Scaling up mRNA-CAR-T cell manufacturing

A work-in-progress master thesis

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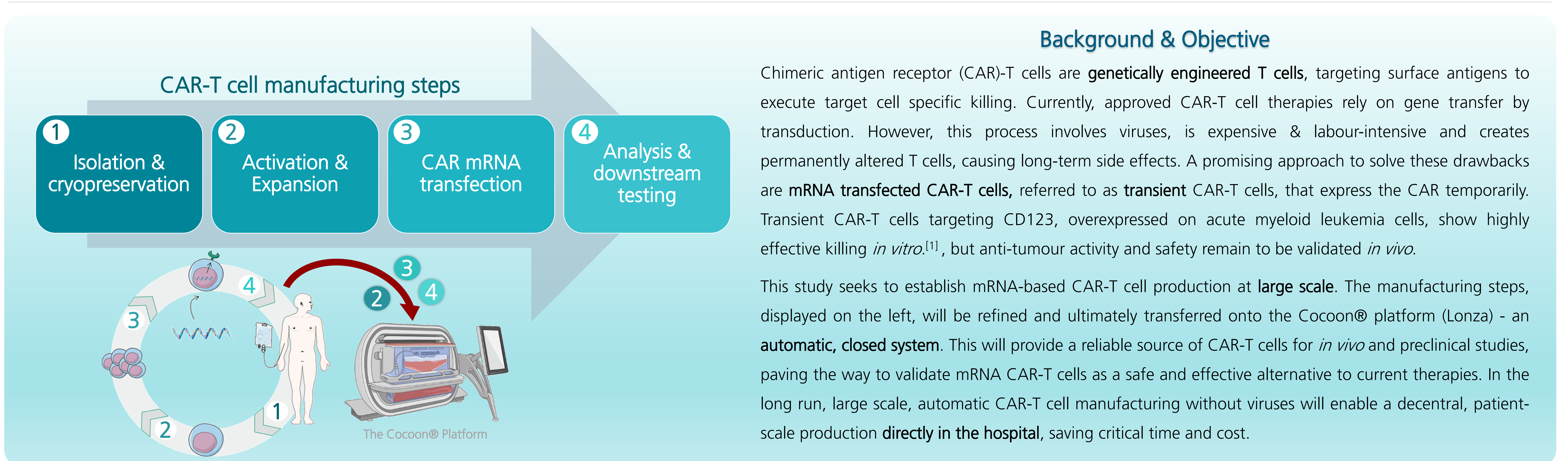


Background & Objective

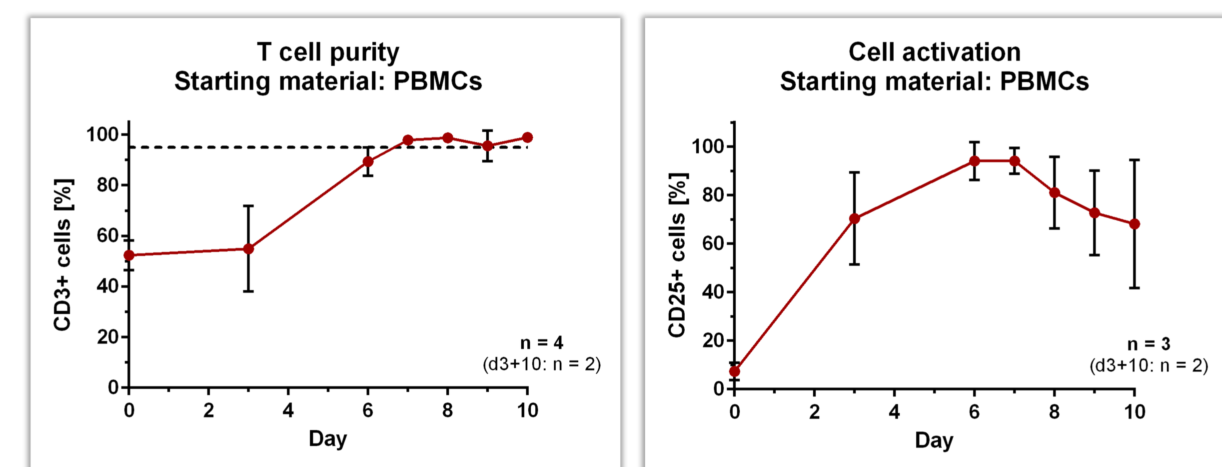
Chimeric antigen receptor (CAR)-T cells are **genetically engineered T cells**, targeting surface antigens to execute target cell specific killing. Currently, approved CAR-T cell therapies rely on gene transfer by transduction. However, this process involves viruses, is expensive & labour-intensive and creates permanently altered T cells, causing long-term side effects. A promising approach to solve these drawbacks are **mRNA transfected CAR-T cells,** referred to as **transient** CAR-T cells, that express the CAR temporarily. Transient CAR-T cells targeting CD123, overexpressed on acute myeloid leukemia cells, show highly effective killing *in vitro*.[1] , but anti-tumour activity and safety remain to be validated *in vivo*.

