Supplementary Information

Administration Sequence- and Formulation-dependent Vaccination using Acid-degradable Polymeric Nanoparticles with High Antigen Encapsulation Capability

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Materials and Methods for the Synthesis of Acid-degradable Monomers and Crosslinkers

Materials. 2-(2-aminoethoxy)ethanol, trimethylamine (TEA), ethyl acetate, hexane, tetrahydrofuran (THF), methylene chloride, silica gel sorbent, 2-methoxypropene, molecular sieve, trifluoroacetate, N-hydroxysuccinimide (NHS), and methyl sulfoxide-d6, ethyl were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Methacryloyl chloride, pyridinium *p*-toluenesulfonate (PPTS), nitrilotriacetic acid (NTA), N,N'-dicyclohexylcarboodiimide (DCC), 4-(dimethylamino)pyridine (DMAP), and Nickel (II) chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Synthesis of Compound 1. 2-(2-aminoethoxy)ethanol (5 g, 47.6 mmol, 1 equiv.) dissolved in 30 mL of DI water was mixed with 25mL of triethylamine (19.27g, 190.4 mmol, 4 equiv.), followed by adding methacryloyl chloride (5 g, 47.6 mmol, 1 equiv.) using a syringe. The mixture was stirred for 5 min at room temperature (RT) and extracted with 3 × 300 mL of ethyl acetate. After the solvent was removed by rotary evaporation, Compound 1 was purified by

silica gel chromatography using 4:1 hexane:ethyl acetate, 3:2 hexane:ethyl acetate, 1:1 hexane:acetate, 2:3 hexane:ethyl acetate, 1:4 hexane:ethyl acetate, and finally ethyl acetate alone as the eluent. The structure of Compound 1 was analyzed by ^{1}H NMR (500 MHz, DMSO): δ 1.84 (s, 3H), δ 3.18-3.54 (m, 8H), δ 5.32 (s, 1H), δ 5.64 (s, 1H), δ 7.89 (s, 1H).

Synthesis of ketal bismethacrylamide cross-linker (KXL). Compound 1 (7 g, 40.4 mmol, 1 equiv.) and pyridinium p-toluenesulfonate (PPTS) (3.0 g, 12.1 mmol, 0.3 equiv.) were dissolved in 300 mL of THF. After 10 min of stirring, 50 g of molecular sieves (5 Å, 1.6 mm pellet) were added, and the mixture was stirred for an additional 10 min followed by addition of 2-methoxypropene (17.5 g, 242.4 mmol, 6 equiv.). The resulting reactant solution was stirred overnight at RT. After the molecular sieves were removed by filtration, the crude mixture was subject to rotary evaporation. The product was purified by silica gel chromatography using 9:1 hexane:ethyl acetate, 8:2 hexane:ethyl acetate, 7:3 hexane:acetate, 6:4 hexane:ethyl acetate, 5:5 hexane:ethyl acetate, and finally 4:6 hexane/ethyl acetate. To avoid hydrolysis of acid-sensitive ketal cross-linker (KXL), 2% trimethylamine (TEA) was added to the eluents. The chemical structure of KXL was analyzed by ^{1}H-NMR (500 MHz, DMSO): δ 1.25 (s, 6H), δ 3.22-3.33 (m, 4H), δ 3.40-3.51 (m, 12H), δ 5.55-5.59 (m, 2H), δ 6.04-6.09 (m, 2H), δ 8.12 (s, 2H).

Synthesis of Compound 2. Triethylamine (19.25 g, 190.2 mmol, 2 equiv.) was added to 2-(2-aminoethoxy)ethanol (10g, 95.1 mmol, 1 equiv.) dissolved in 300 mL of methylene chloride. After adding ethyl trifluoroacetate (16.2 g, 114.12 mmol, 1.2 equiv.), the reaction mixture was stirred overnight at RT. Most of the solvent was removed by rotary evaporation, then the mixture was dissolved in 30 mL of DI water and extracted with 3 × 50 mL of ethyl acetate, followed by evaporation of ethyl acetate. The chemical structure of Compound 2 was analyzed by ^{1}H-NMR (500 MHz, DMSO): δ 3.26-3.65 (m, 8H), δ 4.64 (s, 1H), δ 9.46 (s, 1H).

