Synergistic Effect of Nucleoside Modification and Ionizable Lipid Composition on Translation and Immune Responses to mRNA Vaccines

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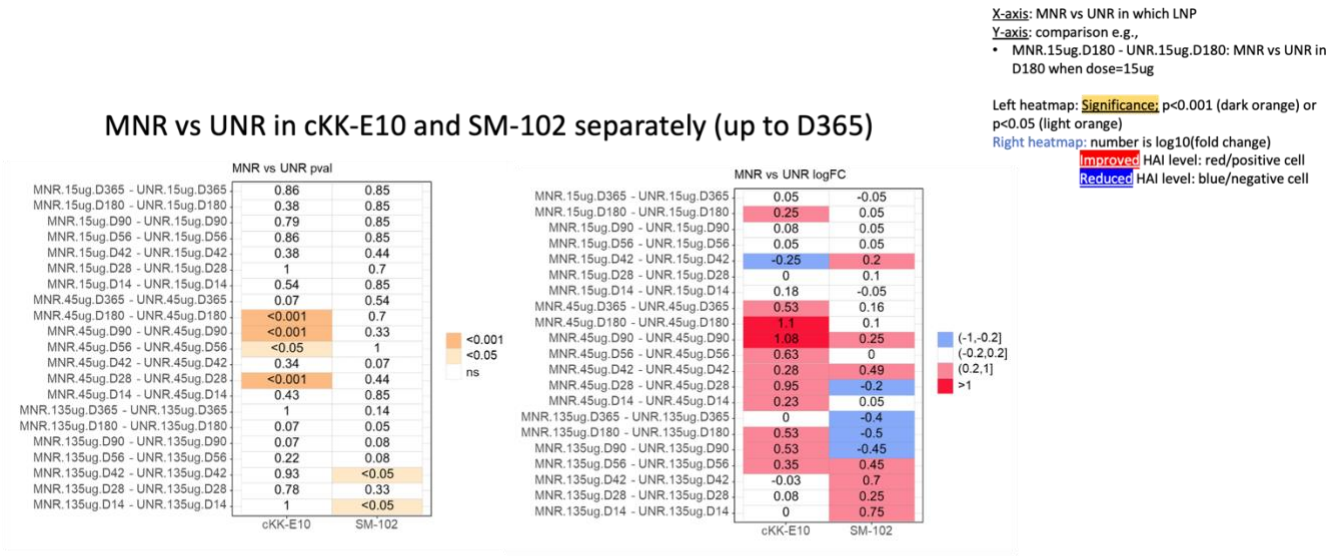
Supplementary Information

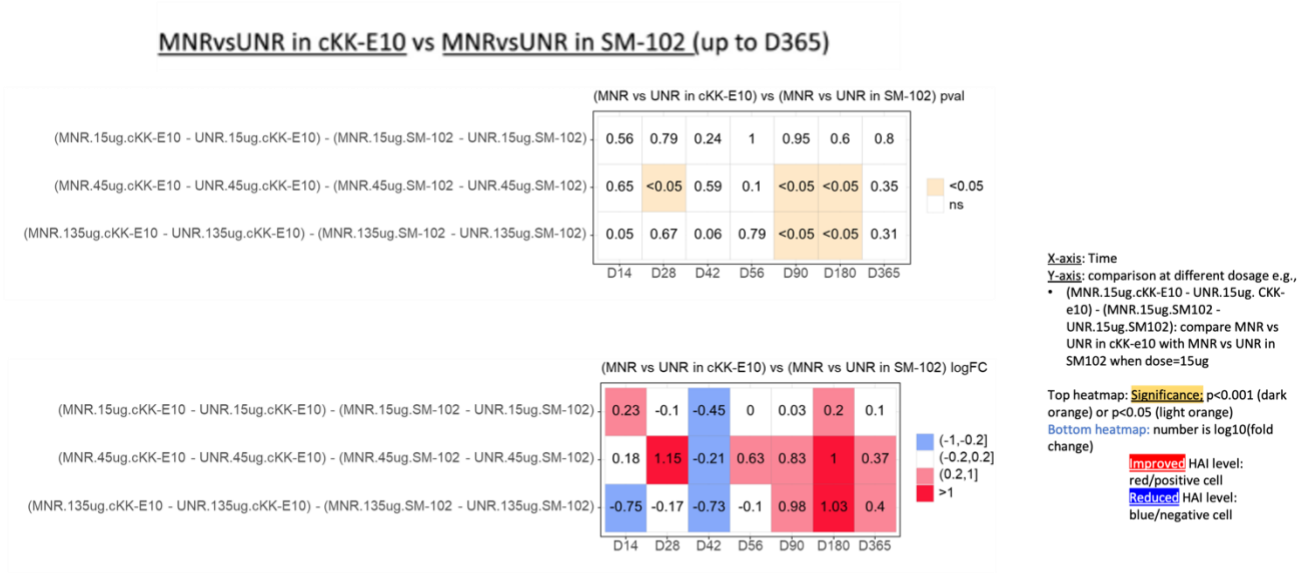
Supplementary Fig. 1 Statistical analysis on serological evaluation of UNR vs. MNR mRNA-LNP vaccines in non-human primates, dose effect. Log_{10}-transformed hemagglutination inhibition (HAI) fold change (FC) modeled by a linear mixed effect model to account for the repeated measurements for each animal across time are provided for UNR vs MNR for SM-102 and cKK-E10 lipid nanoparticles (LNPs). The fixed effects include nucleoside modification type, LNP, time, dosage, and their interactions. The left table shows the significance *p*-values while the right table shows the effect sizes at log_{10} FC. Columns represent LNP formulations used, and rows represent nucleoside modification comparisons (MNR vs UNR) at different times and dosage. Left table: $p <$ 0.001(dark orange) or $p <$ 0.05 (light orange). Right table: red/positive cell represents increased HAI level while blue/negative cell represents decreased HAI level.