This study seeks to establish mRNA-based CAR-T cell production at **large scale**. The manufacturing steps, displayed on the left, will be refined and ultimately transferred onto the Cocoon® platform (Lonza) - an **automatic, closed system**. This will provide a reliable source of CAR-T cells for *in vivo* and preclinical studies, paving the way to validate mRNA CAR-T cells as a safe and effective alternative to current therapies. In the long run, large scale, automatic CAR-T cell manufacturing without viruses will enable a decentral, patient-scale production **directly in the hospital**, saving critical time and cost.

Results

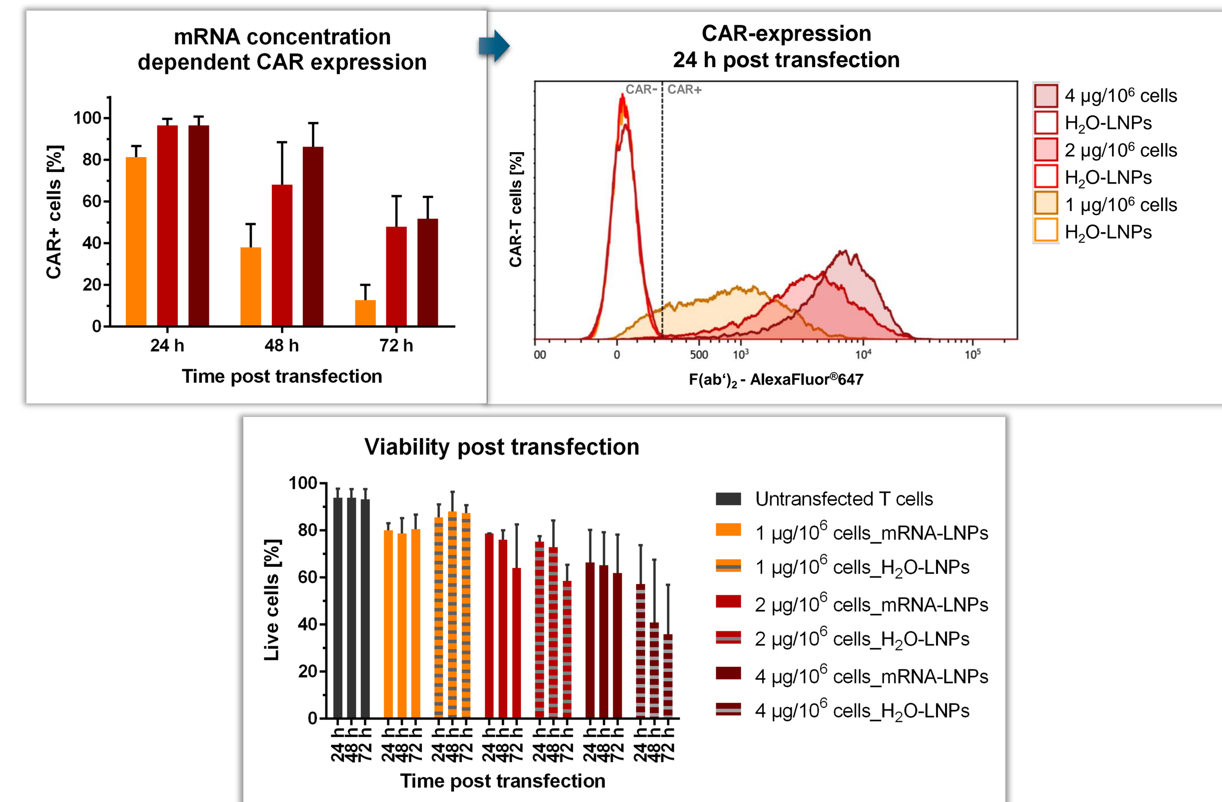
1. PBMCs and T cells are feasible for T cell expansion with TheraPEAK T-VIVO® culture medium

