Supplementary Materials

Pharmaceutics

Title:

The Effect of Cholesterol Content on the Adjuvant Activity of Nucleic-Acid-Free Lipid Nanoparticles

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Supplementary Table S1. Detailed list of materials used in the study

Reagent	Size	Manufacturer	Product number
SM-102	100 mg	Cayman Chemical	33474
COATSOME MC-8080 (DSPC; 1,2-Distearoyl-sn-glycero-3-phosphocholine)	1 g	NOF CORPORATION	MC-8080
Cholesterol	5 g	Sigma Aldrich	C8667-5G
DMG-PEG (1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol) 2000	1 g	NOF CORPORATION	GM-020
Sodium Acetate (CH_3COONa)	500 g	nacalai tesque	C31118-75
MES (2-(*N*-morpholino)ethanesulfonic acid)	100 g	nacalai tesque	02442-44
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	500 g	Dojindo	342-01375
D-PBS (-)	500 mL	nacalai tesque	14249-24
99.5% Ethanol	500 mL	nacalai tesque	14713-95
UltraPure ™ DNase/RNase-Free Distilled Water (DDW)	500 mL	Invitrogen	10977-023
Albumin from chicken white egg	250 mg	Sigma Aldrich	A2512-250MG
Polyoxyethylene Sorbitan Monolaurate (Tween 20)	500 g	nacalai Tesque	35624-15
Fetal Bovine Serum (FBS)	500 mL	Gibco	10270
Goat anti-Mouse IgG-Fc Fragment Antibody HRP Conjugated	1 mL	Bethyl Laboratories	A90-131P
TMB Solution	100 mL	Merck Millipore	CL07-100ML
1mol/l Sulfuric Acid (H_2SO_4)	500 mL	nacalai Tesque	95626-06
Sodium Hydrogen Carbonate ($NaHCO_3$)	500 g	nacalai Tesque	31212-25
Sodium Carbonate (Na_2CO_3)	500 g	nacalai Tesque	31310-35
LEGENDplex™ Mouse Anti-Virus Response Panel (13-plex) with V-bottom plate	100 tests	BioLegend	740622
Isoflurane Inhalation Anesthetic Solution	250 mL	Viatris	1119701G1092
AmiconUltra-4-100K Centrifugal Units	4 mL volume	Merck	UFC810096
AmiconUltra-4-100K Centrifugal Units	15 mL volume	Merck	UFC910096

S2. Methods for supplementary figures

S2-1. Circular dichroism (CD) measurement

LNP_A-D were prepared as described in the main text. The final concentration of samples was 6.6 mM of total lipid. Circular dichroism of these samples was measured using a J-1500 Circular Dichroism Spectrophotometer (JASCO Corporation, Japan). Parameters were as follows: measured range = 192-280 nm, scanning ratio = 50 nm/min, response = 4 sec, band width = 1 nm, accumulations = 4 times, and repeat = 3 times. The obtained CD spectrum was normalized by absorbance at 204 nm.

S2-2. Chemokine/cytokine measurement

Dispersed cholesterol particles, Liposome_C and LNP_A-D, were administered to BALB/c mice via s.c. injection (back of neck). Six hours after the injection, the blood was collected from the vena cava inferior using a 1 mL syringe and a 26G needle, and it then was moved to a 1.5 mL Eppendorf tube. The blood serum was obtained by incubating the blood at room temperature (RT) for 2 hours, followed by centrifugation (4 ^{o}C, 2,000G, 10 min). The chemokine/cytokine quantification was done using a LEGENDplex™ Mouse Anti-Virus Response Panel (13-plex) with a V-bottom Plate kit (#740622, BioLegend, California, US), following the manufacturer's instructions. These data were collected by NovoCyte Flow Cytometer Systems and analyzed using the Data Analysis Software Suite for LEGENDplex™. The chemokine/cytokine levels were determined from the mean fluorescence intensity (MFI) values.

