mRNA Lipid Nanoparticles for *ex vivo* Engineering of Primary T cells for Autoimmunity Applications

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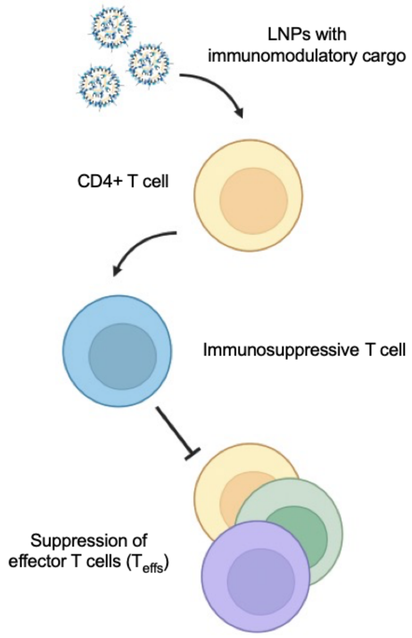
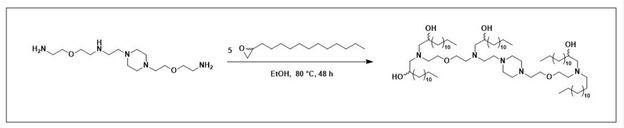
INTRODUCTION

Autoimmune diseases affect approximately 5% of the world's population. Emerging therapies for autoimmune diseases have centered around engineering regulatory T cells (Tregs) – the most well-known suppressive immune cell in the body. However, Tregs account for only ~1-4% of the total immune cells found in the blood, making them difficult to harvest and grow up to numbers suitable for adoptive cell therapies. Thus, scientists have turned to the larger subgroup of CD4+ T cells and have attempted to engineer them by delivering the hallmark transcription factor Forkhead Box Protein 3, or Foxp3, that is known to be of utmost importance in suppressive function.[1] However, Foxp3 is incapable of crossing the cell membrane and the delivery methods being used have high risks of cytotoxicity and genotoxicity. Here, we utilize a lipid nanoparticle (LNP) platform to deliver Foxp3 mRNA to CD4+ T cell to engineer them to be functionally suppressive.



METHODS

Using a well-established SN2-based reaction shown below, a library of 18 novel ionizable lipids was synthesized.[2] This library, shown in **Figure 1A**, consisted of 3 polyamine cores containing alkyl spacers and 3 cores containing ether spacers, which were reacted with three lengths of epoxide tails – C12, C14 and C16.



The ionizable lipids were combined with cholesterol, a helper lipid (DOPE) and lipid-PEG using microfluidic mixing to formulate LNPs encapsulating either mCherry reporter mRNA or Foxp3 (variant 2) mRNA shown in **Figure 1B**.

LNPs with Foxp3 mRNA were utilized to engineer **functional, immunosuppressive T cells.** Functionality was assessed using *ex vivo* suppression assays and enzyme-linked immunosorbent assays (ELISAs).

