Constrained Nanoparticles Deliver siRNA And sgRNA to T-Cells In Vivo Without Targeting Ligands

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T cells are involved in immunological regulation, which makes them an important target for RNA therapies. In the past, nanoparticles carrying RNA have been delivered to T cells in vivo using protein- and aptamer- targeting ligands. However, systemic delivery of RNA remains challenging without the use of targeting ligands. Our approach delivers RNA to T cells using lipid nanoparticles (LNPs) devoid of targeting ligands. Using a novel siGFP-based barcoding system we administered 168 nanoparticles for delivery of siRNA to 9 cells types in vivo. Nanomaterials containing conformationally constrained lipids are shown to form stable LNPs (cLNPs) which are delivered in a mechanism that is dependent on chemical composition and not structure size. cLNPs delivered siRNA and sgRNA to T cells at doses as low as 0.5 mg/kg, thus offering an alternative approach to using targeting ligands.
Constrained Nanoparticles Deliver siRNA and sgRNA to T Cells In Vivo without Targeting Ligands

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T cells help regulate immunity, which makes them an important target for RNA therapies. While nanoparticles carrying RNA have been directed to T cells in vivo using protein- and aptamer-based targeting ligands, systemic delivery to T cells without targeting ligands remains challenging. Given that T cells endocytose lipoprotein particles and enveloped viruses, two natural systems with structures that can be similar to lipid nanoparticles (LNPs), it is hypothesized that LNPs devoid of targeting ligands can deliver RNA to T cells in vivo. To test this hypothesis, the delivery of siRNA to 9 cell types in vivo by 168 nanoparticles using a novel siGFP-based barcoding system and bioinformatics is quantified. It is found that nanomaterials containing conformationally constrained lipids form stable LNPs, herein named constrained lipid nanoparticles (cLNPs). cLNPs deliver siRNA and sgRNA to T cells at doses as low as 0.5 mg kg\(^{-1}\) and, unlike previously reported LNPs, do not preferentially target hepatocytes. Delivery occurs via a chemical composition-dependent, size-independent mechanism. These data suggest that the degree to which lipids are constrained alters nanoparticle targeting, and also suggest that natural lipid trafficking pathways can promote T cell delivery, offering an alternative to active targeting approaches.

T lymphocytes help regulate immune responses, which makes them important drug targets. For example, antibodies that block T cell CTLA-4 or PD-1 signaling can drive antitumor responses. However, antibodies target druggable proteins, which constitute \(\approx 15\%\) of total proteins. By contrast, siRNA can inhibit the translation of any gene; many “undruggable” proteins were recently implicated in T cell–mediated immunity. Nonetheless, antibodies target druggable proteins, which constitute \(\approx 15\%\) of total proteins. By contrast, siRNA can inhibit the translation of any gene; many “undruggable” proteins were recently implicated in T cell–mediated immunity.

Clinically relevant RNA delivery to cells other than hepatocytes remains challenging. However, there have been advances in T cell siRNA delivery. For example, siRNA was delivered to T cells using a single-chain antibody linked to a cationic peptide; this led to gene silencing at 5 mg kg\(^{-1}\). In a second example, nanoparticles were coated with anti-CD4 antibodies, leading to 20% target gene silencing at 1 mg kg\(^{-1}\) doses. More recently, lipid nanoparticles (LNPs) that target hepatocytes were retargeted to T cells by coating them with CD4 antibodies, leading to 50% in vivo T cell gene silencing at 6 mg kg\(^{-1}\) doses. These papers (and others) achieve T cell delivery using peptide-, protein-, or aptamer-based targeting ligands, and more generally, ligand-based targeting is used throughout nanomedicine. However, ligands can make reproducible manufacturing at human scales more challenging.

