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mRNA Lipid Nanoparticles for *Ex Vivo* Engineering of Immunosuppressive T Cells for Autoimmunity Therapies

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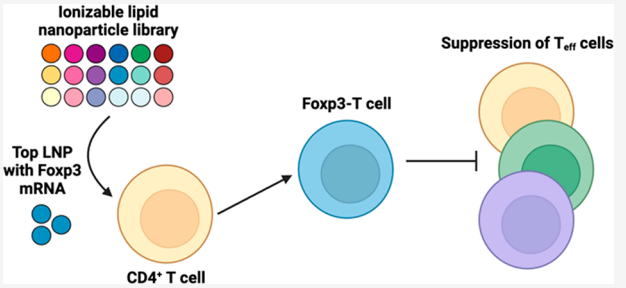
ABSTRACT: Cell-based therapies for autoimmune diseases have gained significant traction, with several approaches centered around the regulatory T (T_{reg}) cell—a well-known immunosuppressive cell characterized by its expression of the transcription factor Foxp3. Unfortunately, due to low numbers of T_{reg} cells available in circulation, harvesting and culturing T_{reg} cells remains a challenge. It has been reported that engineering Foxp3 expression in $CD4^+$ T cells can result in a T_{reg}-like phenotype; however, current methods result in the inefficient engineering of these cells. Here, we develop an ionizable lipid nanoparticle (LNP) platform to effectively deliver Foxp3 mRNA to $CD4^+$ T cells. We successfully engineer $CD4^+$ T cells into Foxp3-T (FP3T) cells that transiently exhibit an immunosuppressive phenotype and functionally suppress the proliferation of effector T cells. These results demonstrate the promise of an LNP platform for engineering immunosuppressive T cells with potential applications in autoimmunity therapies.



KEYWORDS: *lipid nanoparticles, mRNA delivery, T cell engineering, Foxp3, autoimmune diseases*

Affecting approximately 5−7% of the world's population, autoimmune disorders have risen to be among the most prevalent chronic diseases across the globe.[1] These disorders (e.g., type 1 diabetes, myasthenia gravis, rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis) are mediated and propagated by the presence of self-reactive lymphocytes—B and T cells—that act in concert with one another by producing autoantibodies or by triggering self-reactive clonal expansion, respectively.[2−4] As such, autoimmune disorders have a systemic pathology and, therefore, can affect one or multiple organ systems, depending on the disease and progression.

The key downstream pathology that unites autoimmune disorders is the presence of inflammation.[2] As a result, therapies for autoimmune diseases have focused on using immunosuppressive medications such as small molecules, steroids, and biologics to mitigate symptoms of inflammation.[5−15] However, these therapies are highly nonspecific, resulting in systemic immunosuppression and increasing susceptibility for infection, and require frequent dosing, which is both costly and may result in drug resistance.[9−11,16] A safer emerging alternative for autoimmunity therapies is adoptive cell therapy, which utilizes cells derived from the patient's own body to achieve immunosuppression. These therapeutic cells are recognized by the patient's body as "self", limiting extreme side effects such as cytokine release syndrome and macrophage activation syndrome.[17−21] Additionally, these therapies can be engineered using antigen-specific cells to increase suppression specificity and localize the intended therapeutic effect.[22,23]

For autoimmune diseases, adoptive cell therapies have largely centered around the regulatory T (T_{reg}) cell, which engenders immunosuppression through various cell- and cytokine-mediated responses.[24−26] T_{reg} cells are primarily defined by the expression of Forkhead box protein 3 (Foxp3), which is the master transcription factor that mediates the expression of several key genes that confer T_{reg} cells with their suppressive capabilities.[27,28] Although T_{reg} cells offer great potential, utilizing them for therapeutic purposes remains a significant challenge. T_{reg} cells only compose approximately 5−10% of circulating peripheral blood mononuclear cells.[25] Furthermore, although generally considered to be $CD4^+$ and $CD25^+$, T_{reg} cells lack more specific surface markers that differentiate them from other T cell populations.[25,29] These hurdles make it difficult to harvest, purify, and grow T_{reg} cells to therapeutically relevant numbers. Although there are additional tissue-resident T_{reg} cells in nonlymphoid organs

