In vivo Characterization of T-Cell Activating Disulfide Lipids

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PROVIDENCE therapeutics

INTRODUCTION

Lipid nanoparticles (LNPs) have emerged as promising vehicles to deliver a variety of therapeutics *in vivo* for the treatment and prevention of various diseases. mRNA LNPs conventionally contain mRNA encapsulated in a lipid bilayer consisting of an ionizable lipid, a phospholipid, cholesterol, and a pegylated lipid[1,2]. The ionizable lipid generally contains an amino group, which electrostatically interacts with the phosphate groups on the mRNA backbone. The structure of the ionizable lipid impacts the efficacy, stability, and safety of mRNA LNP therapeutics[3]. One of our successfully developed ionizable lipid families integrates a disulfide bond into the main structure. The reduction of disulfide bonds happens in endosomes that facilitate the endosomal escape of mRNA.

The purpose of this experimentation was to formulate four lipids from this family using a tool mRNA and evaluate immune response *in vivo*.

LIPID DEVELOPMENT

A series of ionizable lipids were developed at Providence Therapeutics to incorporate into various mRNA vaccines/therapeutics programs. These lipids were developed from distinct core structures with a library of lipids being derived from each core structure by varying alkyl chain length, degree of unsaturation, number of alkyl chains, and the spacer between the cationic head and hydrophobic tails. These lipids were screened *in vivo* to identify the right candidates for individual applications.

METHODOLOGY

Lipid nanoparticle preparation

For particle formation, aqueous mRNA and ethanolic lipid solutions were turbulently mixed in a t-mixer while a neutral pH buffer was introduced inline for dilution to aid particle maturation[1]. The mRNA encapsulated encoded model antigenic epitopes from LCMV glycoprotein (LCMV-gp). Lipid solutions contained PEG-2K, DSPC, cholesterol, and one of the four disulfide ionizable lipids (Lipid 1, Lipid 2, Lipid 3, and Lipid 4). Ultrafiltration and diafiltration was carried out using Amicon Ultra 15 centrifugal units (Merck Millipore Ltd., Tullagreen, IRL). The final products were then sterile filtered and particle characteristics measured.

Particle characteristics – analytical methods

Particle size and polydispersity index (PDI) were measured with the Malvern Zetasizer Nano ZS (Malvern Panalytical, Malvern, UK). Encapsulation efficiency was determined via ribogreen assay, with fluorescence reading performed using a GloMax Discover plate reader (Promega, Madison, USA). mRNA purity was tested by high-performance liquid chromatography (HPLC).

Particle stability

LNPs were stored at -20°C and -80°C for six months. Particle characteristics were assessed for samples stored at both temperatures at 3- and 6-month time points and compared to the characteristics at the time of manufacturing.

In vivo testing

Female C57BL/6 mice (4 mice per group) were immunized with approximately 50 µL of formulation (concentration 0.25 mg/mL) in the biceps-femoris of the left leg on day 0 and right leg on day 0 + 4. A positive control formulated using the same method and mRNA with Dlin-MC3 as the ionizable lipid was also injected. Day 8 peripheral blood cells were stained for CD8+ T cells and gp-33 or gp-34 tetramers[4].

PARTICLE CHARACTERISTICS RESULTS

Preliminary formulation results (n=1 for each ionizable lipid, Table 1) showed sizes ranging from 58 to 86 nm at the time of manufacturing, with PDI less than 0.25, encapsulation >92%, and mRNA purity > 97%. At -20°C, Lipid 3 showed particle growth (86 nm at time 0, 188 nm at 3 months and 253 nm at 6 months) and drop in encapsulation (93% at 3 months and 89% at 6 months).

The LNPs formed with Lipid 1 and Lipid 2 were disc-shaped, while Lipid 4 formed spheres and Lipid 3 formed particles with larger water pockets present (Figure 1).