Synthesis of Compound 3. Compound 2 (15 g, 74.6 mmol, 1 equiv.) was dissolved in 300 mL of THF and pyridinium *p*-toluenesulfonate (PPTS) (5.6 g, 22.4 mmol, 0.3 equiv.) was added. After 10 min of stirring, 250 g of molecular sieves (5 Å, 1.6 mm pellet) were added and the mixture was stirred for 10 min. Then 2-methoxypropene (32.3 g, 448 mmol, 6 equiv.) was added before additional stirring for 4 h at RT. After removing the molecular sieves by filtration, the solvent was evaporated. The mixture was dissolved in 30 mL of DI water and extracted with 3 × 50 mL ethyl acetate, and the ethyl acetate solvent was evaporated. After evaporating the solvent, the product was purified by silica gel column chromatography using 9:1 hexane:ethyl acetate, 8:2 hexane:ethyl acetate, 7:3 hexane:ethyl acetate, 6:4 hexane:ethyl acetate, 5:5 hexane:ethyl acetate, and finally 4:6 hexane:ethyl acetate. To avoid hydrolysis of

Compound 3, 2% trimethylamine (TEA) was added to the eluents. The product's structure was analyzed by ^{1}H-NMR (500 MHz, DMSO): δ 1.24 (s, 6H), δ 3.07 (s, 3H), δ 3.26-3.65 (m, 8H), δ 9.40 (s, 1H).

Synthesis of Compound 4. Compound 1 (3.07 g, 17.13 mmol, 1 equiv.) was dissolved in 300 mL of THF with 150 g of molecular sieves (5 Å, 1.6 mm pellet), and pyridinium *p*-toluenesulfonate (PPTS) (0.9 g, 5.3 mmol, 0.2 equiv.) was added, followed by stirring for 10 min. Then compound 3 (5 g, 19.5 mmol, 1.1 equiv.) was added, and the mixture was stirred overnight at RT. After removing the molecular sieves by filtration, the solvent was evaporated. After evaporating the solvent, the product was purified by silica gel column chromatography using 9:1 hexane:ethyl acetate, 8:2 hexane:ethyl acetate, 7:3 hexane:ethyl acetate, 6:4 hexane:ethyl acetate, and finally 5:5 hexane:ethyl acetate. To avoid hydrolysis of acid-sensitive Compound 4, 2% triethylamine (TEA) was added to the eluents. The chemical structure of ketal methacrylamide monomer was analyzed by ^{1}H-NMR (500 MHz, DMSO): δ 1.23 (s, 6H), δ 1.87 (s, 3H), δ 3.28 (s, 2H), δ 3.33-3.69 (m, 14H), δ 5.35 (s, 1H), δ 5.66 (s, 1H), δ 7.90 (s, 1H).

Deprotection of Compound 4 to obtain ketal methacrylamide monomer (KM). Compound 4 (2 g) was dissolved in 10 mL of 6M NaOH in water and stirred for 2 h. After extraction with 3 × 30 mL of methylene chloride and solvent evaporation, ketal methacrylamide monomer was obtained as a pale-yellow oil. The chemical structure of ketal methacrylamide monomer was analyzed by ^{1}H-NMR (500 MHz, DMSO): δ 1.26 (s, 6H), δ 1.84 (s, 3H), δ 3.26 (s, 2H), δ 3.33-3.65 (m, 14H), δ 5.31 (s, 1H), δ 5.64 (s, 1H), δ 7.94 (s, 1H).

Synthesis of Compound 5. Nitrilotriacetic acid (NTA) (3 g, 15.7 mmol, 1.2 equiv.) was stirred in 30 mL DI water along with 10 mL TEA to completely dissolve it. The mixture of *N*-hydroxysuccinimide (NHS) (1.51 g, 13.08 mmol, 1 equiv.) dissolved in 5 mL DI water, *N,N'*-dicyclohexylcarbodiimide (DCC) (2.7 g, 13.08 mmol, 1 equiv.) dissolved in 5 mL THF, and 4-(dimethylamino)pyridine (DMAP) (0.08 g, 0.65 mmol, 0.05 equiv.) dissolved in 0.5 mL DI water was slowly added dropwise to the NTA solution, followed by stirring at RT under N_2 gas for 24 h. The solution was filtered to remove dicyclohexylurea (DCU) and evaporated to remove the organic solvent before lyophilization for 2 days.