RESULTS and DISCUSSION

1. FORMULATION OF NOVEL LNP LIBRARY

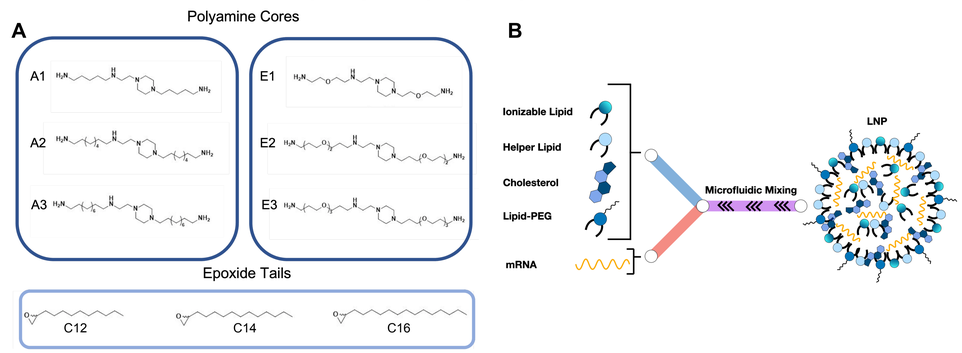


Figure 1. Ionizable lipid library and LNP formulation. (A) A library of 18 unique ionizable lipids composed of 6 polyamine cores with either alkyl (A1-A3) or ether (E1-E3) spacers, each reacted with one of three epoxide tails of lengths C12, C14 or C16. (B) Schematic of LNP formulation. The ionizable lipids, helper lipid (DOPE), cholesterol and lipid-anchored PEG were combined with mRNA using microfluidic mixing to form LNPs ranging from 65-100 nm in diameter.

2. EX VIVO SCREEN REVEALS TOP-PERFORMING LNP IN CD4+ T CELLS

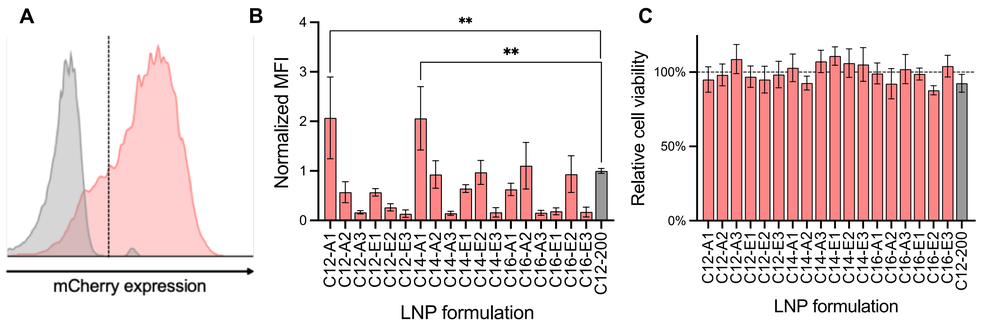


Figure 2. LNP screening in CD4+ T cells *ex vivo*. (A) Representative histogram of mCherry expression assessed via flow cytometry. (B) Screen of LNP library reveals two formulations resulting in above a 2-fold increase in mCherry MFI compared to C12-200 control. **p-value < 0.01 after a one-way ANOVA with Holms-Šídák correction (C) Relative cell viability after dosing with LNP library.

Led to the identification of **C14-A1 as the top-performing LNP**, which was used in the subsequent studies.

3. TOP-PERFORMING LNP LEADS TO GENERATION OF FOXP3+ T CELLS (FP3T)

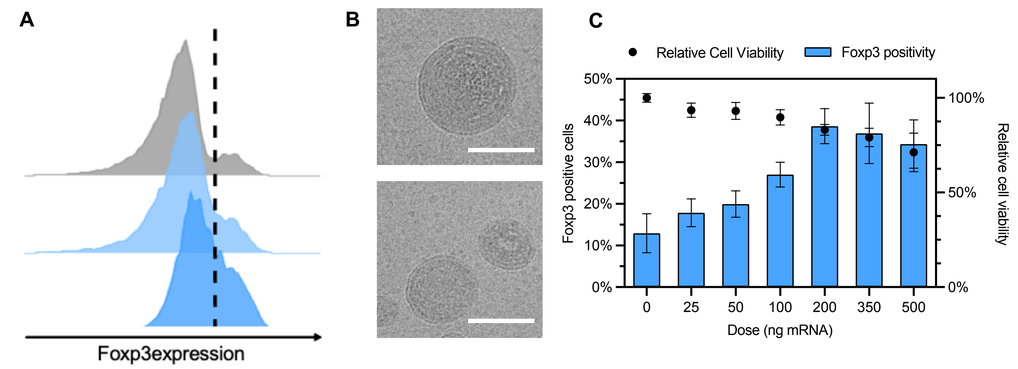


Figure 3. Generation of Foxp3+ T cells *ex vivo*. (A) Representative histogram of Foxp3 expression at doses of 0, 50 and 350 ng mRNA/100,000 cells. (B) Cryo-TEM images of LNPs reveal no major changes in morphology when LNPs are formulated with Foxp3 mRNA (bottom) instead of mCherry mRNA (top). Scale bar is 50 nm. (C) Intracellular expression of Foxp3 at doses ranging from 0-500 ng/100,000 cells and the corresponding viability.