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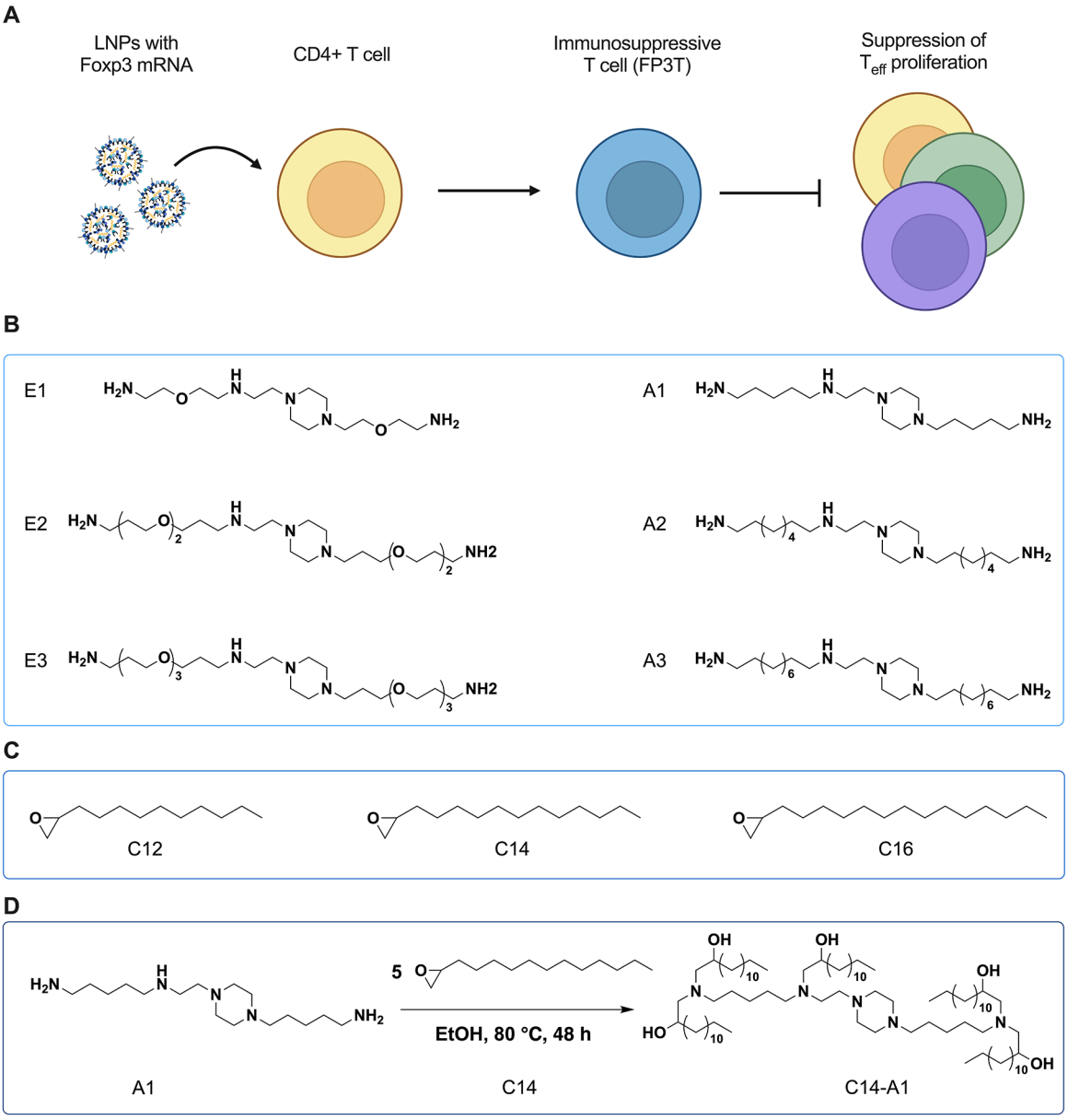

Figure 1. Development of a mRNA lipid nanoparticle (mRNA-LNP) platform for immunosuppressive Foxp3-T (FP3T) cell engineering. (A) Schematic of Foxp3 mRNA-LNPs delivered to $CD4^+$ T cells to generate Foxp3-T (FP3T) cells that can suppress effector T cell proliferation. (B) Chemical structures of six polyamine cores with either alkyl (A1−A3) or ether (E1−E3) spacers. (B) Chemical structures of epoxide tails with three varying carbon chain lengths—C12, C14, or C16. (C) Scheme of the S_N2 reaction chemistry used to synthesize each unique ionizable lipid.

such as in skeletal muscle and visceral adipose tissue, these T_{reg} cells are severely inaccessible and low in number.[30]

Several groups have attempted to engineer T_{reg} cells by delivering Foxp3 protein to $CD4^+$ T cells to achieve a T_{reg}-like immunosuppressive cell.[31−33] However, achieving adequate delivery of Foxp3 protein is difficult as it does not readily cross the cell membrane due to its complex tertiary organization and large size.[34−36] Although there have been attempts to engineer Foxp3 protein to be cell-permeable, delivering Foxp3 intranuclearly where it can readily perform its function as a transcription factor remains challenging.[33,37,38] Researchers have utilized gene therapy approaches to achieve Foxp3 delivery and expression, in particular using lentiviruses and adeno-associated viruses (AAVs) to either transduce T cells or genetically edit the Foxp3 locus.[31,32,39,40] While effective, viral delivery poses a number of challenges, including limited cargo capacity, off-target genomic integration and genotoxicity, and potential adverse immunological effects if administered *in vivo*.[39,41,42]