Synthesis of Compound 6. Under N_2 gas, ketal methacrylamide monomer (0.5 g, 1.5 mmol, 1 equiv.) was reacted with compound 5 (0.54 g, 1.9 mmol, 1.27 equiv.) in 10 mL DI water in the presence of TEA for 24 h to produce compound 6. The product solution was evaporated to

remove TEA and lyophilized for 3 days prior to use it.

Synthesis of Ni-NTA ketal methacrylamide monomer (NKM). Compound 6 (0.5 g, 1 mmol, 1 equiv.) was dissolved in 5 mL DI water and stirred before TEA was added to dissolve Compound 6 completely. Then nickel(II) chloride ($NiCl_2$) (0.13 g, 1 mmol, 1 equiv.) dissolved in 1 mL DI water was slowly added dropwise and the mixture was reacted for 12 h before lyophilization. The structure of Ni-NTA ketal methacrylamide monomer was analyzed by ^{1}H-NMR (500 MHz, DMSO): δ 1.19 (s, 6H), δ 1.84 (s, 3H), δ 3.03 (s, 2H), δ 3.21-3.67 (m, 20H), δ 5.33 (s, 1H), δ 5.65 (s, 1H), δ 7.92 (s, 1H).

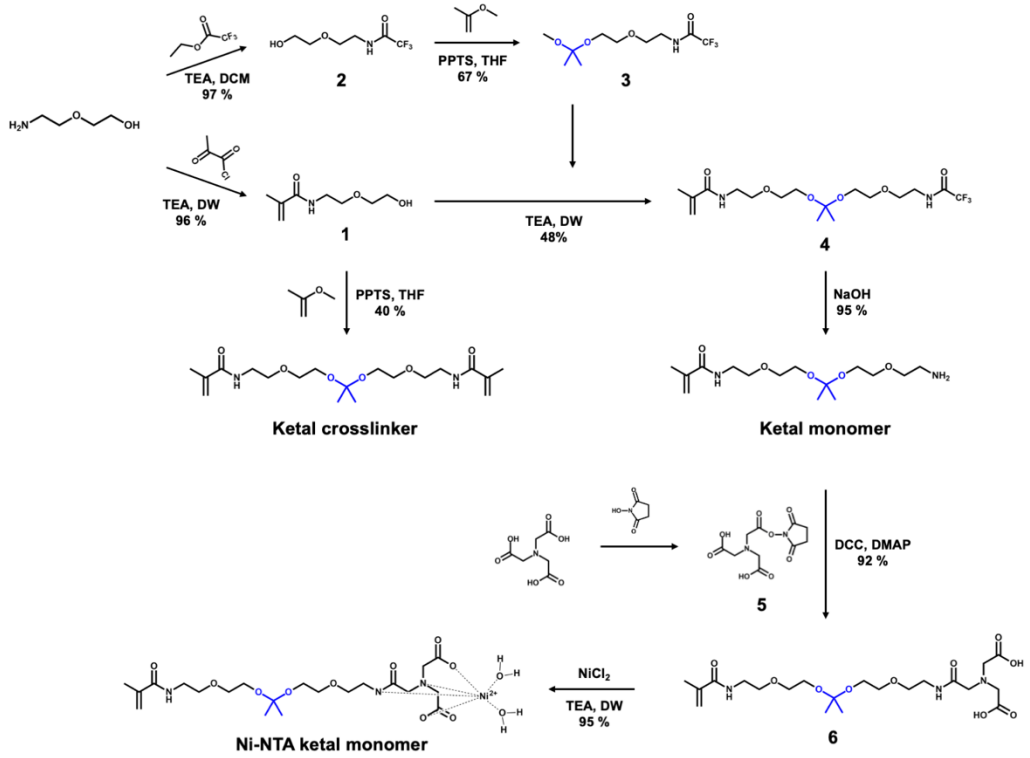

Figure S1. Synthesis scheme of acid-cleavable amino ketal methacrylamide monomer (KM), Ni-NTA ketal methacrylamide monomer (NKM), and ketal bismethacrylamide cross-linker (KXL).