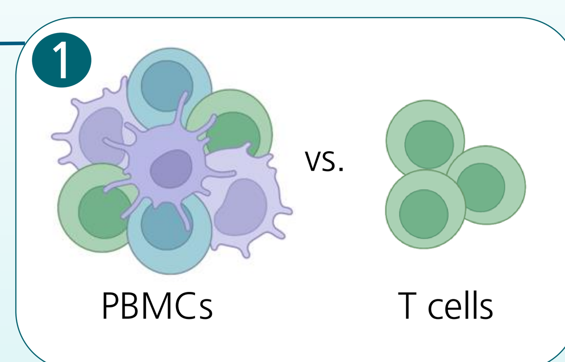
TheraPEAK® T-VIVO® medium allows T cell selection and expansion from thawed **peripheral blood mononuclear cells** (PBMCs), eliminating the need for prior T cell selection. As shown below, a **T cell purity >95%** (left, dashed line) is reached **by day 7**. Cell activation was assessed by analyzing CD25 expression, which peaked on days 6 and 7. Consequently, day 7 is an efficient time point for T cell transfection when using PBMCs. Starting with preselected T cells, a **high activation state and a high cell number** (data not shown) were key parameters to define transfection on **day 6** at the earliest.[2] The Cocoon allows to use both starting materials, but with PBMCs a monocyte depletion may be necessary. Therefore, experiments were continued with pre-isolated, **thawed T cells**.



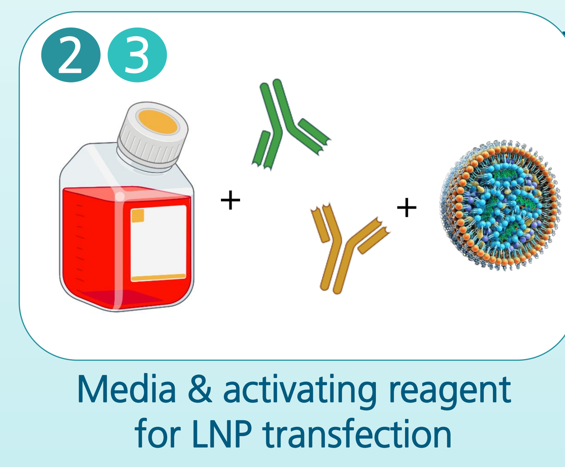
3. mRNA optimization and concentration-dependent CAR expression

To optimize an established anti-CD123 CAR construct for large scale mRNA manufacturing, the DNA template was modified to enable *in vitro* **transcription, 5' capping, and 3' polyadenylation** in a single reaction. Additionally, **untranslated regions** (UTRs) were integrated to enhance mRNA stability.[4] Adapting to clinical standards, **high performance liquid chromatography** (HPLC) was used to purify the mRNA. Concentration dependent viability (bottom, n = 2) and CAR expression (top, n = 2) was tested. As the best compromise with respect to a high viability paired with high and durable CAR expression, **2 µg/10⁶ cells** will be used for subsequent upscaling experiments.