A potentially promising strategy to induce safe, efficient, and potent expression of Foxp3 is to use messenger RNA (mRNA).[38,43] mRNA is transiently expressed and does not pose any risk of genomic integration, unlike vector-based strategies.[44,45] Importantly, mRNA is translated in the cytoplasm and therefore does not require nuclear delivery in order to achieve a therapeutic effect. In terms of synthesis, mRNA can be customized using *in vitro* transcription and chemically modified to achieve stable and potent expression.[45,46] Finally, the size, charge, and relatively low complexity of mRNA allows it to be easily packaged into effective delivery systems such as ionizable lipid nanoparticles (LNPs). LNPs have proven to be a powerful delivery platform, particularly for

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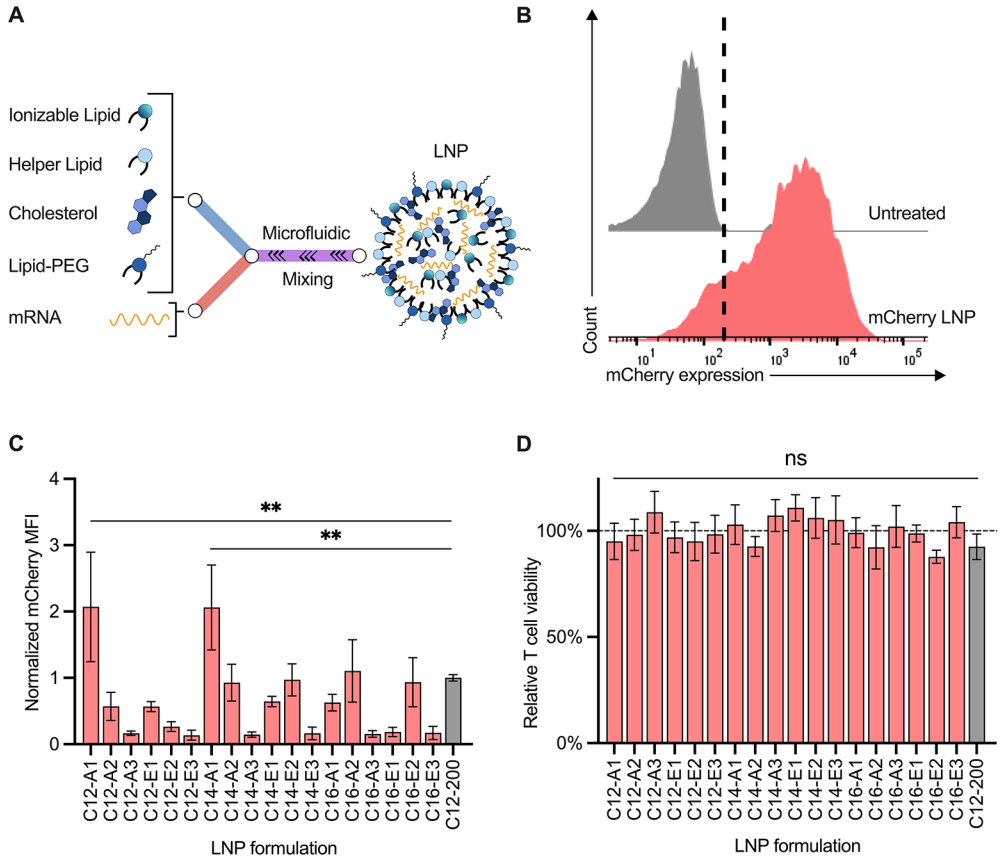

Figure 2. LNP formulation and evaluation of mRNA delivery to primary human CD4$^+$ T cells *ex vivo*. (A) Schematic of LNP formulation via microfluidic mixing, combining a lipid phase consisting of ionizable lipid, helper lipid (DOPE), cholesterol, and lipid-anchored PEG with an aqueous phase containing mRNA. (B) Representative histograms of mCherry fluorescence in primary human CD4$^+$ T cells 24 h post-transfection at an mRNA dose of 333 ng per 100,000 cells. (C) Screen of LNP library reveals two ionizable lipids (C12-A1, C14-A1) resulting in over a 2-fold increase in the mCherry MFI compared to the C12-200 LNP control. $^{**}p < 0.01$ in *post hoc t* tests with the Holm−Šídák correction. (D) Relative primary human CD4$^+$ T cell viability 24 h after transfection with LNPs at a dose of 333 ng per 100,000 T cells. Data represent $n = 3$ biological replicates for all experiments.

mRNA, as demonstrated by the recent success of the COVID-19 mRNA vaccines.[47−49] LNPs offer several benefits, including the ability to tune their composition, size, and charge, modify their surface with targeting ligands, and effectively encapsulate a variety of cargoes.[50−54] By tuning these parameters, LNPs can be modified based on their specific disease or therapeutic application.[55−60] Thus, LNPs offer a modular strategy to achieve efficient mRNA delivery for the desired application of T cell immunomodulation.

