ADVANCED MATERIALS

Supporting Information

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Nanomaterials-Mediated Co-Stimulation of Toll-Like Receptors and CD40 for Antitumor Immunity

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Supplementary Methods

Synthesis of Resiquimod derived amino lipids

1. *Synthesis of compound **3***

To a solution of resiquimod (100 mg, 0.32 mmol) and TEA (0.115 mL, 0.83 mmol) in DCM (2 mL) was added Trityl chloride (106.4 mg, 0.35 mmol) at 0 ℃. Then the mixture was allowed to warm to RT and stirred overnight. DCM was removed under reduced pressure. Then cold MeCN (5 mL) was added to the residue, **3** precipitated and was isolated by filtration at 0 ℃, washed with cold MeCN. The filtrate was further purified by silica gel chromatography (0%–30% [mixture of 3% NH_4OH, 22% MeOH in dichloromethane] in dichloromethane) to give 160 mg of compound **3** as a white powder, yield 89.9%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.95 (d, *J* = 8.4 Hz, 1H), 7.50 (d, *J* = 7.6 Hz, 6H), 7.36 – 7.15 (m, 12H), 7.05 (s, 1H), 4.89 (s, 2H), 4.73 (s, 2H), 3.71 (q, *J* = 6.8 Hz, 2H), 3.46 (s, 1H), 1.39 – 1.26 (m, 9H). MS (*m/z*): $[M+H]^+$ calcd. For $C_{36}H_{37}N_4O_2$, 557.29; found: 557.25.

2. *Synthesis of compound **4***

Amino lipid **1** was synthesized according to methods reported previously.[1] To a solution of amino lipid **1** (44.8 mg, 0.065 mmol) and TEA (0.027 mL. 0.195 mmol) in 2 mL of dry Toluene was added 2,4,6-Trichlorobenzoyl Chloride (21.8 mg, 0.129 mmol), and stirred at RT for 1 h. The solution was then allowed to be warmed to 40 ºC and stirred for 2 h. Then a solution of **3** (36 mg, 0.065 mmol) and DMAP (15.7 mg, 0.065 mmol) in 1 mL of anhydrous Toluene was added to the above mixture, the obtained cloudy mixture was stirred for 12 h at 40 ºC. Then the reaction mixture was diluted with 20 mL of water and extracted with DCM (15 mL* 3 times). The organic phase dried over anhydrous Na_2SO_4. The solution was filtered and the solvent was removed under reduced pressure. The residue was purified by silica gel chromatography (0%–20% [mixture of 3% NH_4OH, 22% MeOH in dichloromethane] in dichloromethane) to give 28 mg of desired product was obtained as a yellow oil, yield 35.0%. HRMS (*m/z*): $[M+H]^+$ calcd. For $C_{81}H_{127}N_6O_3$, 1232.9964; found: 1231.9900.

3. *Synthesis of compound **RAL1***

To a solution of **4** (28 mg, 0.0227 mmol) in 2 mL of DCM was added Trifluoroacetic acid (0.5 mL) dropwise at 0 ºC. The solution was then allowed to warm to rt and stirred for 2 h. The reaction was quenched by addition of saturated aqueous $NaHCO_3$ solution, and extracted with DCM (15 mL* 3 times). The organic phase dried over anhydrous Na_2SO_4. The solution was filtered and the solvent was removed under reduced pressure. The filtrate was further purified by silica gel chromatography (0%–30% [mixture of 3% NH_4OH, 22% MeOH in dichloromethane] in dichloromethane) to give 12.0 mg of **RAL1** as a yellow oil, yield 53%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.15 (dd, *J* = 8.4, 1.4 Hz, 1H), 7.80 (dd, *J* = 8.4, 1.2 Hz, 1H), 7.49 (ddd, *J* = 8.4, 6.9, 1.2 Hz, 1H), 7.37 – 7.27 (m, 1H), 5.43 (s, 2H), 4.93 (s, 2H), 3.56 (q, *J* = 6.9 Hz, 2H), 2.45 – 2.27 (m, 12H), 1.89 (t, *J* = 7.5 Hz, 2H), 1.58 – 1.51 (m, 2H), 1.46 - 1.35 (m, 8H), 1.33 - 1.23 (m, 65H), 1.13 - 1.05 (m, 2H), 0.88 (t, *J* = 6.9 Hz, 9H). HRMS (*m/z*): $[M+H]^+$ calcd. For $C_{62}H_{113}N_6O_3$, 989.8869; found: 989.8864.