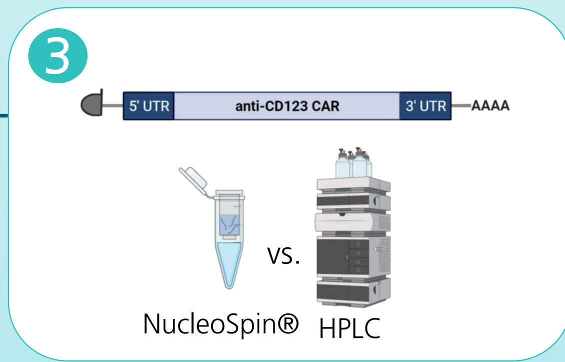




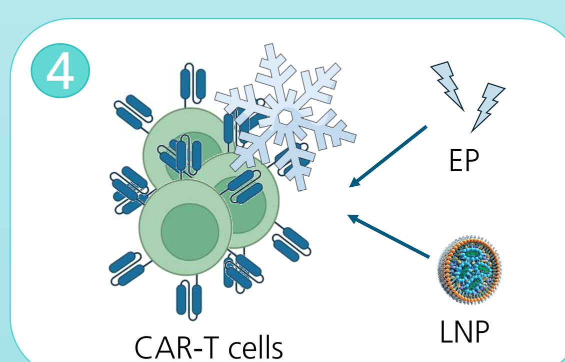
Starting material



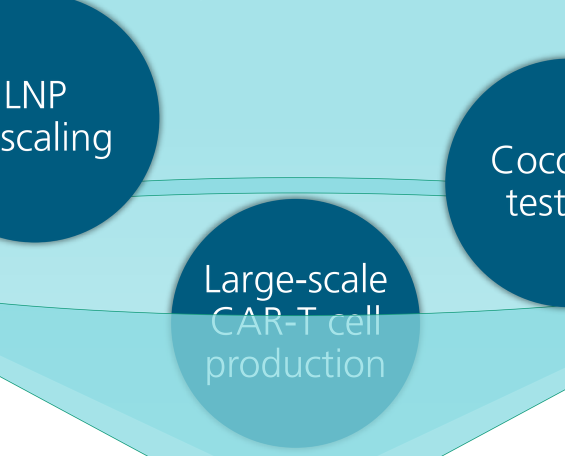
Media & activating reagent for LNP transfection