Supplementary Figure S1

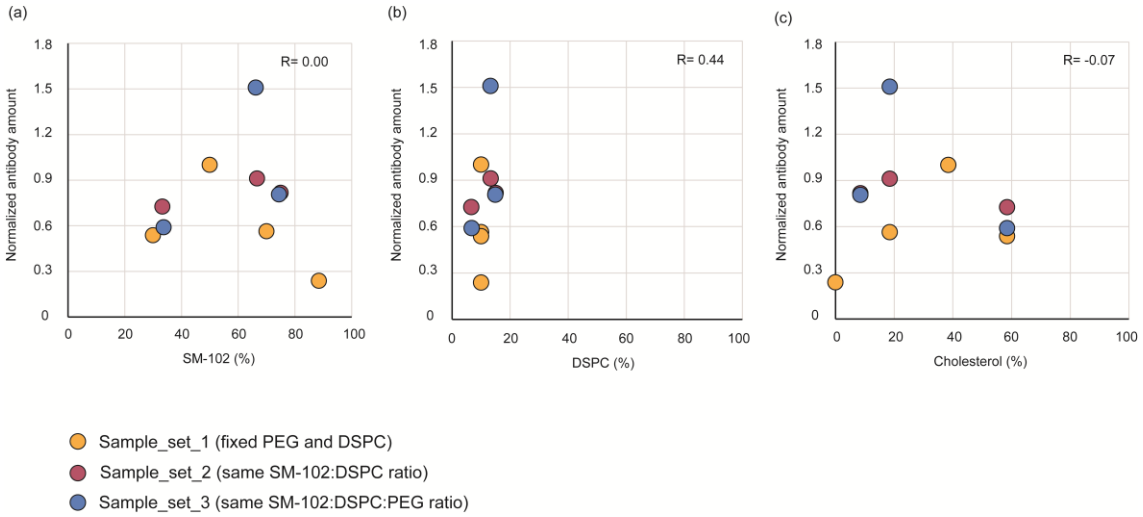

Figure S1. Correlation of lipid content and antibody production: Antibody production 14 days after boost vaccination correlated with **(a)** ionizable lipid SM-102, **(b)** DSPC, and **(c)** cholesterol content within the LNP. No significant correlation was found between antibody production and lipid content.

Supplementary Figure S2

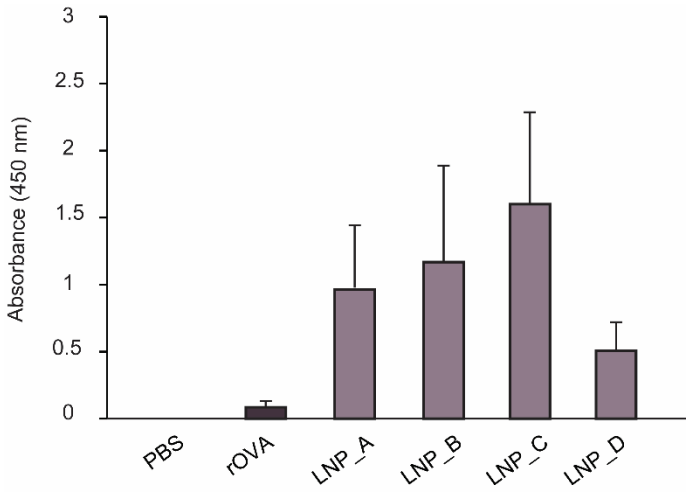

Figure S2. Antibody production after separate administration of LNPs and antigens: The antibody production of LNP_A-D administered separately with recombinant OVA proteins is shown. Doses of 200 nmol of empty-LNPs were administered subcutaneously (s.c.) into BALB/c mice, followed by the administration (s.c.) of 10 µg of rOVA 6 hours later. The treatment was performed twice with a 14-day interval. Later, the blood of each mouse was collected, and the serum was used for anti-OVA total IgG quantification by enzyme-linked immunosorbent assay (ELISA). The antibody production of LNPs did not depend on their ionizable lipid content. Mean ± SD (*n* = 1-5).