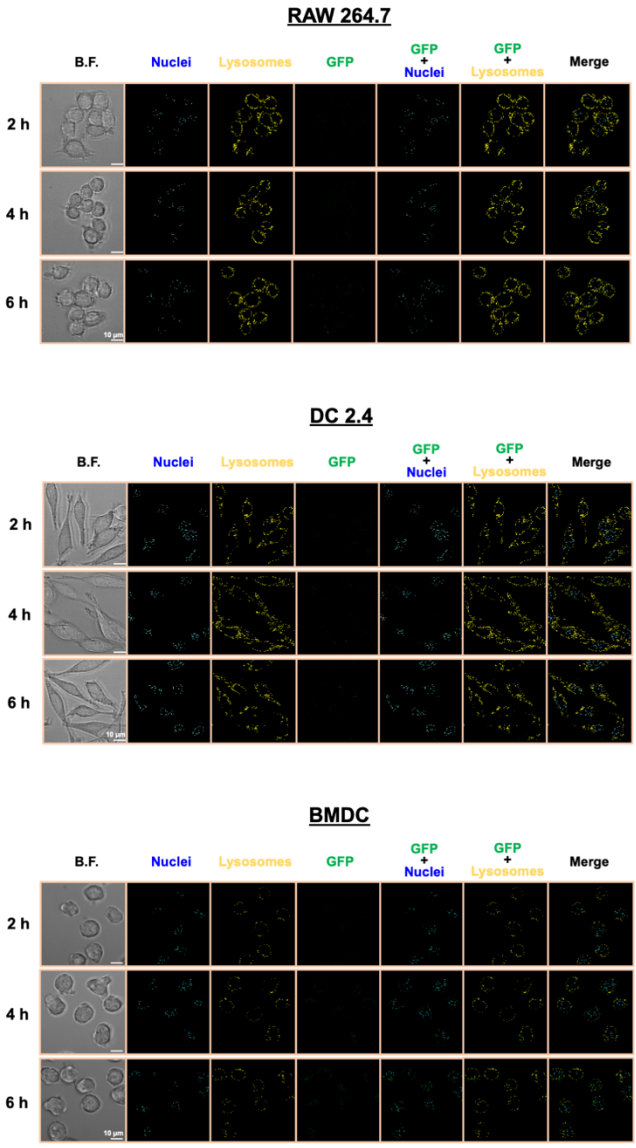

Figure S2. Intracellular distributions of free GFP in DC 2.4 cells for 2, 4, and 6 h. The fluorescence of GFP in the cells was imaged by super resolution fluorescence microscopy.

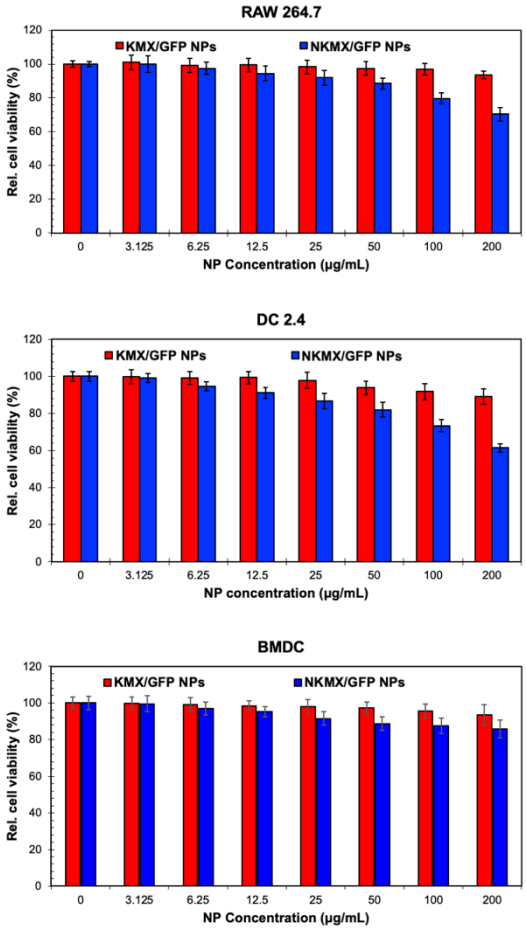

Figure S3. Relative viabilities of RAW 264.7, DC 2.4, and BMDC incubated with KMX/GFP and NKMX/GFP NPs at varying concentrations for 24 h. The data are represented as the mean ± standard deviation (SD) (n=6).