mRNA optimization

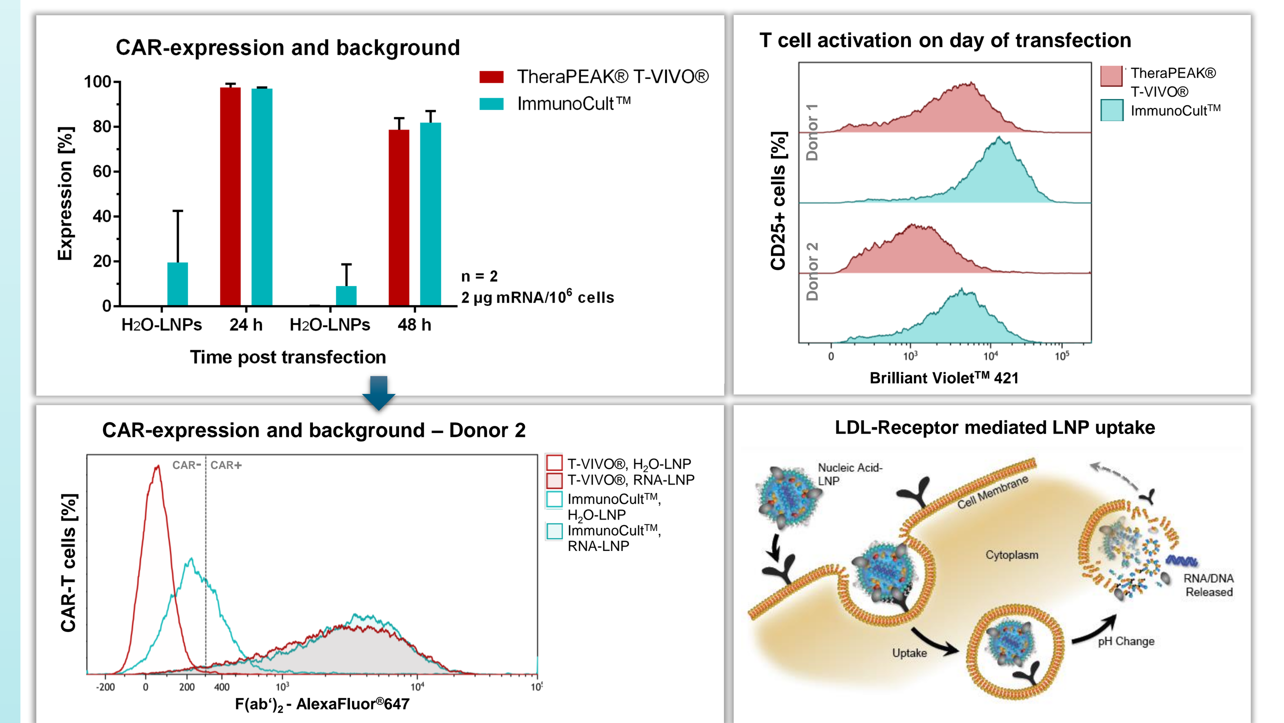


Cryopreservation



2. The optimal culture medium for mRNA-LNP transfection

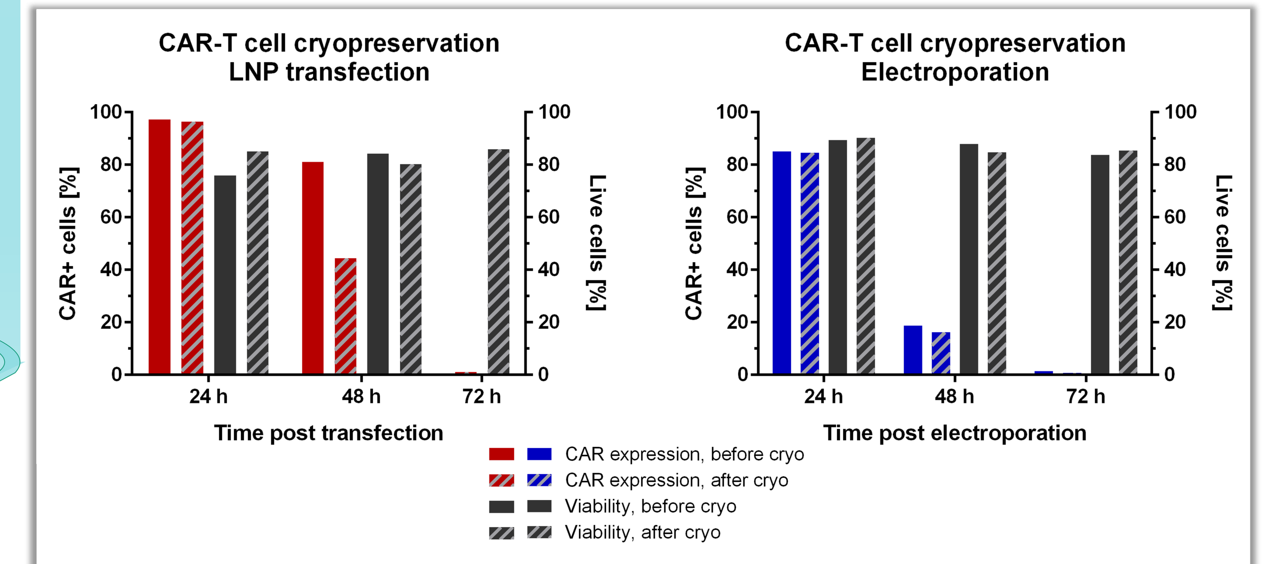
Lipid nanoparticles (LNPs) were shown to be the superior, more gentle CAR-mRNA delivery method when compared to electroporation (EP).[1] LNP transfection relies on the **uptake of mRNA loaded LNPs** via the low-density lipoprotein receptor (LDL-R), followed by endosomal escape of the mRNA (right, bottom).[3] Consequently, a **high cell activation** on the day of transfection is crucial as it correlates with increased lipid metabolism and therefore with a high LDL-R density, promoting LNP uptake. Two different media with the recommended activating reagents were compared for LNP transfection. Based on a similarly high CAR expression (left, top) and a comparable T cell activation (right, top), our findings indicate that both protocols are suitable for LNP transfection. However, based on the absence of a **CAR background signal** (left, top & bottom), **superior viability** (data not shown) and **GMP-compliance**, TheraPEAK® T-VIVO® is preferred for upscaling.