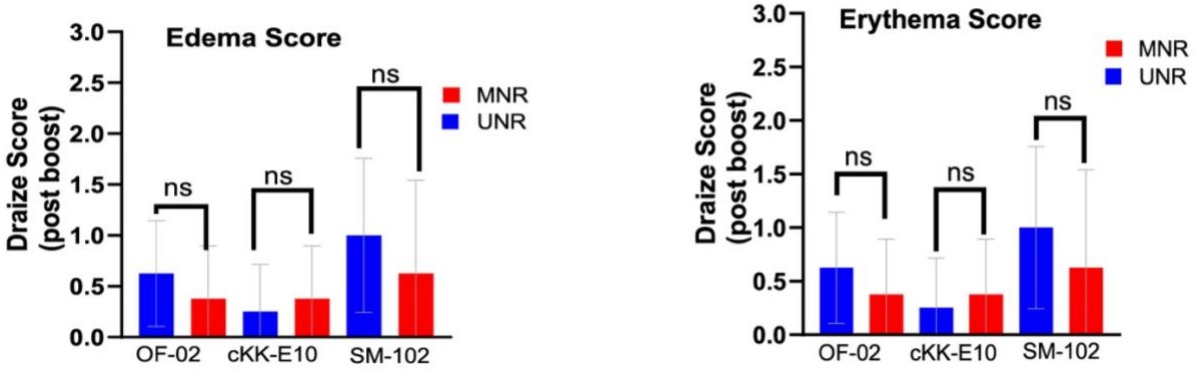
Analysis description: log10-transformed HAI fold change is modeled by a linear mixed effect model to account for the repeated measurements for each subject across time. The fixed effects include nucleoside modification type, time, dosage, and their interactions.

Heatmap description: X-axis is LNP. Y-axis represents nucleoside modification comparisons (MNR vs UNR) at different time and dosage. The left heatmap shows the significance p-values while the right heatmap shows the effect sizes.

Supplementary Fig. 2 Statistical analysis on serological evaluation of UNR vs. MNR mRNA-LNP vaccines in non-human primates, duration of immune response. Log_{10}-transformed hemagglutination inhibition (HAI) fold change modeled by a linear mixed effect model to account for the repeated measurements for each subject across time are provided for UNR vs MNR for SM-102 and cKK-E10 lipid nanoparticles (LNPs). The fixed effects include nucleoside modification type, LNP, time, dosage, and their interactions. Columns represent time and rows represent the comparison of LNP specific nucleoside modification effects at different dosages. The top table shows the significance *p*-values; $p < 0.001$(dark orange) or $p < 0.05$ (light orange). The bottom table shows the effect sizes at log_{10} fold change; red/positive cell represents increased HAI level while blue/negative cell represents decreased HAI level.