Herein, we engineered an LNP platform to deliver a variant of Foxp3 mRNA to CD4$^+$ T cells to generate immunosuppressive Foxp3-T (FP3T) cells for applications in autoimmunity (Figure 1A). We first screened a novel LNP library to identify a top-performing LNP formulation for the delivery of mRNA to primary human CD4$^+$ T cells. We then reformulated this LNP to deliver Foxp3 mRNA to primary human CD4$^+$ T cells to generate FP3T cells. Finally, we demonstrated using a coculture suppression assay that FP3T cells exhibit the desired immunosuppressive phenotype and functionally suppress effector T cell proliferation. Together, our results demonstrate the potential of using mRNA LNPs to engineer immunosuppressive cell-based therapies for applications in autoimmune disease therapies.

DESIGN AND CHARACTERIZATION OF LNP LIBRARY

To engineer FP3T cells, we first investigated ionizable lipid nanoparticles (LNPs) as a platform for mRNA delivery to CD4$^+$ T cells by screening a novel library of ionizable lipids with structural analogues that previously resulted in potent mRNA delivery to immune cells.[53,61−63] We synthesized 18 unique ionizable lipids for this library using a well-established S_N2-based reaction mechanism, where six polyamine cores containing either ether or alkyl spacers were reacted individually with one of three epoxide-terminated alkyl chains of three different lengths (Figure 1B−D). These ionizable lipids were then combined with three common lipid excipients—dioleoylphosphoethanolamine (DOPE), cholesterol, and lipid-anchored polyethylene glycol (PEG). Each excipient assists with overall LNP structure and stability; specifically, DOPE strengthens the lamellar structure and promotes endosomal escape, cholesterol enhances LNP membrane stability, and PEG allows for reduced LNP aggregation.[55,64−,66] The molar ratio of excipients—35%, 16%, 46.5%, and 2.5% of ionizable lipid, DOPE, cholesterol, and PEG, respectively—was selected based on an optimized LNP formulation previously shown to potently deliver mRNA.[67] We solubilized these four lipid components in ethanol and combined them with mRNA using a microfluidic

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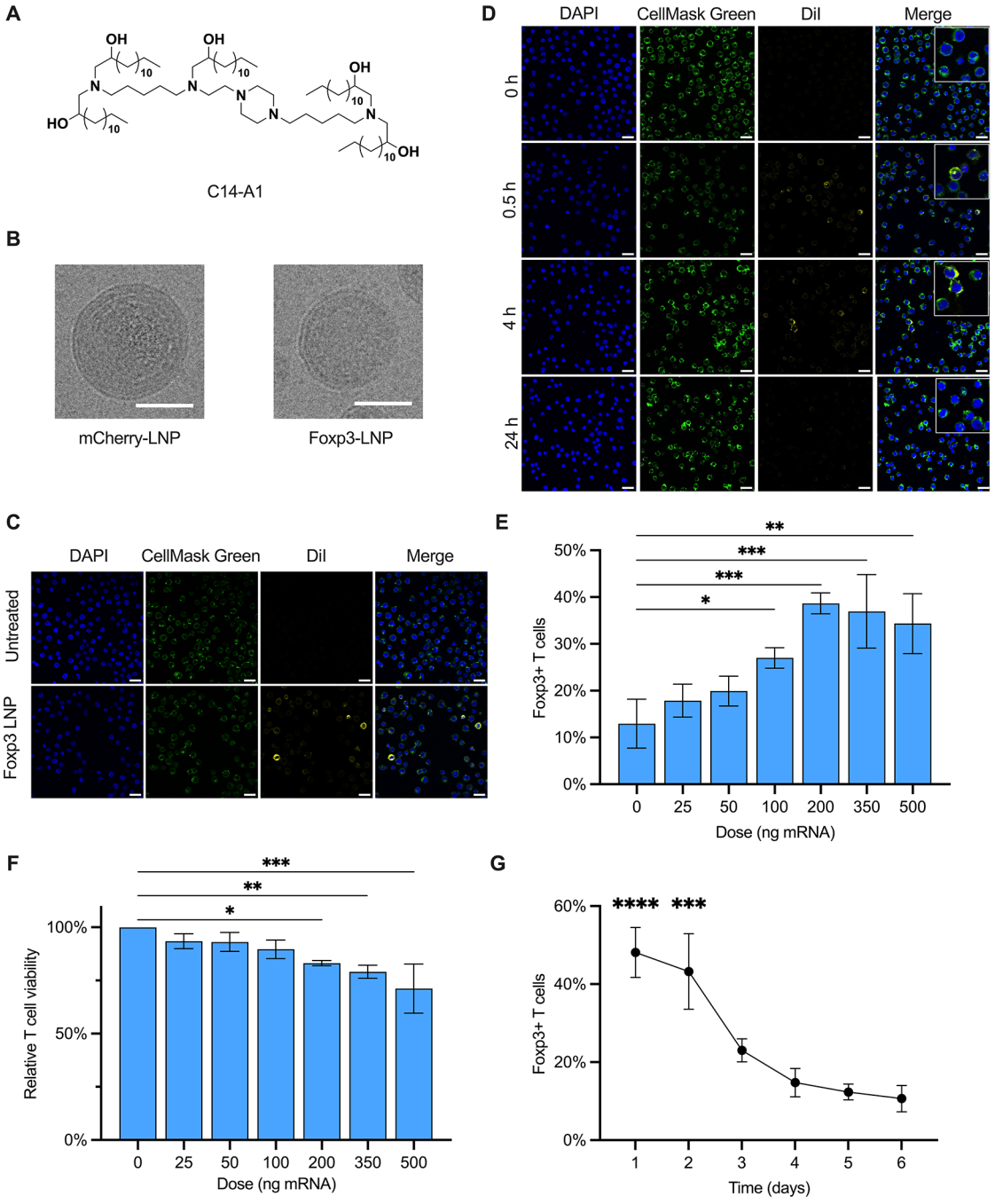

