs were characterized with dynamic light scattering and the cargo mRNA loading efficiency by RiboGreen assay. Nanobody conjugation efficiency was evaluated with SDS-PAGE. LNP targeting and delivery were studied *in vitro* using human peripheral blood mononuclear cells (hPBMCs) and *in vivo* using humanized NSG mice.

RESULTS

Nanobody density on the LNP surface can be controlled by varying the amount of DSPE-PEG2000-azide (Fig. 2)

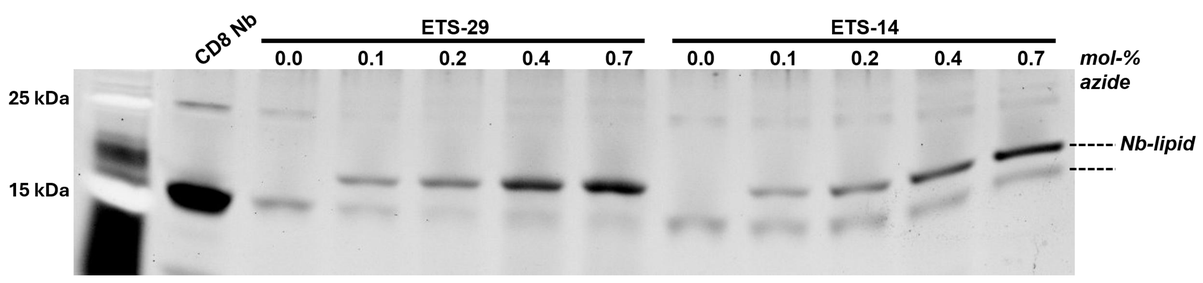

Figure 2. *Evaluation of the nanobody (Nb) conjugation on the LNP surface using SDS-PAGE and SYPRO Ruby staining. 1:1 mass ratio of Nb:mRNA. 2h conjugation at room temperature, followed by removal of free Nb using Capto Core 700 beads.* ***The extent of conjugation increases as a function of azide mol-% in the LNPs.*** *ETS-14 LNPs demonstrate more unspecific Nb binding as seen in the higher amount of free Nb.*

Nanobody-conjugation does not compromise the quality of the LNPs (Fig. 3)

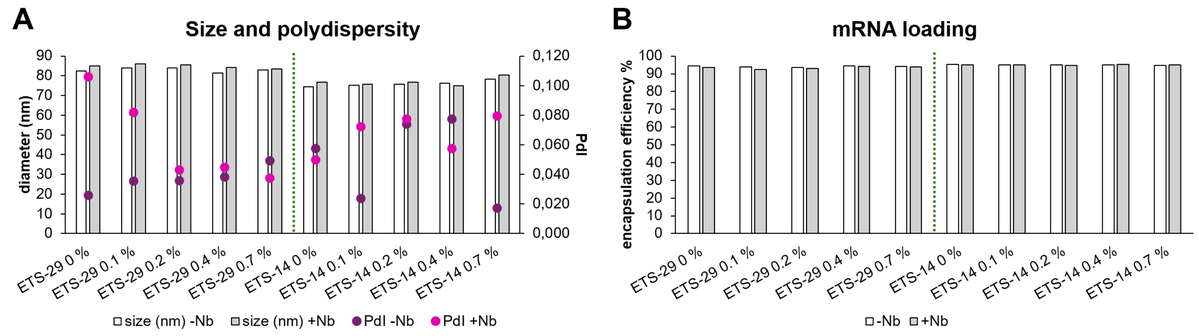

Figure 3. *LNP quality after the nanobody conjugation.* ***A.*** *LNP size and polydispersity and* ***B.*** *mRNA cargo loading into LNPs before and after nanobody (Nb)-conjugation. PdI = polydispersity index.*

Nanobody-decorated LNPs specifically target CD8+ *in vitro* independent of the nanobody density (Fig. 4)

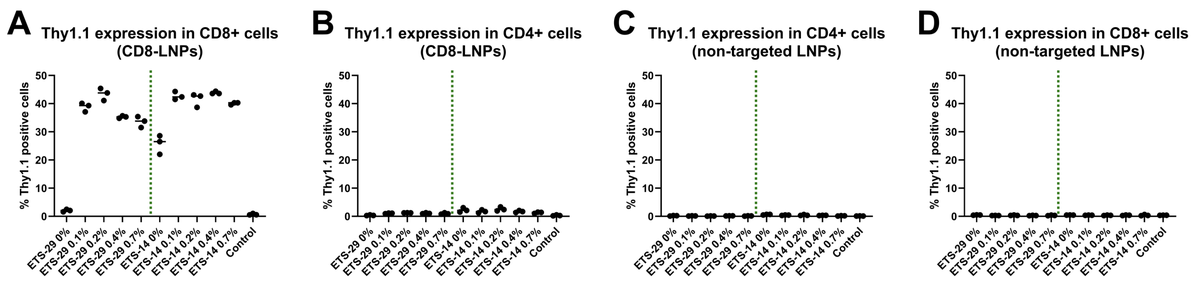

Figure 4. *The functionality of targeted LNPs in vitro.* ***A.*** *The % of Thy1.1 positive CD8+ T-cells after 24h treatment with CD8-LNPs.* ***B.*** *The % of Thy1.1 positive CD4+ T-cells after 24h treatment with CD8-LNPs.* ***C.*** *The % of Thy1.1 positive CD8+ T-cells after 24h treatment with non-decorated LNPs.* ***D.*** *The % of Thy1.1 positive CD4+ T-cells after 24h treatment with non-decorated LNPs. Thy1.1 mRNA was used as a model cargo. % = mol-% DSPE-PEG2000-azide. Dose 300 ng/10^5 cells. N = 3.*