One alternative to active targeting is to exploit endogenous lipid trafficking; notably, the only FDA-approved RNA nanoparticle therapy utilizes LNPs without ligands that are trafficked to hepatocytes via endogenous cholesterol transport. Natural trafficking has not been exploited to promote nanoparticle delivery to T cells, yet these cells can interact with viruses and lipoprotein particles, which can have diameters similar to LNPs. By combining high-throughput in vivo analyses and bioinformatics, we found that a new class of materials, named conformationally constrained lipids, can form stable LNPs. We also found that these “constrained LNPs” (cLNPs) can deliver siRNA to T cells in vivo. These data demonstrate that the conformational state of lipids can alter LNP tropism and provide intriguing preliminary evidence that natural trafficking can promote T cell delivery, offering a potential alternative to active targeting.
We synthesized 13 chemically diverse lipids containing amines or boronic acid. The library was constructed to investigate whether the structure of the 1) head groups and 2) lipid tail affected delivery. We purified a "scaffold" containing the unsaturated lipid linoleate and two ester bonds (Figure 1A; Figure S1A, Supporting Information). This scaffold did not have any ionizable components; we attached head group variants to the reactive sites, in order to create chemical diversity. At reactive site 1, we added 11 head groups (labeled 1–11) via esterification, resulting in head groups linked by ester or carbonate linkages, respectively (Figure 1B). At reactive site 2, we added three lipid tails (labeled A–C) with diverse structures (Figure 1C) via esterification. Tail A contained adamantane, a constrained lipid with a defined “armchair” structure. Tail B contained two lipid tails, bringing the total number of tails to 3. The control tail, C, was linoleate; this created a construct with two identical lipid tails. After synthesis, we confirmed the chemical structure of all 13 lipids using high-resolution mass spectrometry or $^1$H NMR (Figure S1B–D, Supporting Information). We named each lipid with the nomenclature “head group number–tail letter” (e.g., 11-A).

We then investigated whether the 13 ionizable lipids formed stable LNPs. We measured the hydrodynamic diameter of LNPs carrying an siRNA targeting GFP[12] (siGFP) as well as a DNA barcode[15]; the LNPs were formulated using microfluidics.[16] The siGFP was chemically modified to reduce immunostimulation and enhance on-target silencing via preferential antisense RISC loading (Figure S2A, Supporting Information). To minimize the chance that our results were affected by other constituents added to the LNP, we added previously validated constituents: C14PEG2000, unmodified cholesterol, and either 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) or 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) (Figure 1D). As a control to ensure that our results were not affected by the molar ratio of the 4 components, we formulated each of the 13 lipids with 2 phospholipids and 4 molar ratios, producing a total of 104 chemically distinct LNPs (Figure 1D). Encouragingly, 100 of the 104 LNPs formed small, monodisperse populations, as evidenced by hydrodynamic diameter and polydispersity index (PDI); these 100 LNPs were pooled together. The diameter for individual LNPs varied between 30 and 170 nm. As a control, we also measured the diameter and polydispersity of the pooled LNPs. We found them to be 76 nm (Figure 1E) and 0.23 (Figure 1F), respectively, which were within the range of the individuals comprising the pool. We then analyzed the hydrodynamic diameter as a function of ionizable lipid (Figure S2B, Supporting Information), molar ratio of the four constituents (Figure S2C, Supporting Information), and the type of phospholipid (DSPC/DOPE) added to the formulation (Figure S2D, Supporting Information). In all cases, the average diameter
varied between 50 and 100 nm. These data led us to conclude that these lipids could form LNPs with hydrodynamic diameters similar to those of lipoproteins and viruses.

We evaluated how each LNP delivered siRNA to target cells (in this case, T lymphocytes) as well as eight off-target cell types in vivo (Figure S3, Supporting Information). Our approach utilizes DNA barcodes and siGFP, to evaluate how many distinct LNPs functionally delivered siGFP, in any combination of target cells, in a single mouse. We formulated LNP-1, with chemical structure 1, to carry siGFP and DNA barcode 1; we separately formulated LNP-N, with chemical structure N, to carry siGFP and DNA barcode N (Figure 2A). We included naked barcodes as a negative control,[14] since DNA does not readily enter cells. We pooled the LNPs together, and intravenously injected them into mice that constitutively express GFP under a CAG promoter (Figure 2B). The GFP acted as the functional delivery readout; LNPs that functionally delivered siGFP into the cytoplasm would have lower GFP protein expression. Thus, 3 days after injecting mice, we isolated GFPLow cells using FACS, and deep sequenced the DNA barcodes in GFP Low cells. In this way, we isolated barcodes colocalized with cells in which GFP protein silencing occurred. After sequencing the barcodes, we...
calculated normalized delivery, i.e., the number of barcodes for each individual barcode, divided by the total number of barcodes within that sample. Normalized delivery is analogous to counts per million in RNAseq experiments. Since GFP is expressed in all cell types, this assay allows us to 1) compare GFP knockdown in any combination of on-/off-target cells and 2) identify LNPs that colocalized in GFP<sup>Low</sup> cells.