Figure 3. mRNA-LNP-mediated engineering of Foxp3 T (FP3T) cells from primary human T cells *ex vivo*. (A) Chemical structure of the lead ionizable lipid C14-A1 used to formulate Foxp3 mRNA-LNPs. (B) Cryo-TEM images of LNPs reveal no changes in gross morphology when LNPs are formulated with Foxp3 mRNA versus mCherry mRNA. Scale bar: 50 nm. (C) Representative confocal images at 20× magnification showing uptake of Foxp3 mRNA-LNPs in primary human $CD4^+$ T cells. T cells were treated with DiI-labeled LNPs for 0.5 h before staining with CellMask Green and DAPI. Scale bar: 20 μm. (D) Representative confocal images showing the kinetics of Foxp3 mRNA-LNP uptake and dissociation in primary human T cells. T cells were treated with DiI-labeled LNPs for 0, 0.5, 4, and 24 h before staining with CellMask Green and DAPI. Scale bar: 20 μm. (E) Intracellular expression of Foxp3 protein 24 h following transfection at doses ranging from 0 to 500 ng of Foxp3 mRNA per 100,000 cells and (F) the corresponding cell viability data. (G) Intracellular Foxp3 protein expression kinetics in primary human T cells transfected with Foxp3 mRNA-LNPs over a duration of 6 days. Statistical comparisons are relative to untreated T cells. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ in *post hoc t* tests with the Holm−Šídák correction. Data represent $n = 3$ biological replicates for all experiments.

mixing device with a staggered herringbone mixer structure to formulate our LNP library for screening (Figure 2A).[68,69] An additional LNP formulated with C12-200 ionizable lipid—shown previously to be a highly potent ionizable lipid—was used as a positive control, resulting in a complete library of 19 LNPs.[70,71]

We characterized the LNP library on the basis of several physicochemical properties that have been shown to impact delivery efficacy. We measured the LNP hydrodynamic size (diameter) and polydispersity index (PDI), which is a measure of the heterogeneity of the LNPs based on their size distribution, using dynamic light scattering (DLS). Overall,

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the LNPs ranged from 56.74 to 108.77 nm in diameter with PDIs ranging from 0.10 to 0.27, indicating that the LNPs formulated were relatively small and had homogeneous size distributions (Table S1).[62,72] All observed mRNA encapsulation efficiencies were above 70%, indicating that the LNP formulations were able to encapsulate mRNA efficiently (Table S1). Finally, the apparent pK_a values were measured to be in the range of 5.7−7.2. At pK_a values between 5.0 to 7.0, LNPs become stably ionized in acidic endosomal environments, disrupt the endosomal membrane, and promote endosomal escape.[73] Thus, our measured pK_a values suggested that the majority of our LNP library had a strong potential to facilitate efficient mRNA delivery (Table S1). Taken together, the characterization data suggest that all ionizable lipids tested produced well-formulated LNPs.

SCREEN IDENTIFIES TOP-PERFORMING IONIZABLE LIPID FOR MRNA DELIVERY TO PRIMARY CD4$^+$ T CELLS EX VIVO

To identify lead ionizable lipids that resulted in potent mRNA delivery, we utilized LNPs encapsulating mCherry mRNA as reporter cargo. This mRNA contained a complete substitution of uridine residues with N1-methyl-pseudouridine ($m^1\psi$), which has been demonstrated to improve mRNA transfection by enabling better encapsulation and boost mRNA translation by increasing ribosome pausing and density on the mRNA intracellularly.[74] Moreover, $m^1\psi$ modification enabled the recent success of the mRNA LNP COVID-19 vaccines formulated by Moderna and Pfizer-BioNtech.[47,48,74] Here, we encapsulated $m^1\psi$-modified mCherry mRNA in the LNP library and transfected primary human CD4$^+$ T cells to assess mRNA transfection efficiency. We activated primary T cells using anti-CD3/anti-CD28 Dynabeads before treating them with our LNP library at a previously established mRNA dose of 333 ng per 100,000 cells.[61,62] After 24 h, we evaluated mCherry expression using flow cytometry (Figure 2B,C).