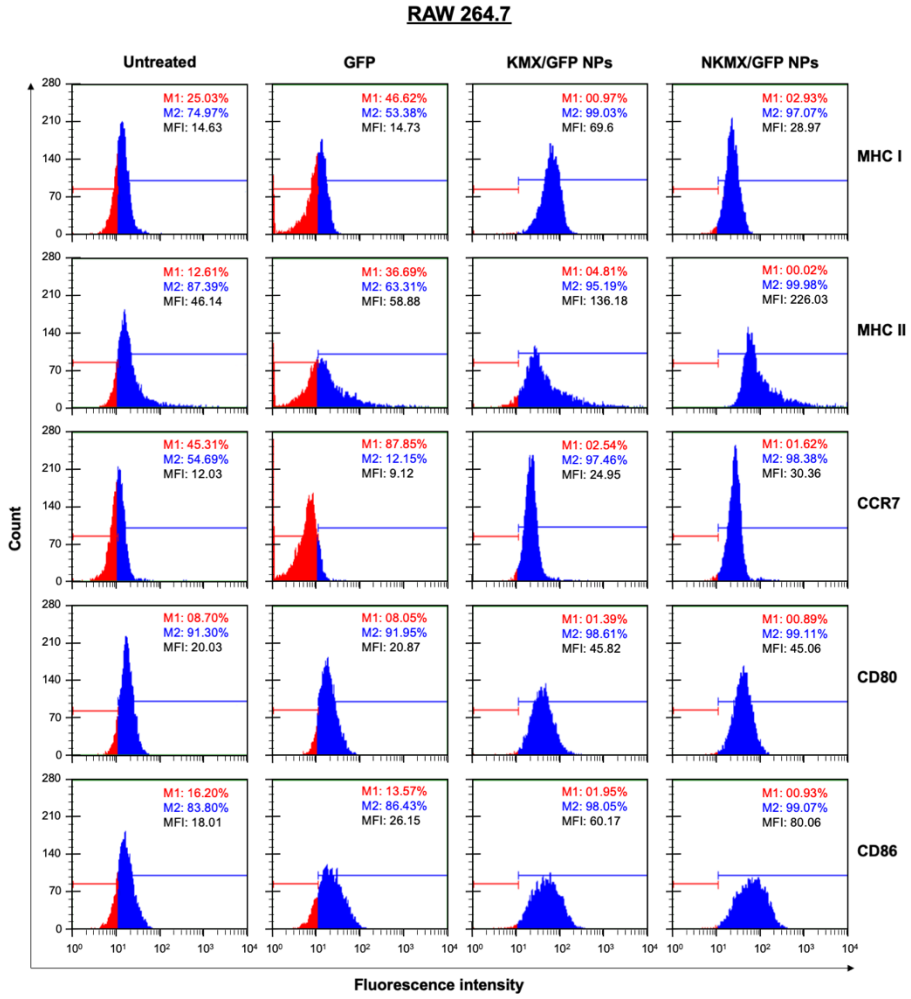

Figure S4. Changes on the cell surface markers after RAW 264.7 cells were incubated with free GFP, KMX/GFP NPs, and NKMX/GFP NPs for 6 h.