Three days after injecting a total dose of 1.5 mg kg<sup>−1</sup> into mice (100 distinct LNPs, 0.015 mg kg<sup>−1</sup> per particle on average), we quantified GFP silencing in 9 cell types. Compared to phosphate-buffered saline–treated mice, there was an increased number of GFP<sup>Low</sup> splenic B cells and splenic T cells (Figure 2C). The average GFP protein silencing, quantified by mean fluorescent intensity, was greatest in splenic T cells, followed by liver immune cells, splenic B cells, and lung endothelial cells (Figure 2D). Surprisingly, we found no evidence of silencing in hepatocytes (Figure 2C,D), which are preferentially targeted by many LNPs. We then monitored the controls included in our data; we sequenced the GFP<sup>Low</sup> splenic T cells as well as lung endothelial cells, splenic B cells, and liver immune cells. In all four cell types, the normalized delivery of both negative controls (naked barcodes) was lower than barcodes delivered by LNPs, as expected (Figure 2E).

We then performed a large in vivo structure–function analysis, using the DNA sequencing data to evaluate whether any nanoparticle material properties promoted delivery to splenic T cells. First, we calculated the enrichment for different nanoparticle properties (Figure S4, Supporting Information). Briefly, we calculated the odds a nanoparticle with a particular property would show up by chance in: 1) particles that performed in the top 10% and, separately, 2) particles that performed in the bottom 10%. Nanoparticles formulated with DSPC were enriched in effective particles (i.e., particles with high normalized delivery), whereas nanoparticles formulated with DOPE were enriched in particles that performed poorly (Figure 2F). To validate these results, we then compared the normalized delivery for all LNPs formulated with DSPC and DOPE, respectively, and found that DSPC-containing LNPs outperformed DOPE-containing LNPs (Figure S5A, Supporting Information). As an additional validation, we calculated the normalized delivery of “paired” LNPs, i.e., LNPs that had the same molar ratios and ionizable lipids (but different phospholipids). We found that DSPC LNPs outperformed their paired DOPE containing LNP (Figure S5B, Supporting Information). Based on these data, we concluded that the phospholipid contained within the LNP affected splenic T cell delivery. We therefore limited future chemical analysis to DSPC-containing formulations.

We then analyzed enrichment for the 13 ionizable lipids, in order to evaluate the effect of the lipid tail and head group, and found that 3 ionizable lipids were enriched (Figure 2G). As a control, we plotted enrichment of each head group versus effects driven by the molar ratio of the components, we formulated all 16 ionizable lipids with 4 molar ratios, for a total of 64 LNPs (Figure 3D). Each LNP carried siGFP and a unique barcode. Fifty-five LNPs formed small, monodisperse populations, based on the hydrodynamic diameter and polydispersity index, and were therefore pooled together. The diameter for individual LNPs varied between 20 and 200 nm, with an average of 92 nm (Figure 3E) and an average PDI of 0.20 (Figure 3F). We plotted hydrodynamic diameter for each ionizable lipid (Figure S7A, Supporting Information) and each molar ratio (Figure S7B, Supporting Information), and found that all the compositions formed LNPs within the 20–200 nm range.

We then administered all 55 LNPs intravenously to mice at a total dose of 1.5 mg kg<sup>−1</sup>. Three days later, we quantified GFP silencing in nine cell types. Once again, we: 1) found GFP<sup>Low</sup> splenic T cells (Figure 3G), 2) measured GFP protein silencing in splenic T cells (Figure 3H), 3) found no evidence of silencing in hepatocytes (Figure 3G,H), and 4) found no relationship between nanoparticle size and delivery (Figure S7C, Supporting Information). As a control, we quantified the normalized delivery of both negative controls (naked barcodes), and found that they were lower than barcodes delivered by LNPs (Figure 3I). We then evaluated whether the molecule added to reactive site 3 (Figure 3B) altered delivery when adamantane was present. Using enrichment (Figure 3J), we found that reactive groups D, I, and N were enriched. We were unable to identify why these variants performed well. However, compared to the top-performing cLNP from screen 1, we found that delivery was not greatly improved with any alteration to the reactive site 3 molecule (Figure 3K). It is interesting to note that the top-performing cLNP from screen 1 (11-A-M) was enriched more than any other cLNP (Figure 3J). These data provided additional evidence to support the hypothesis that LNPs can deliver siRNA to T cells without targeting ligands.