We found that these LNPs generally delivered mRNA efficiently, with all of the observed transfection efficiencies above 40% (Figure S1). Although none of the formulations significantly improved percent transfection compared to the C12-200 industry standard LNP, we noted that two formulations—C12-A1 and C14-A1—resulted in a statistically significant ($^{**}p < 0.01$) increase in median fluorescence intensity (MFI) of mCherry by ∼2−3-fold (Figure 2C). MFI is a measure of how mRNA is being translated and expressed as a protein; therefore, although none of the LNPs in the library boosted transfection efficiency in T cells, some facilitated greater intracellular mRNA expression. We did not notice any significant toxicity from the LNPs; however, of the two top-performing LNPs, C12-A1 had a slightly lower average cell viability than C14-A1 (Figure 2D). Therefore, we determined C14-A1 to be the top-performing ionizable lipid for *ex vivo* delivery of mRNA to CD4$^+$ T cells.

LNP-MEDIATED FOXP3 MRNA DELIVERY TO ENGINEER FOXP3-T (FP3T) CELLS

Having confirmed the efficiency of our mRNA LNP formulations using reporter genes, we next aimed to formulate our top-performing LNP with Foxp3 mRNA and deliver it to primary CD4$^+$ T cells. Foxp3 is the master transcription factor that regulates the multifaceted suppressive functions of T_{reg} cells.[27,28] Thus, we hypothesized that by delivering Foxp3 mRNA to CD4$^+$ T cells, we could generate Foxp3-T (FP3T) cells and potentially induce a T_{reg}-like immunosuppressive phenotype. To engineer FP3T cells, we utilized mRNA encoding the Foxp3 transcript variant 2 which is a splice variant of Foxp3 found in T_{reg} cells.[75−77] The full-length Foxp3 protein contains a nuclear localization signal (NLS) and a nuclear export signal (NES). However, the Foxp3 transcript variant 2 lacks the NES, allowing for better nuclear retention of Foxp3 protein and thereby facilitating its primary functions as a transcription factor.[77]

We formulated the $m^1\psi$-modified Foxp3 transcript variant 2 (hereafter termed Foxp3) mRNA into our C14-A1 LNP and found no significant changes in LNP physicochemical characteristics (Figure 3A and Table S2). To visually confirm this, we performed cryo-transmission electron microscopy (cryo-TEM) on both mCherry and Foxp3 mRNA-LNPs (Figure 3B). The cryo-TEM images confirmed that there were no morphological changes in the LNP structure resulting from switching the mRNA cargo. We next confirmed Foxp3 mRNA-LNP uptake by primary human CD4$^+$ T cells using confocal fluorescence microscopy (Figure 3C). We further examined various time points of 0, 0.5, 4, and 24 h to elucidate the kinetics of LNP uptake and dissociation in the cytosol. We observed LNP internalization and uptake at 0.5 and 4 h, as evidenced by the intracellular presence of DiI-labeled LNPs (Figure 3D). However, we observed a minimal DiI-LNP signal at 24 h, indicating that the LNPs were successfully able to escape from endosomes at longer time scales (Figure 3D).

After confirming LNP uptake, we aimed to corroborate that the Foxp3 mRNA delivered was being translated and expressed as Foxp3 protein. As dosing schemes can be highly variable based on mRNA characteristics such as size, charge, modifications and protein being expressed, we first aimed to establish an optimal dosing scheme for Foxp3 mRNA-LNPs.[63,64] We treated LNPs at doses ranging from 0 to 500 ng of mRNA per 100,000 cells based on previous work and measured Foxp3 protein expression via flow cytometry (Figure S2). As Foxp3 is an intranuclear protein, we utilized intranuclear staining to assess expression. Compared to the untreated control, treatments in the range of 100−500 ng of mRNA resulted in a statistically significant increase in Foxp3 expression, with up to 45% of the cells expressing Foxp3 at a dose of 350 ng of mRNA (Figure 3E). Cytotoxicity analysis showed that treatments above 200 ng resulted in statistically significant reductions in cell viability, with the 350 and 500 ng treatments resulting in 80% and 71% mean cell viability, respectively (Figure 3F). Based on previous *ex vivo* T cell engineering studies and the fact that the application of this platform is also *ex vivo* T cell engineering, we reasoned that 80% cell viability was acceptable. Thus, we proceeded with the treatment of 350 ng of mRNA to study Foxp3 expression kinetics in CD4$^+$ T cells over the course of 6 days. We observed the highest expression 1 day post-transfection, with roughly 50% of cells expressing Foxp3. As expected, this expression decayed and plateaued 3 days post-transfection with approximately 18% of cells expressing Foxp3 (Figure 3G). This experiment validated the ability of our lead LNP to deliver Foxp3 mRNA to primary human CD4$^+$ T cells *ex vivo* and generate FP3T cells.