GenVoy-ILM - Non-Viral LNP Delivery of RNA (precisionnanosystems.com), 03.11.2023

4. Transient CAR expression can be cryopreserved

Cryopreservation experiments were performed to assess **storage** possibilities and plan subsequent *in vivo* studies. LNP transfected CAR-T cells were compared with electroporated CAR-T cells. First findings suggest that transient CAR expression can be cryopreserved if frozen 24 h after LNP (left) or EP (right) for a duration of three to four weeks. However, the CAR expression appears to **decrease more rapidly after thawing**, possibly as a result of additional handling steps before and after cryopreservation. Further testing is required to validate the results.



Summary & Outlook

Based on the experiments presented, preselected frozen T cells were chosen as the starting material (1.) and TheraPEAK® T-VIVO® T cell medium with associated reagents for T cell culture and transfection (2.) to scale up mRNA-CAR-T cell production. Additionally, an anti-CD123-CAR construct was optimized to facilitate and accelerate mRNA production, and the mRNA to cell ratio was evaluated for subsequent experiments (3.). Consequently, the next steps will involve:

- **Enlarging data sets** to adjust for biological variability, validate the results and prove reproducibility
- Performing a **T cell expansion run on the Cocoon® Platform**, monitoring cell viability, expansion and activation
- **Scaling up mRNA-LNP production** from the NanoAssemblr® Spark™ onto the NanoAssemblr® Ignite™ (Precision NanoSystems)

➢ Automated, closed-system CAR-T cell manufacturing on the Cocoon® Platform with analysis of CAR-expression & *in vitro* cytotoxicity to compare small- and large-scale efficacy

Illustrations created with BioRender.com

[1] Kitte, R.; Rabel, M.; Geczy, R. et al.: **Lipid Nanoparticles (LNPs) outperform Electroporation in mRNA-based CAR-T cell Engineering.** *Molecular Therapy - Methods & Clinical Development* (2023), S. 101139. DOI: *10.1016/j.omtm.2023.101139.*

[2] Auw, N. von; Serfling, R.; Kitte, R. et al.: **Comparison of two lab-scale protocols for enhanced mRNA-based CAR-T cell generation and functionality.** *Scientific reports* (2023) 13 (1), S. 18160. DOI: *10.1038/s41598-023-45197-x.*

[3] **GenVoy-ILM - Non-Viral LNP Delivery of RNA** (2023). *https://www.precisionnanosystems.com/platform-technologies/genvoy-platform/genvoy-ilm,* checked on 03.11.2023.

[4] Orlandini von Niessen, A. G.; Poleganov, M. A.; Rechner, C. et al.: **Improving mRNA-Based Therapeutic Gene Delivery by Expression-Augmenting 3' UTRs Identified by Cellular Library Screening.** *Molecular therapy : the journal of the American Society of Gene Therapy* (2019) 27 (4), S. 824–836. DOI: *10.1016/j.ymthe.2018.12.011.*

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