Like all high-throughput screening systems, the value of this siGFP/DNA barcode assay is related to its ability to make
Figure 3. A second high-throughput siRNA screen suggests that adamantanes can deliver siRNA to T cells. A,B) Fifteen cLNPs can be formed with the top-performing head group (A) and 15 tail variations (B). C) The top-performing constrained lipid from screen 1 was included. D) Using 4 molar ratios, each of the 16 ionizable lipids were formulated with cholesterol, lipid-PEG, and DSPC to create 64 distinct LNPs. E) Hydrodynamic diameter and F) polydispersity index of all formulated LNPs, measured individually. G) Percent GFPLow cells in nine cell types. Two-way ANOVA, \( *P < 0.05 \). H) Normalized decrease in GFP MFI in nine cell types. I) Normalized DNA delivery in lung endothelial cells, splenic B and T cells, and liver Kupffer cells. J) Enrichment for each of the 16 ionizable lipids. K) Normalized DNA delivery of each LNP formulated.
predictions. We therefore evaluated whether the top-ranked LNPs from our first (11-A-M) and second (1-A-N) screens delivered siRNA in vivo (Figure 4A). We formulated each LNP with siGFP and analyzed physical traits; each LNP had similar hydrodynamic diameter, polydispersity, and pKa (Figure 4B).

We then intravenously injected mice with 1.5 mg kg\(^{-1}\); 3 days later, we isolated cells and measured GFP protein expression. When compared to mice treated with a nontargeting, chemically modified siRNA (siLuc), we found that 11-A-M silenced GFP more than 1-A-N (Figure 4C). To validate the activity of 1-A-N, we formulated it with siCD45, and quantified CD45 silencing 3 days after injecting mice with 1.5 mg kg\(^{-1}\); we found statistically significant silencing in T cells (Figure S7D,E, Supporting Information). Based on these head-to-head data, we focused on compound 11-A-M. We performed an siRNA gene silencing dose response in vivo, and found that 11-A-M silenced GFP at doses as low as 0.5 mg kg\(^{-1}\) (Figure 4D). We quantified silencing in subsets of T cells, focusing on CD4\(^+\) and CD8\(^+\) cells, respectively, and observed more potent protein silencing in CD8\(^+\) T cells (Figure 4E). During this experiment, we also evaluated whether 11-A-M delivered siGFP to other common “off-target” cell types by quantifying GFP silencing. Recapturing observations made in both screens, we observed no significant silencing at doses as high as 1.5 mg kg\(^{-1}\) in other cell types, including hepatic T cells, bone marrow T cells, and splenic macrophages (Figure S8A–H, Supporting Information). We then quantified on- and off-target biodistribution using QUANT, a highly sensitive digital droplet PCR-based method we recently reported.\(^{[15]}\) We formulated 11-A-M to carry the chemically modified QUANT barcode and injected 1.0 mg kg\(^{-1}\) intravenously. Twenty-four hours later, we isolated cell types using FACS, and measured biodistribution in eight cell types. We found that 11-A-M distribution was highest in splenic CD8\(^+\) T cells, CD4\(^+\) T cells, and B cells. Distribution was lower in splenic macrophages as well as endothelial cells, Kupffer cells, hepatocytes, and T cells isolated from the liver (Figure S9A,B, Supporting Information). We also quantified biodistribution mediated by 1-A-N in all eight cell types, and found that splenic macrophages acted as the primary “sink” for this LNP (Figure S9C,D, Supporting Information). These data suggest that the 11-A-M cLNP may preferentially silence genes in splenic CD8\(^+\) T cells.