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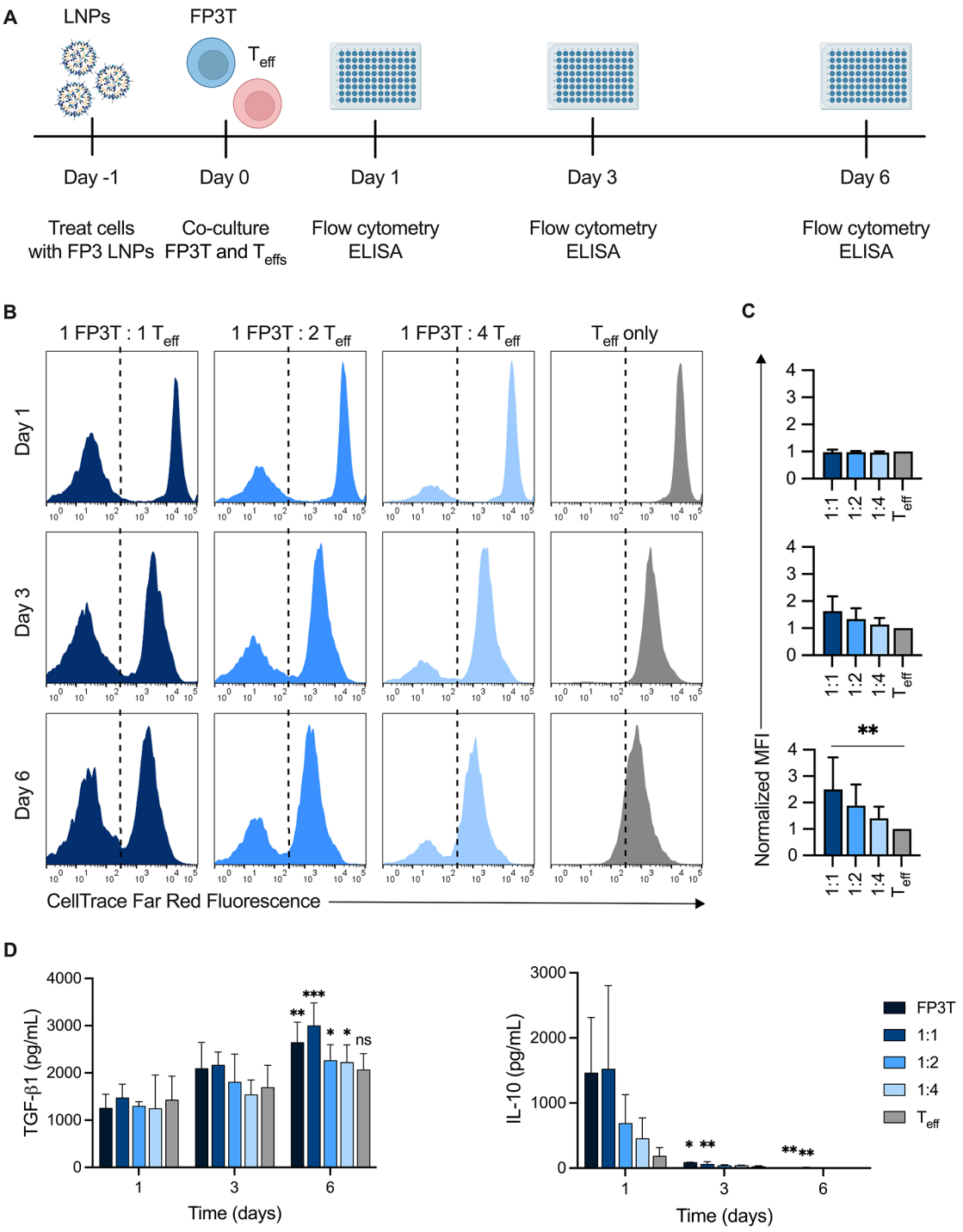

Figure 4. mRNA-LNP engineered FP3T cells functionally suppress T_{eff} cell proliferation *ex vivo*. (A) Timeline of coculture suppression assay testing ratios of 1:1, 1:2, and 1:4 of FP3T:T_{eff} cells ($CD4^+/CD8^+$ T cells). (B) Representative histograms of shifting CellTrace Far Red fluorescence and (C) overall normalized CellTrace Far Red MFI values. $^{**}p < 0.01$ in *post hoc t* tests with the Holm−Šídák correction. (D) TGF-β1 and IL-10 levels detected in the suppression assay supernatant as measured by ELISA. Statistical annotations denote comparisons to the observed levels at 1 day post-coculture. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ in *post hoc t* tests with the Holm−Šídák correction. Data represent $n = 3$ biological replicates for all experiments.