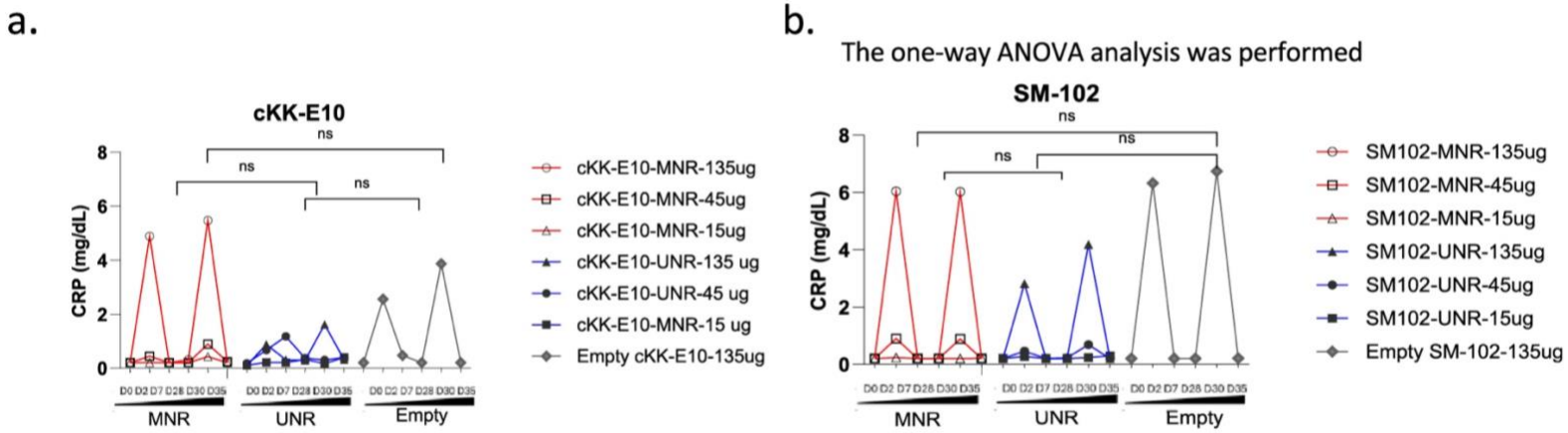


Supplementary Fig. 3 Site of injection reactions in mice immunized with mRNA-LNP vaccines. BALB/c mice (*n* = 8 per group) were immunized twice intramuscularly (IM), 3 weeks apart with different Sing16 HA mRNA-LNP formulations. IM injection sites were monitored in mice receiving 1 μg of different Sing16 HA mRNA-LNP formulations. Injection site reactions for edema (left panel) and erythema (right panel) were observed one day following the booster dose and graded according to the Draize Grading Scale shown in the table below. The one-way ANOVA analysis was performed. ns, non significant.