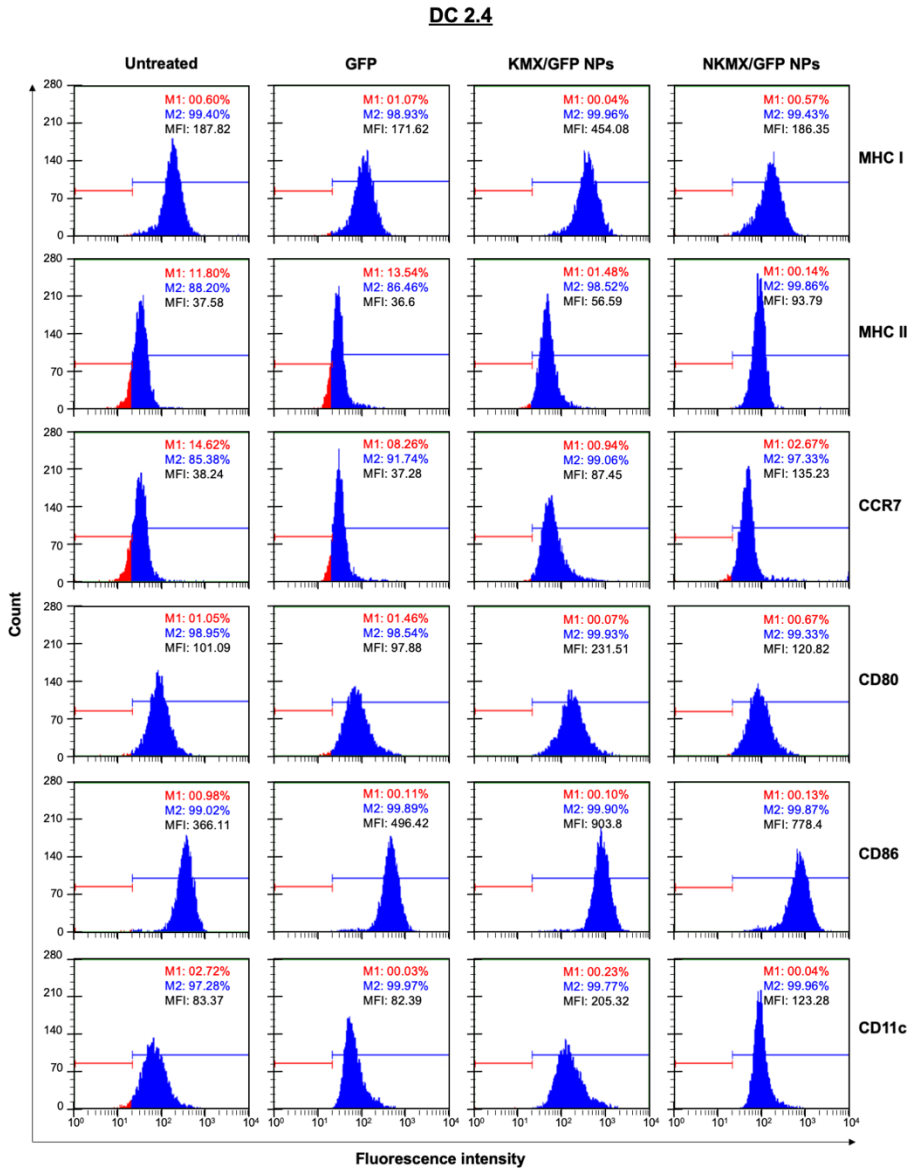

Figure S5. Changes on the cell surface markers after DC 2.4 cells were incubated with free GFP, KMX/GFP NPs, and NKMX/GFP NPs for 6 h.

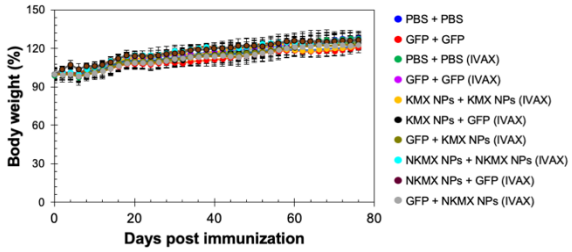

Figure S6. Body weight changes of C57BL/6 mice subcutaneously injected with combinations of GFP, KMX/GFP NPs, and NKMX/GFP NPs for prime and booster. Data are expressed as the means ± SD (n=5).

Statistical analyses for **Figure 6**

Day 10

	PBS PBS –	GFP GFP –	PBS PBS +	GFP GFP +	KMX NPs KMX NPs +	KMX NPs GFP +	GFP KMX NPs +	NKMX NPs NKMX NPs +	NKMX NPs GFP +	GFP NKMX NPs +
PBS PBS –	–	ns	ns	ns	ns	ns	$p < 0.05$	ns	ns	ns
GFP GFP –	ns	–	ns	ns	ns	ns	ns	ns	ns	ns
PBS PBS +	ns	ns	–	ns	ns	ns	$p < 0.05$	ns	ns	ns
GFP GFP +	ns	ns	ns	–	ns	ns	ns	ns	ns	ns
KMX NPs KMX NPs +	ns	ns	ns	ns	–	ns	ns	ns	ns	ns
KMX NPs GFP +	ns	ns	ns	ns	ns	–	ns	ns	ns	ns
GFP KMX NPs +	$p < 0.05$	ns	$p < 0.05$	ns	ns	ns	–	ns	ns	ns
NKMX NPs NKMX NPs +	ns	ns	ns	ns	ns	ns	ns	–	ns	ns
NKMX NPs GFP +	ns	ns	ns	ns	ns	ns	ns	ns	–	ns
GFP NKMX NPs +	ns	ns	ns	ns	ns	ns	ns	ns	ns	–