Nanobody-decorated LNPs specifically target CD8+ cells *in vivo* and induce tumor cell killing in a mouse model (Fig. 5)

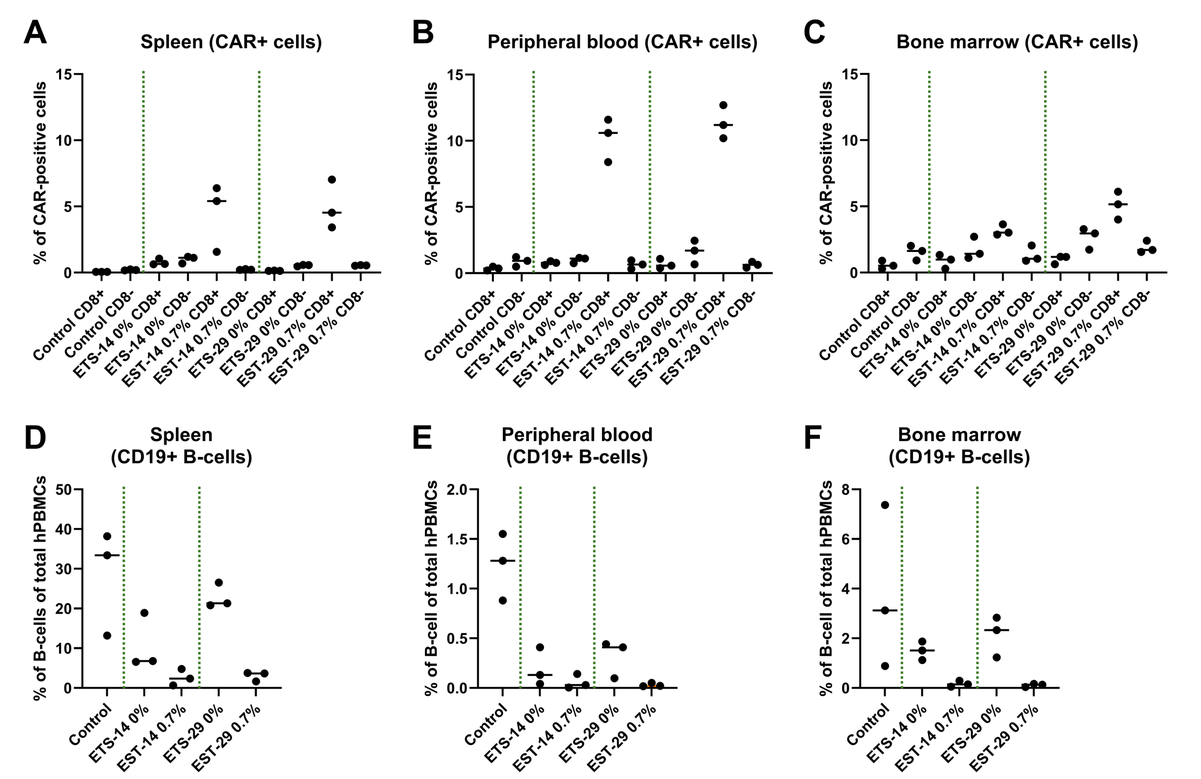

Figure 5. *The functionality of targeted LNPs in vivo. The % of CAR-positive T-cells (CD8-/+) in* ***A.*** *spleen,* ***B.*** *peripheral blood and* ***C.*** *bone marrow after 24h of LNP administration i.v. at 1 mg/kg dose of CD19-CAR mRNA. CAR-mediated killing of CD19+ B-cells in* ***D.*** *spleen,* ***E.*** *peripheral blood and* ***F.*** *bone marrow. B-cell depletion is more pronounced with targeted LNPs and the ETS-29 lipid composition. Nb = nanobody. N = 3.*

CONCLUSIONS

- **The amount of targeting ligands** on the LNP surface **can be controlled by varying the mol-% of the click-chemistry handles** in the LNP composition
- **Nanobody conjugation on** the LNP surface at various densities **does not compromise the LNP quality**
- Anti-CD8 **nanobody conjugated LNPs** deliver their cargo **specifically** to CD8+ T-cells **independent of the nanobody density**
- The presented strategy leads to **highly specific delivery and tumor cell killing** followed by systemic administration ***in vivo*** **in mice**

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REFERENCES: [1] A Bot, A Scharenberg, K Friedman, L Guey et al. *Nat Rev Drug Discov.* 2026 Feb;25(2):116-137.

Contact: miina.ojansivu@ki.se