Supplementary Figure S3

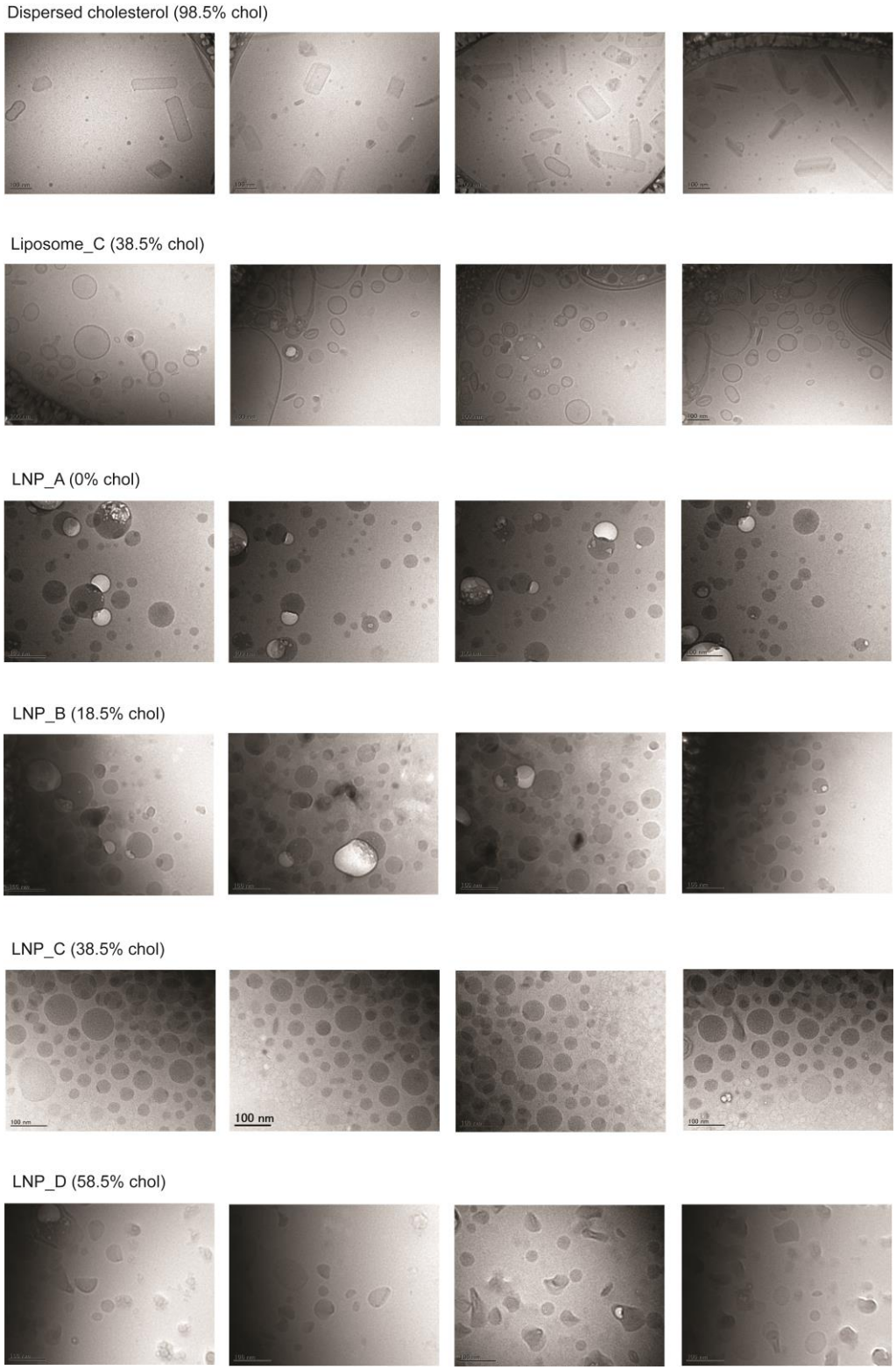

Figure S3. Additional images of particle morphology observed using Cryo-TEM: Additional Cryo-TEM images of dispersed cholesterol, Liposome_C, LNP_A, LNP_B, LNP_C, and LNP_D are shown. Each bar indicates 100 nm.