ns: not significant

Day 28

	PBS PBS –	GFP GFP –	PBS PBS +	GFP GFP +	KMX NPs KMX NPs +	KMX NPs GFP +	GFP KMX NPs +	NKMX NPs NKMX NPs +	NKMX NPs GFP +	GFP NKMX NPs +
PBS PBS –	–	ns	$p < 0.05$	$p < 0.001$	ns	$p < 0.01$	ns	ns	$p < 0.05$	ns
GFP GFP –	ns	–	$p < 0.05$	$p < 0.001$	ns	$p < 0.01$	ns	ns	$p < 0.05$	ns
PBS PBS +	$p < 0.05$	$p < 0.05$	–	$p < 0.001$	ns	$p < 0.01$	ns	$p < 0.05$	$p < 0.05$	ns
GFP GFP +	p < 0.001	$p < 0.001$	$p < 0.001$	–	$p < 0.001$	ns	$p < 0.05$	$p < 0.001$	ns	$p < 0.01$
KMX NPs KMX NPs +	ns	ns	ns	$p < 0.001$	–	$p < 0.01$	ns	ns	$p < 0.05$	ns
KMX NPs GFP +	$p < 0.01$	$p < 0.01$	$p < 0.01$	ns	$p < 0.01$	–	ns	$p < 0.01$	ns	ns
GFP KMX NPs +	ns	ns	ns	$p < 0.05$	ns	ns	–	ns	ns	ns
NKMX NPs NKMX NPs +	ns	ns	$p < 0.05$	$p < 0.001$	ns	$p < 0.01$	ns	–	$p < 0.05$	ns
NKMX NPs GFP +	$p < 0.05$	$p < 0.05$	$p < 0.05$	ns	$p < 0.05$	ns	ns	$p < 0.05$	–	ns
GFP NKMX NPs +	ns	ns	ns	$p < 0.01$	ns	ns	ns	ns	ns	–

ns: not significant

Day 50

	PBS PBS –	GFP GFP –	PBS PBS +	GFP GFP +	KMX NPs KMX NPs +	KMX NPs GFP +	GFP KMX NPs +	NKMX NPs NKMX NPs +	NKMX NPs GFP +	GFP NKMX NPs +
PBS PBS –	–	ns	$p < 0.05$	$p < 0.001$	ns	$p < 0.001$	ns	ns	$p < 0.05$	ns
GFP GFP –	ns	–	ns	$p < 0.001$	ns	$p < 0.001$	ns	$p < 0.05$	$p < 0.05$	ns
PBS PBS +	$p < 0.05$	ns	–	$p < 0.001$	ns	$p < 0.001$	ns	$p < 0.001$	$p < 0.05$	ns
GFP GFP +	$p < 0.001$	$p < 0.001$	$p < 0.001$	–	$p < 0.001$	$p < 0.05$	$p < 0.05$	$p < 0.001$	ns	$p < 0.01$
KMX NPs KMX NPs +	ns	ns	ns	$p < 0.001$	–	$p < 0.001$	ns	ns	$p < 0.05$	ns
KMX NPs GFP +	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.05$	$p < 0.001$	–	ns	$p < 0.001$	ns	ns
GFP KMX NPs +	ns	ns	ns	$p < 0.05$	ns	ns	–	ns	ns	ns
NKMX NPs NKMX NPs +	ns	$p < 0.05$	$p < 0.001$	$p < 0.001$	ns	$p < 0.001$	ns	–	$p < 0.05$	ns
NKMX NPs GFP +	$p < 0.05$	$p < 0.05$	$p < 0.05$	ns	$p < 0.05$	ns	ns	$p < 0.05$	–	ns
GFP NKMX NPs +	ns	ns	ns	$p < 0.01$	ns	ns	ns	ns	ns	–

ns: not significant

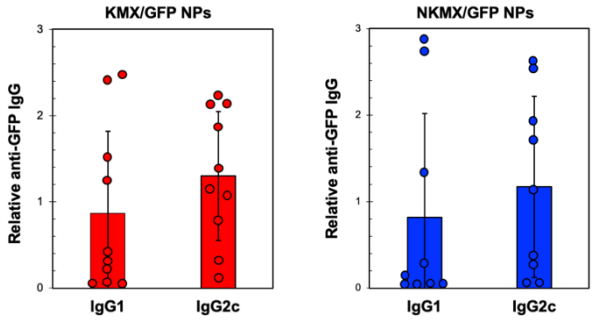

Figure S7. Quantification of IgG1 vs IgG2c in the mice vaccinated with KM/GFP NPs or NKMX/GFP NPs as the prime and free GFP as the booster injections.