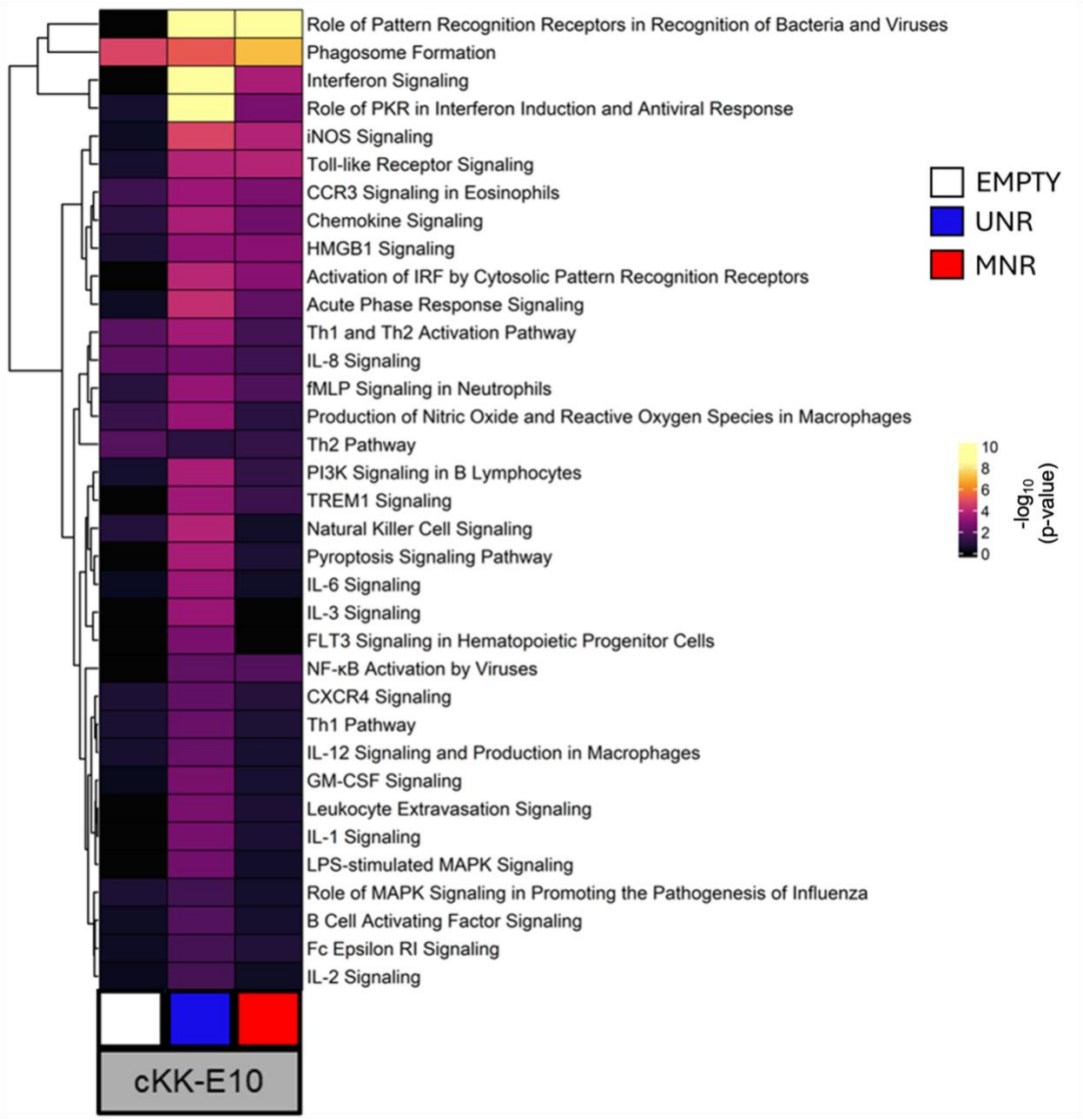


Score	Grade	Edema	Erythema
0	None	No swelling	Normal color
1	Minimal	Slight swelling (barely perceptible)	Light pink (barely perceptible)
2	Mild	Defined swelling (distinct)	Bright pink/Pale red
3	Moderate	Defined swelling (raised)	Bright red
4	Severe	Pronounced swelling	Dark red

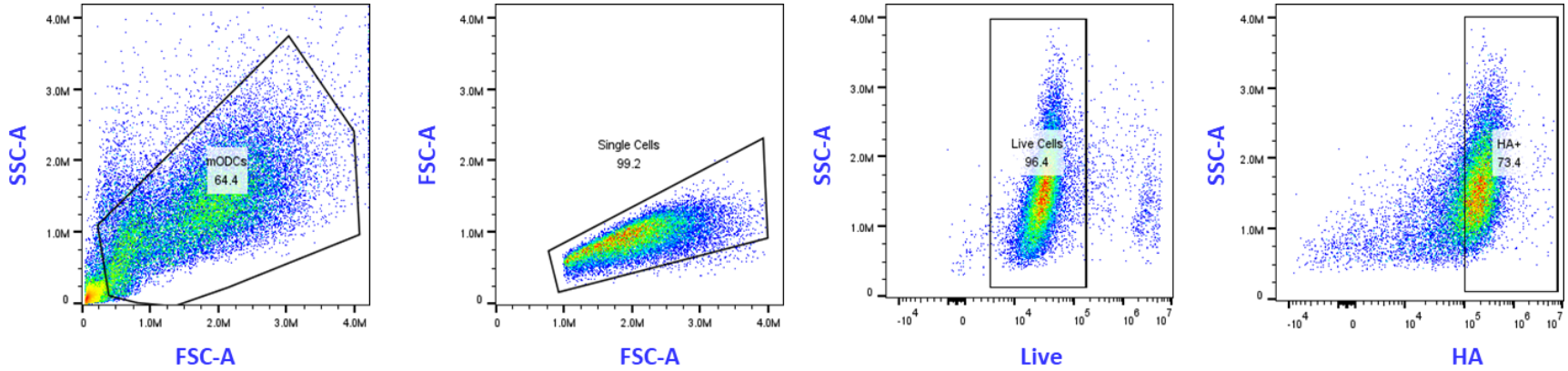
Supplementary Fig. 4 Serum CRP values in NHPs treated with the various UNR and MNR LNPs. Cynomolgus macaques (*n* = 4-6 per group) were immunized at Day 0 and Day 28 by intramuscular route with 135, 45, or 15 µg of different mRNA-LNP formulations encoding for the H3-HA protein from the A/Singapore/INFIMH-16-0019/2016 strain of influenza. Serum CRP levels tested at D0, D2, D7(after prime) and D28, D30, D35 (after boost) are presented for **a)** Sing16 HA-cKK-E10 **b)** Sing16 HA-SM-102



Supplementary Fig. 5 Top enriched canonical pathways following mRNA vaccination in non-human primates. Heatmap depicting overrepresentation of canonical pathways enriched in whole blood of NHPs following a 135 µg dose of mRNA-LNP (cKK-E10) or empty-LNP vaccines. The color bar below the heatmap denotes the mRNA modification class (empty-LNP: white; UNR: blue; MNR: red). The color of each cell refers to the -$\log_{10}$ (*p*-value) and indicates the significance of the pathway enrichment (see color key).