Supplementary Figure S4

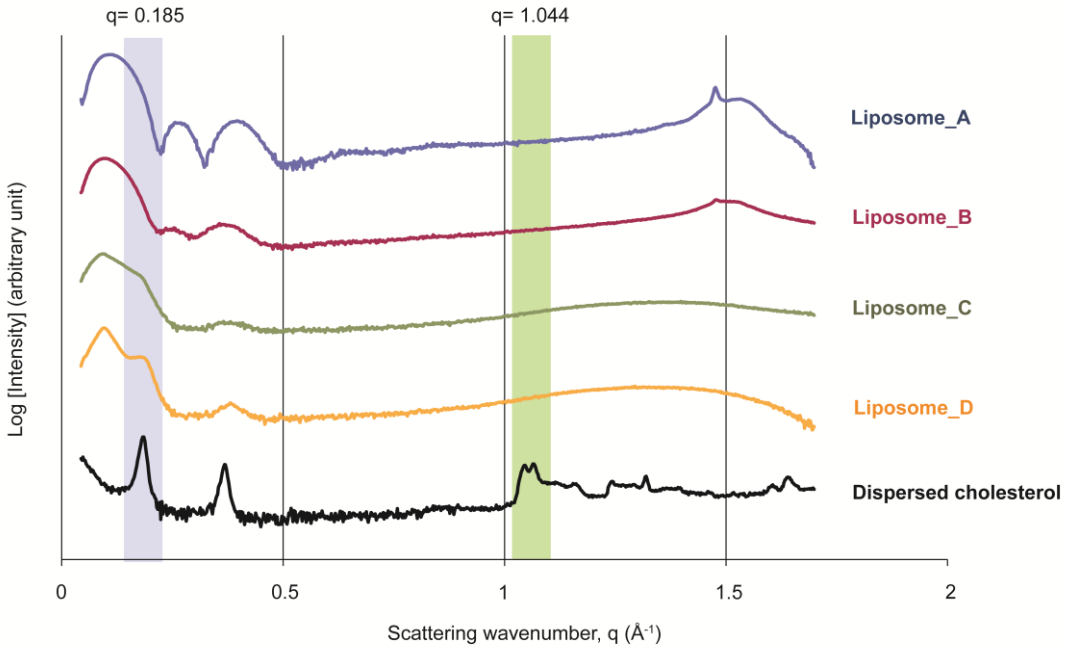

Figure S4. The SAXS profile of Liposomes_A-D: The SAXS profiles of Liposome_A-D are shown. The SAXS profile of dispersed cholesterol particle is included for comparison.