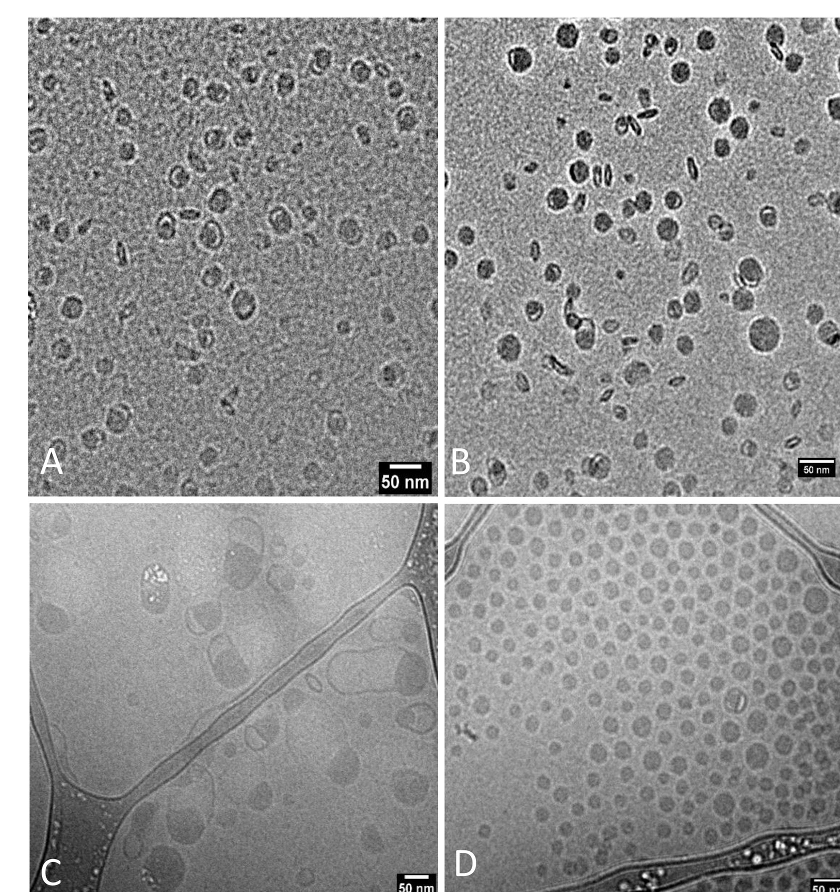

Figure 1: Cryo-TEM images of A. Lipid 1, B. Lipid 2, C. Lipid 3, and D. Lipid 4

Stability Results	Ionizable Lipid	Manufacturing	-20°C 3 months	-20°C 6 months	-80°C 3 months	-80°C 6 months
Size (nm)	Lipid 1	60	61	62	61	61
	Lipid 2	61	82	86	70	63
	Lipid 3	86	188	253	89	105
	Lipid 4	65	64	66	67	68
PDI	Lipid 1	0.18	0.17	0.19	0.19	0.20
	Lipid 2	0.20	0.22	0.26	0.20	0.19
	Lipid 3	0.16	0.26	0.28	0.11	0.12
	Lipid 4	0.22	0.20	0.21	0.24	0.21
EE%	Lipid 1	93	86	88	92	90
	Lipid 2	98	98	98	98	98
	Lipid 3	95	93	89	92	88
	Lipid 4	99	99	99	99	99
mRNA Purity	Lipid 1	98	98	97	98	98
	Lipid 2	97	85	85	91	91
	Lipid 3	98	95	94	96	98
	Lipid 4	98	97	96	98	98

Table 1: Particle characteristics for LNPs formed with disulfide lipids at release and 3 and 6 months of storage at -20 and -80°C

IN VIVO TESTING RESULTS

LNPs with Lipid 4 were the most effective in expanding gp-33 and gp-34 specific T-cells, followed by Lipid 3, five-fold and three-fold the buffer (Figure 2). LNPs with lipid 1 to 3 were all one or less than one-fold over the control. LNPs with lipid 4 activated expansion of antigen-specific T-cells to more than 10% of total circulating CD8+ T-cells.