By assessing the dose-dependent expression of Foxp3 intracellularly and comparing it to the relative cell viability, we were able **the optimal Foxp3 mRNA LNP dose** that was used in the subsequent functional assays.

4. FP3T CELLS SUPPRESS PROLIFERATION OF T_{EFF} CELLS

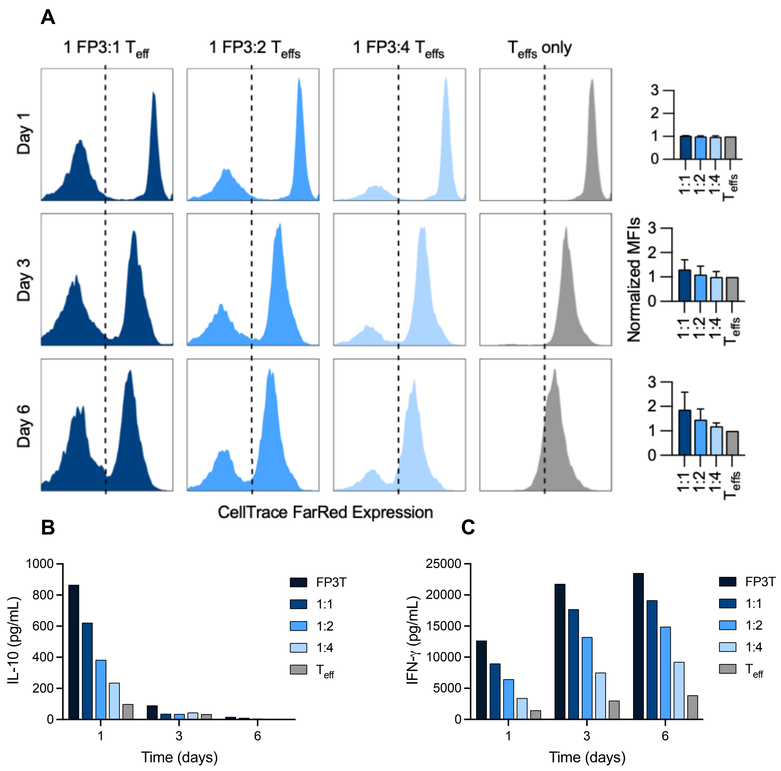


Figure 4. Testing the functional suppressive ability of FP3T cells. (A) Co-culture suppression assay of varying ratios of FP3T cells to T_{effs} (CD4/CD8 T cells) with normalized MFIs plotted on the right. (B and C) Concentrations of IL-10 (B) and IFN-ɣ (C) obtained from suppression assay supernatants.

High IL-10 and IFN-ɣ concentrations in the FP3T cell population indicate that FP3T cells may **possibly be using cytokine-mediated suppression** to suppress effector T cell growth.

CONCLUSIONS and FUTURE WORK

Here, we were able to:

- **Develop an LNP platform** for efficient transfection of primary CD4+ T cells
- Deliver a transcription factor mRNA to **modulate immune cell function**
- **Engineer a functional, immunosuppressive T cell** with potential applications in autoimmunity therapies, allergies treatments, and beyond.

Future work will examine the effects of these immunosuppressive cells on other immune cells (DCs, macrophages) and expand to adoptive cell therapies into autoimmunity disease models

REFERENCES

[1] Janssens, et al. Cytotherapy 2022, 24(6), 659-672
[2] Billingsley et al. Nano Letters 2020 Mar 11, 20(3), 1578-1589

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