Supplementary Figure S5

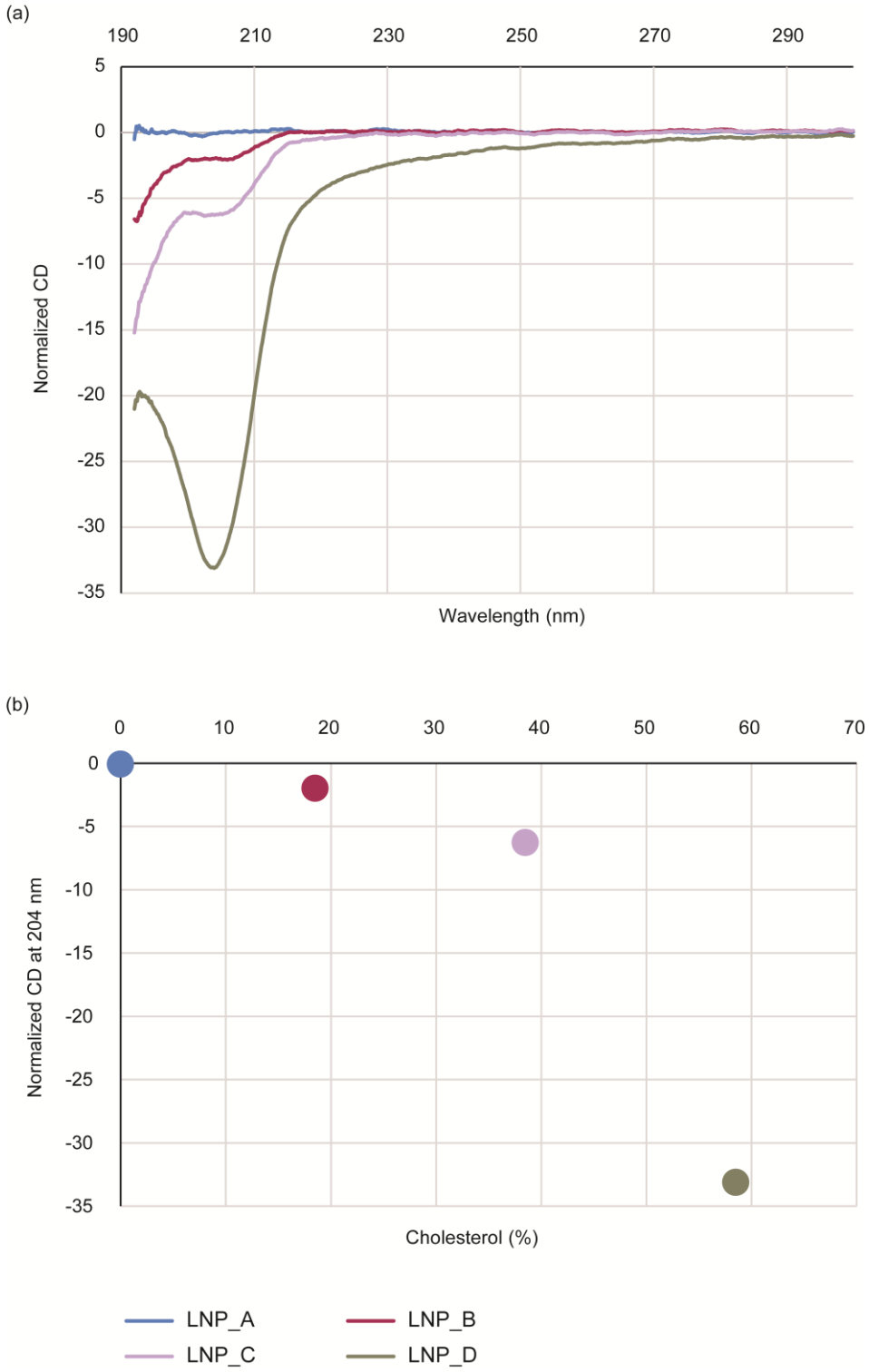

Figure S5. Circular dichroism (CD) spectra: (a) The CD spectra of LNP_A-D normalized at an absorbance wavelength of 204 nm; **(b)** The CD of LNP_A-C show a linear relationship with cholesterol content, while that of LNP_D shows a large absolute value compared with that of the other LNPs.