Supplementary Fig. 6 Flow gating strategy for dendritic cells transfected with mRNA LNP and expressing HA protein: DCs were transfected with mRNA LNP for 24 hrs. The cells were washed, stained with live dead stain and HA antibody. Dendritic cell events acquired by flow cytometer were gated by FSC-A and SSC-A. The percentage of HA positive cells was determined from live dendritic cells.



Supplementary Table 1 Attributes of mRNA-LNPs used in different experimental models. Size and PDI of LNP were measured using dynamic light scattering, dsRNA impurities were quantified by ELISA, and % encapsulation was measured using a fluorescent assay.

	Cell culture and Mice				NHPs		
mRNA/LNP format	**Size (nm)**	**PDI**	**% EC**	**dsRNA drug Substance**	**Size (nm)**	**PDI**	**% EC**
UNR Sing16 HA cKK-E10	113	0.15	95	0.251±0.006	119	0.106	90
MNR Sing16 HA cKK-E10	114	0.123	93	0.083±0.007	95	0.12	93
UNR Sing16 HA SM-102	83	0.106	98	0.251±0.006	94	0.116	98
MNR Sing16 HA SM-102	100	0.122	98	0.083±0.007	128	0.145	97
UNR Sing16 HA OF-02	115	0.108	96	0.251±0.006	-	-	-
MNR Sing16 HA OF-02	118	0.084	96	0.083±0.007	-	-	-
UNR Wisconsin HA OF-02	116	0.106	96	0.414±0.012	-	-	-
MNR Wisconsin HA OF-02	126	0.088	93	0.203±0.022	-	-	-
UNR Tasmania HA OF-02	119	0.133	95	0.175±0.025	-	-	-
MNR Tasmania HA OF-02	126	0.091	88	0.216±0.007	-	-	-
UNR Washington HA OF-02	123	0.057	91	0.312±0.028	-	-	-
MNR Washington HA OF-02	128	0.058	84	0.722±0.011	-	-	-
UNR Austria HA OF-02	126	0.004	86	0.687±0.043	-	-	-
MNR Austria HA OF-02	128	0.039	84	0.253±0.005	-	-	-

Notes: EC = % Encapsulation; PDI= Polydispersity index. DSPC = 1,2-distearoyl-sn-glycero-3-phosphocholine; DOPE= Dioleoylphosphatidylethanolamine
All mRNA used had a % Cap-1 >95% and %RNA integrity > 77%
LNP **composition (molar ratios)** DMG-PEG2k: CL:Cholesterol:DOPE for OF-O2 and cKK-E10 are 1.5: 40: 28.5: 30; and DMG-PEG2k: CL:Cholesterol:DSPC for SM-102 are 1.5:50:38.5:10
pKa of OF-O2 LNP formulation is 6.5; cKK-E10 LNP formulation is 6.6; SM-102 formulation is 6.5

Supplementary Table 2: GeneBank Accession numbers for protein and nucleotide sequence for influenza Hemagglutinins used in the study.

Strain	GISAID	Protein GenBank	GenBank Strain	Nucleotide GenBank
A/Singapore/INFIMH-16-0019/2016	EPI_ISL_1164036	QQY97257.1	A/Singapore/INFIMH-16-0019/2016	SUB15434744 A/Singapore/INFIMH-16-0019/2016 PV874231
A/Wisconsin/588/2019	EPI_ISL_1661758	WMW30925.1	A/Wisconsin/588/2019	SUB15434744 A/Wisconsin/588/2019 PV874232
A/Tasmania/503/2020	EPI_ISL_1752480	WMW30850.1	A/Tasmania/503/2020	SUB15434744 A/Tasmania/503/2020 PV874233
B/Washington/02/2019	EPI_ISL_1368874	AZY31612.1	B/Texas/7671/2018	SUB15435090 B/Washington/02/2019 PV874264
B/Austria/1359417/2021	EPI_ISL_1845793	QXN15658.1	B/New Hampshire/01/2021	SUB15435090 B/Austria/1359417/2021 PV874265