FP3T CELLS FUNCTIONALLY SUPPRESS PRIMARY EFFECTOR T CELL PROLIFERATION EX VIVO

After confirming the successful engineering of transient FP3T cells, we elucidated the suppressive capabilities of FP3T cells by assessing their effect on target cell proliferation. We conducted a coculture suppression assay of FP3T cells cocultured with $CD4^+$ and $CD8^+$ effector T (T_{eff}) cells and tracked T_{eff} cell proliferation over a 6-day period (Figure 4A). Briefly, FP3T cells were evaluated for their Foxp3-positivity and coplated at varying ratios with T_{eff} cells. These T_{eff} cells, which consisted of $CD4^+$ and $CD8^+$ primary T cells combined in a 1:1 ratio, were stained with CellTrace Far Red fluorescent tracking dye. We then quantified T cell proliferation by a dye dilution analysis, with increased proliferation resulting in lower

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MFI values and decreased proliferation resulting in higher MFI values. We hypothesized that the inclusion of FP3T cells would result in transient immunosuppression and thus hinder T_{eff} cell proliferation, resulting in higher MFI values. By 6 days post-coculture, we observed that the normalized MFI values observed for groups including FP3T cells were indeed higher than the T_{eff} cells only group (Figure 4C). This was evident in the flow cytometry histograms, where the population of fluorescent cells showed a more delayed shift toward the left with the inclusion of FP3T cells compared to that without (Figure 4B). Moreover, the normalized MFI values displayed a stepwise increase with increasing suppressor:effector ratio, and the flow cytometry histograms showed a gradual shift delay with the inclusion of more FP3T cells (higher ratios).

To further validate FP3T cell immunosuppression, we employed enzyme-linked immunosorbent assays (ELISAs) that gauged the levels of secreted suppressive and proliferative/inflammatory cytokines. Compared to the untreated T_{eff} group, we observed significantly increased TGF-β1 and IL-10 levels, both of which are immunosuppressive cytokines known to be released by T_{reg} cells, in groups containing FP3T cells (Figure 4D). We noted a steady increase in TGF-β1 by 6 days post-coculture, whereas IL-10 spiked on 1 day post-coculture and drastically decreased by 6 days post-coculture. Notably, we observed a steady increase in IFN-γ levels over time, which was unsurprising, as one of the known mechanisms through which T_{reg}-like cells induce suppression is through release of IFN-γ (Figure S3).[78] We also observed a gradual decrease in IL-2 levels across all coculture groups over time; however, the FP3T-only group and the 1:1 FP3T:T_{eff} group exhibited a smaller decrease in IL-2 levels. This posits that the T cells in these groups are not continuously consuming IL-2 in order to proliferate, reinforcing that the cells in these groups are indeed being functionally suppressed. Through our functional studies, we were able to confirm that the engineered FP3T cells exhibit an immunosuppressive phenotype and can functionally suppress the proliferation of T_{eff} cells.

In this work, we developed a potent and safe LNP platform to deliver Foxp3 mRNA to $CD4^+$ T cells to engineer immunosuppressive FP3T cells. We first screened a library of 18 unique LNPs in primary human T cells and identified a top-performing formulation. Using this formulation, we then delivered Foxp3 mRNA to $CD4^+$ human T cells to generate FP3T cells, confirming the transience of Foxp3 expression with kinetic studies. Finally, we validated the suppressive capabilities of FP3T cells using coculture suppression assays and investigated the mechanisms of suppression by assaying cytokine secretion. Thus, our lead mRNA LNP platform has the potential to be used for *ex vivo* engineering of immunosuppressive T cells for future application in systemic-cell-based immunotherapies for autoimmune disorders.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.nanolett.3c02573.

Materials and methods utilized, graphs and tables showing the characterization data of the LNP library, characterization of the Foxp3-containing LNP, relative transfection efficiencies of the LNP library, representative flow cytometry histograms of Foxp3 expression, and additional cytokine quantification from coculture suppression assays (PDF)

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Author Contributions

A.S.T. and M.J.M. conceived the project and designed the experiments. The experiments were performed by A.S.T., A.G.H., B.E.N., A.J.M., M.M.B., R.P., and K.L.S. and interpreted by all authors. A.S.T and M.J.M. wrote the manuscript, and A.S.T. prepared the figures in Biorender and Prism. All authors edited the manuscript and figures and approved the final version for submission.

Notes

The authors declare the following competing financial interest(s): M.J.M., A.S.T., and A.J.M. have filed a patent application based on this work. M.J.M., M.M.B., and K.L.S. are inventors on patents related to this work filed by the Trustees of the University of Pennsylvania (PCT/US20/56252).

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