Supplementary Figure S6

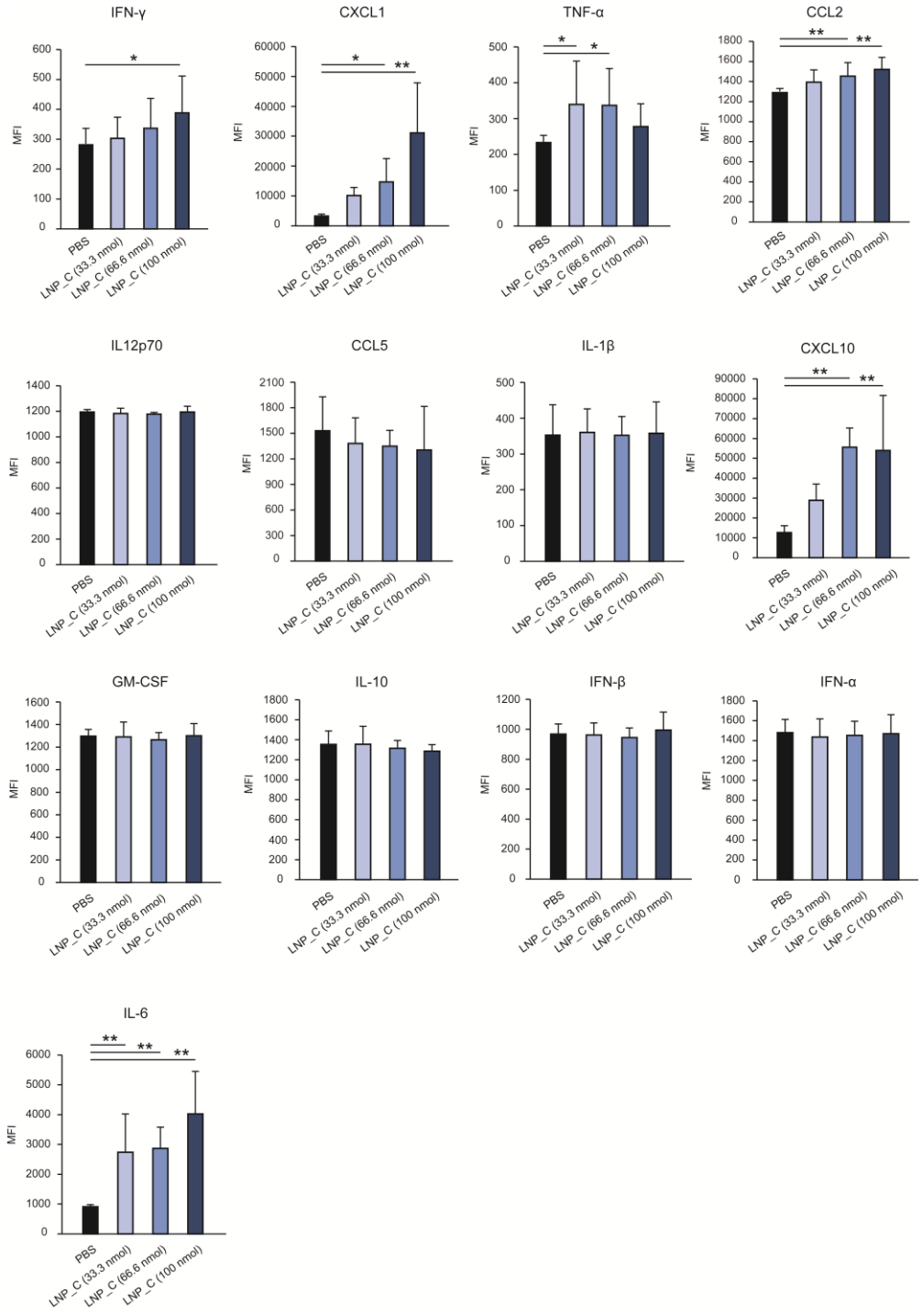

Figure S6. Dose dependent chemokine/cytokine production: The chemokines/cytokines induced by LNP_C are shown with doses of 33.3 nmol, 66.6 nmol, and 100 nmol of total lipid concentration. The respective doses (33.3, 66.6, 100 nmol) of empty-LNPs were administered subcutaneously to the BALB/c mice. After 6 hours, the blood serum was collected and used to quantify the chemokine/cytokine levels. Mean with SD (n = 3), * p<0.05, ** p<0.01 (one-way ANOVA followed by Bonferroni against PBS).