Finally, we utilized 11-A-M to facilitate in vivo gene editing in T cells. We formulated it to carry a chemically modified sgRNA targeting GFP (Figure S10, Supporting Information) into mice\(^{[18]}\) constitutively expressing Cas9 and GFP. Five days
after administration, we quantified GFP expression in CD3+ T cells as well as in CD4+ and CD8+ T cells. We observed a similar tropism; GFP protein was silenced more robustly in CD8+ than in CD4+ T cells (Figure 4F). We did not observe changes in GFP expression when a control sgRNA targeting the gene ICAM-2 was administered to the mice (Figure 4F). Notably, cLNPs did not lead to weight loss 24 h after administration in any experiment (Figure S10, Supporting Information). Taken together, these data led us to conclude that cLNPs without targeting ligands can deliver siRNA and sgRNA to splenic T cells. Finally, we analyzed additional traits of 11-A-M cLNPs formulated with siGFP using transmission electron microscopy. We found that the cLNPs formed small, monodisperse spherical structures (Figure 4G) and that cLNPs encapsulated ≈75% of the formulated RNA (Figure S11, Supporting Information).

Nanoparticles that deliver RNA systemically to nonhepaticocytes are difficult to design,[4] in large part because: 1) there is no high-throughput method to study nanoparticle siRNA delivery in vivo and 2) natural trafficking mechanisms to non-liver cells remain elusive. This universal problem in nanomedicine slows the development of all RNA therapies; currently, scientists perform high-throughput nanoparticle assays in vitro, even though cell culture does not recapitulate all the factors that affect delivery in vivo. Notably, the results from our first siGFP screen predicted that preferential T cell delivery would occur; these data were confirmed by the second siGFP screen, and by several in vivo experiments with cLNPs selected from the library. These data suggest that high-throughput in vivo siRNA screens can identify nanoparticles with novel tropism. The screening data (Figures 2C,D and 3G,H) suggested that other immune cell subsets may be targeted with LNPs. Although our current data do not allow us to predict how endosomal escape varies between T cells and other immune cells, we believe future studies utilizing traditional cell signaling techniques may elucidate genes and pathways that govern (and differentiate) endosomal escape in subsets of immune cells. Notably, evidence suggests that a given immune cell “type” actually encompasses a spectrum of transcriptionally and phenotypically distinct cells.[19] We therefore hypothesize that the relationship between uptake and cytoplasmic delivery will vary along this spectrum.

It is important to acknowledge the limitations of our work. First, the siGFP system will not work with unstable nanoparticles. It is critical to: 1) analyze the size and polydispersity of each individual nanoparticle before pooling, 2) include the naked DNA barcode control, 3) use untreated GFP mice to gate during FACS, and 4) individually confirm any lead candidates identified by the screen. Second, although we observed protein silencing in T cells at 0.5 mg kg−1 doses, we will need to reduce this dose more than 30-fold before it matches the potency of an FDA-approved siRNA delivery vehicle in mice.[20] We anticipate that iterative in vivo approaches we recently reported[21] may further improve T cell LNP potency. Finally, we did not identify the natural trafficking pathways that promoted delivery to T cells. However, we believe that identifying the genes or pathways that promote LNP delivery to T cells without ligands constitutes an exciting scientific opportunity. In this way, we hope future work on cLNPs will lead to more effective, scalable RNA immunotherapies,[22] as well as fundamental advances in our understanding of T cell lipid trafficking. More generally, these data may inspire other efforts to identify natural mechanisms to target cells that—to date—have only been targeted with active ligands.

**Experimental Section**

Lipids were attached to the scaffold via esterification. Nucleic acids were diluted in citrate buffer; nanoparticle components were diluted in 100% ethanol. The phases were mixed together via microfluidics.[16] LNP hydrodynamic diameter was measured using dynamic light scattering. PKa was calculated using the TNS assay, as previously reported.[21] Mice were purchased from Jackson Laboratory; all were 5–8 weeks old. N = 4–5 mice per group were injected intravenously via the lateral tail vein. All animal experiments were performed in accordance with the Georgia Tech IACUC. Sequencing was performed on Miniseq using Illumina protocols.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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M.P.L. and C.D.S. contributed equally to this work.

**Conflict of Interest**

J.E.D. and C.D.S. are co-founders of GuideRx. J.E.D., C.D.S., and Z.G. have filed patent applications related to this work.

**Keywords**

CRISPR, DNA barcoded nanoparticles, immunotherapy, lipid nanoparticles, RNAi, siRNA, T cells

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