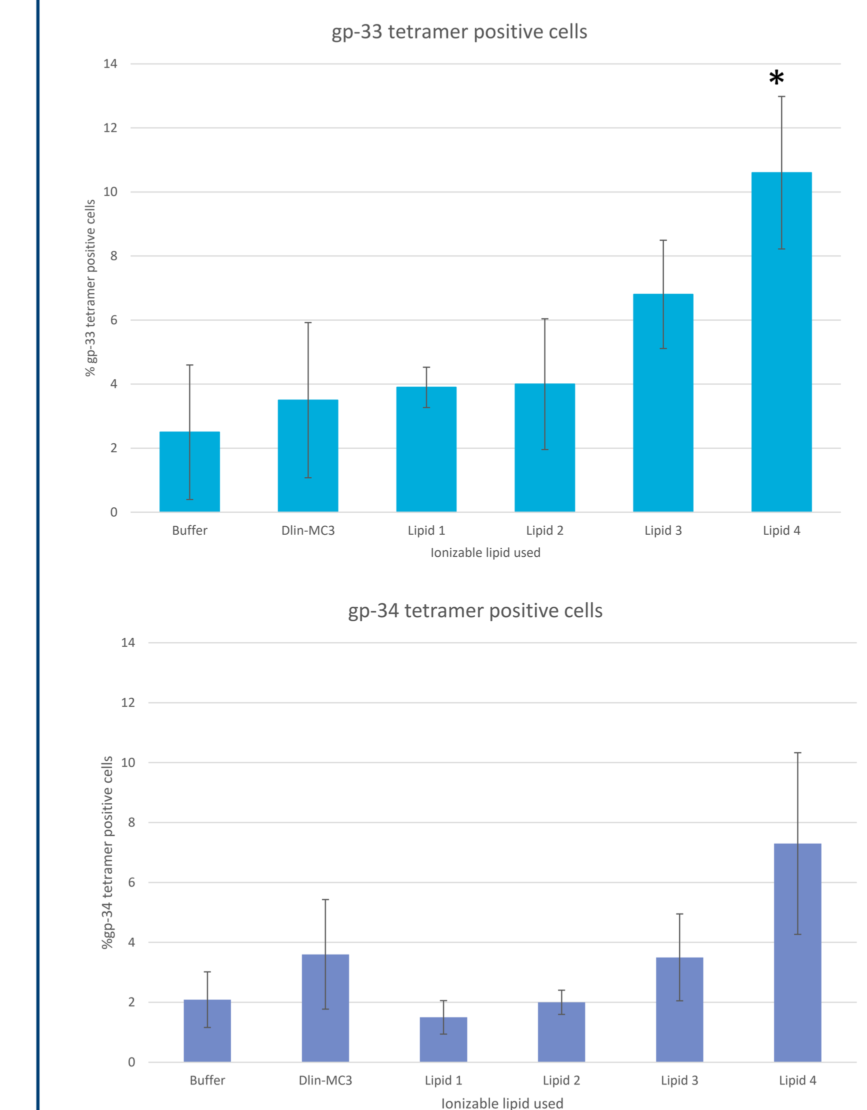

Figure 2: Percentage of gp-33 and gp-34 positive cells day 8 post injection with LNPs formed by various lipids. Formulation buffer used as the negative control and Dlin-MC3 used as the positive (size 83 nm, PDI 0.12, EE 93%, and mRNA purity 89%). *p<0.05 after t-Test (paired two sample) comparing with Dlin-MC3 performed in excel.

CONCLUSIONS

In vivo, these LNPs with novel lipids induced high T-cell activation – overall showing a greater immune response than the positive control. This indicates that disulfide lipids could potentially eliminate the necessity for ultra-low temperature storage of mRNA vaccines at the same time as triggering a strong immune response.

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