Supplementary Figure S7

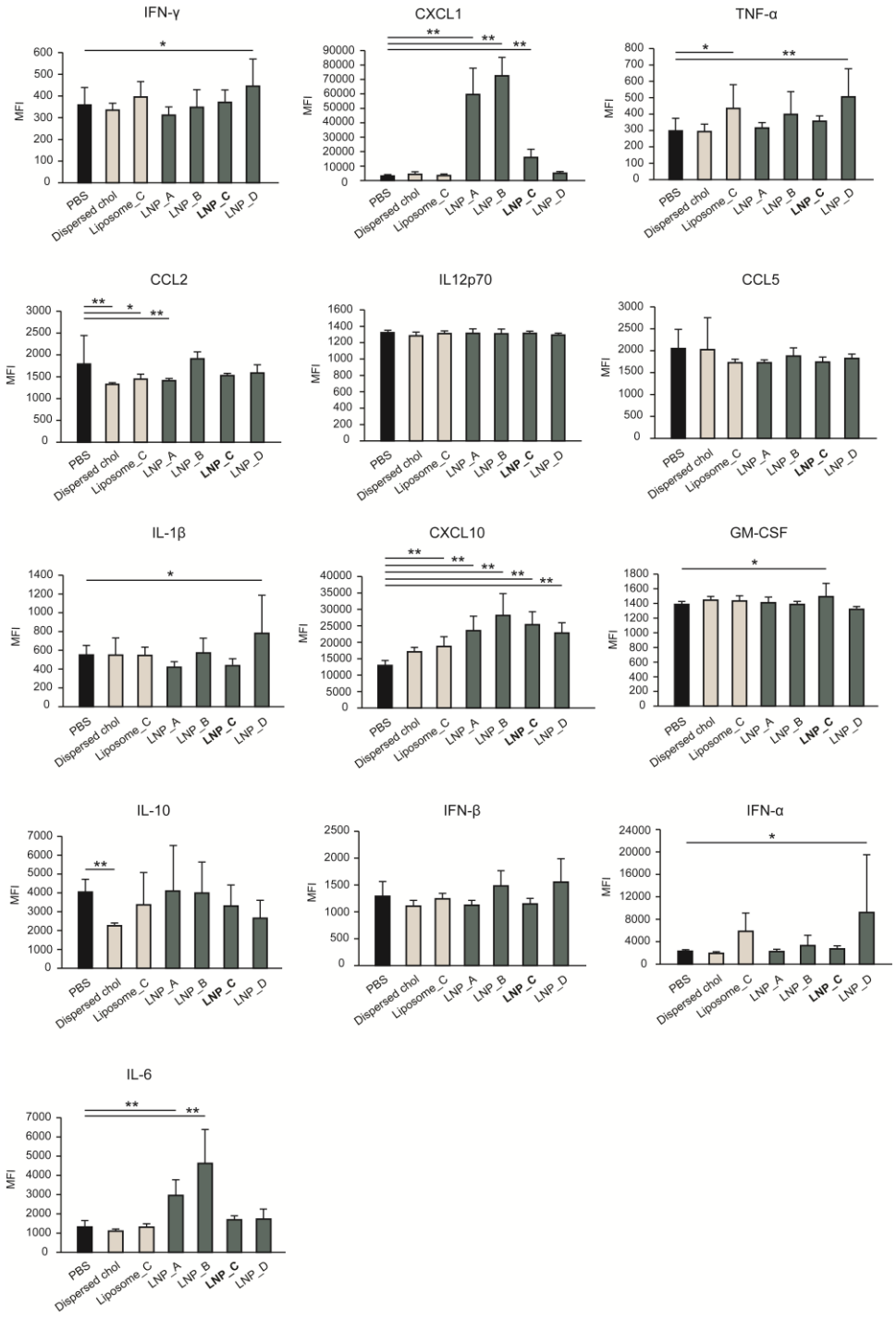

Figure S7. Chemokines/cytokines production of different nanoparticles: The chemokines/cytokines induced by dispersed cholesterol, Liposome_C, and LNP_A-D are shown. Doses of 100 nmol of empty LNPs were administered subcutaneously to the BALB/c mice; after 6 hours, the blood serum was collected and used to quantify the chemokine/cytokine levels. Mean with SD (n = 3), * p<0.05, ** p<0.01 (one-way ANOVA followed by Bonferroni against PBS).