mRNA Lipid Nanoparticles That Activate NLRP3 Inflammasomes Reduce mRNA Transfection Efficiency

James Forster III[1], Dipika Nandi[1,2], Ashish Kulkarni[1,2,3]*

1. Department of Chemical Engineering 2. Department of Veterinary and Animal Sciences 3. Center for Bioactive Delivery at Institute for Applied Life Sciences, University of Massachusetts, Amherst

Introduction: In the last several years, countless developments have been made to engineer mRNA lipid nanoparticles to induce efficient endosomal escape, an important hurdle in developing effective mRNA delivery systems like the COVID-19 vaccines. However, these approaches have primarily been through modifying the structures and compositions of key lipids, resulting in new biophysical characteristics. It is concurrently being elucidated that nanoparticle biophysical effects also play an essential role in the activation of cellular stress and damage signals that induce immunogenicity. However, few approaches have categorized mRNA lipid nanoparticle immunogenicity in detail. To gain mechanistic insights into the lipid composition dependent activation of innate immune responses, we synthesized a panel of six mRNA lipid nanoparticle formulations and studied their effect on NLRP3 inflammasome activation; a key intracellular protein complex that controls various inflammatory responses.

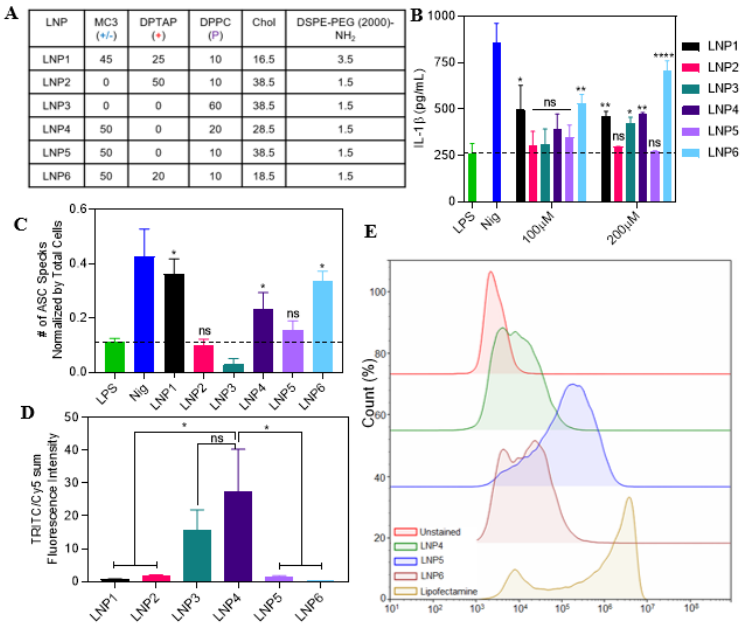

Figure 1: (**A**) Table of LNP mole % compositions studied. (**B**) IL-1β release from NLRP3 activation after 4-hr LPS priming and 24-hr LNP treatment. (**C**) Number of ASC-specks per cell, indicative of NLRP3 complex oligomerization. (**D**) Live cell imaging of LysoTracker signal reduction caused by LNP lysosomal rupture. (**E**) Flow cytometry histogram showing GFP transfection fluorescence by LNP4, 5, and 6. Statistics were conducted using one-way ANOVA followed by Tukey's test.

Materials and Methods: To adequately assess inflammasome activation dependence on lipid composition, we synthesized six lipid nanoparticle formulations (LNPs) via the ethanol dilution method, varying the molar ratios of ionizable lipid DLin-MC3-DMA, cationic lipid DPTAP, phospholipid DPPC, PEGylated lipid DSPE-PEG (2000)-Amine, and cholesterol (**Figure 1A**). We screened the LNPs for NLRP3 activation in LPS-primed iBMDMs by measuring IL-1β cytokine release via ELISA (**Figure 1B**), NLRP3 oligomerization via ASC speck live cell imaging (**Figure 1C**), and activation of inflammasome proteins gasdermin-D and active-caspase-1 via immunoblotting. To discern the mechanism causing inflammasome activation, we categorized the reduction of lysosome signal due to lysosomal rupture using live cell imaging (**Figure 1D**). Finally, mRNA transfection efficiency of select LNPs was determined by encapsulating 5 μg/mL doses of GFP mRNA in the nanoparticles, transfecting in iBMDMs, and running flow cytometry (**Figure 1E**).

Results and Discussion: We report a strong dependence of LNP formulation on NLRP3 inflammasome activation, with LNP6, 1, and 4 activating in that order, confirmed by potent IL-1β release and ASC speck formation indicative of inflammasome complex assembly (**Figure 1B, C**). We also discerned that LNP1 and 6 induced very strong lysosomal rupturing (**Figure 1D**). We found inflammasome activation to negatively correlate with mRNA transfection efficiency in **Figure 1E**, with LNP 6 and 4 showing strong reductions in transfection. It is hypothesized that these formulations exhibit delayed lipid ionization and membrane fusion until the lysosome stage, inducing degradation of viable mRNA and lysosomal rupture induced inflammasome activation.

Conclusion: We provide a molecular-level analysis into the innate immunological responses to mRNA lipid nanoparticle delivery, identifying a strong dependence of principally ionizable, cationic, and cholesterol lipid compositions as activating signals for NLRP3 inflammasomes. Our results provide a new leverage for controlling early mRNA release and inflammasome activation and could be used to develop future self-adjuvant vaccines.