4. *Synthesis of compound **7***

Compound **5** was synthesized according to the method reported previously.[1] Compound **5** (475 mg, 1.5 mmol) was dissolved in CH_2Cl_2 (8 mL) which was followed by the addition of Boc_2O (0.39 g, 1.8 mmol) and Et_3N (0.25 mL, 1.8 mmol) at RT and the mixture was stirred for 2 h. After completion of the reaction, 10 mL of water was added, organic phase was separated, aqueous layer was extracted with CH_2Cl_2 (3 × 20 mL). Organic phases were combined, washed with brine, dried over anhydrous Na_2SO_4 and concentrated to get crude product, which was used directly in the next step without further purification. To a solution of **6** (1.5 mmol) obtained in the previous step in THF (6 mL) and EtOH (1.5 mL) was added 2 N aq. NaOH solution (3 mL) at RT. The reaction mixture was stirred for 3 h at RT. The pH of reaction mixture was adjusted to 2 with 1N HCl aq. solution, then extracted with DCM (25 mL * 3 times), the organic phase was combined and washed with 60 mL of 1M $NaHCO_3$, and dried over anhydrous Na_2SO_4. The solution was filtered and the solvent was removed under reduced pressure. The filtrate was further purified by silica gel chromatography (0%–30% [mixture of 3% NH_4OH, 22% MeOH in dichloromethane] in dichloromethane) to give 507 mg of **7** as a pale yellow oil, yield 81%. ^1H NMR (400 MHz, $CDCl_3$) δ 10.59 (s, 1H), 5.29 (s, 1H), 3.22 (s, 2H), 3.18 – 3.01 (m, 4H), 2.33 (t, *J* = 7.6 Hz, 2H), 1.64 (p, *J* = 7.6 Hz, 4H), 1.56 - 1.50 (m, 2H), 1.44 (s, 9H), 1.43 (s, 9H), 1.35 - 1.27 (m, 2H). HRMS (*m/z*): $[M+Na]^+$ calcd. For $C_{19}H_{36}N_2NaO_6$, 411.2466; found: 411.2467.

5. *Synthesis of amino lipid **2***

Aldehyde **9** was synthesized according to methods reported previously.[2] To a solution of compound **7** (250 mg, 0.6 mmol) in CH_2Cl_2 (7 mL) was added trifluoroacetic acid (2.3 mL). The mixture was allowed to warm to rt, stirred at RT for 1 h and monitored with thin layer chromatography (TLC). Upon completion of the reaction, the solvent was evaporated and the residue was dissolved in MeOH and concentrated, the residue solid **8** was used in the next step without further purification. To a solution of salt **8** (0.6 mmol) in THF (10 mL) was added TEA (0.22 mL, 1.56 mmol) and the mixture was kept stirring for 30 min at RT. Then aldehyde **9** (0.72 g, 2.53 mmol) was added and stirred for another 30 min. $Na(OAc)_3BH$ (0.76 g, 3.60 mmol) was added to above solution, and the resulting mixture was stirred for 12 h. The reaction was quenched with 30 mL of water. The aq. phase was extracted with EA (60 ml × 3 times), the organic phase was combined and dried over anhydrous Na_2SO_4. The residue was purified by silica gel chromatography (0%–40% [mixture of 3% NH_4OH, 22% MeOH in dichloromethane] in dichloromethane) to give 420 mg of amino lipid **2** as a yellow oil, yield 70.5%. ^1H NMR (400 MHz, $CDCl_3$) δ 4.81 (ddd, *J* = 12.4, 6.8, 5.6 Hz, 3H), 2.72 (d, *J* = 8.4 Hz, 6H), 2.45 (q, *J* = 9.6, 7.6 Hz, 6H), 2.28 (t, *J* = 7.6 Hz, 6H), 2.21 (t, *J* = 6.8 Hz, 2H), 1.72 (t, *J* = 7.6 Hz, 2H), 1.68 – 1.42 (m, 26H), 1.42 – 1.16 (m, 46H), 0.93 – 0.80 (m, 18H). HRMS (*m/z*): $[M+H]^+$ calcd. For $C_{60}H_{117}N_2O_8$, 993.8805; found: 993.8782.

6. *Synthesis of **10***

To a solution of amino lipid **2** (440 mg, 0.355 mmol) and TEA (0.174 mL, 0.97 mmol) in 2 mL of dry Toluene was added 2,4,6-Trichlorobenzoyl Chloride (141 mg, 0.49 mmol), and stirred at rt for 1 h. The solution was then allowed to warm to 40 °C and stirred for 2 h. Then a solution of **3** (180 mg, 0.323 mmol) and DMAP (78.9 mg, 0.646 mmol) in 2 mL of anhydrous Toluene was added to the above mixture, the obtained cloudy mixture was stirred for 12 h at 40 °C. Then the reaction mixture was diluted with 20 mL of water and extracted with DCM (15 mL * 3 times). The organic phase dried over anhydrous Na_2SO_4. The solution was filtered and the solvent was removed under reduced pressure. The reaction mixture was diluted with 20 mL of water and

extracted with DCM (15 mL* 3 times). The organic phase dried over anhydrous Na_2SO_4. The solution was filtered and the solvent was removed under reduced pressure. The residue was purified by silica gel chromatography (0%–30% [mixture of 3% NH_4OH, 22% MeOH in dichloromethane] in dichloromethane). 250 mg of **10** was obtained, 50.5% yield. HRMS (*m/z*): $[M+H]^+$ calcd. For $C_{96}H_{151}N_6O_9$, 1533.1571; found: 1533.1530.

7. *Synthesis of* ***RAL2***

To a solution of **10** (250 mg, 0.163 mmol) in 6 mL of DCM was added Trifluoroacetic acid (2 mL) dropwise at 0 ^oC. The solution was then allowed to warm to rt and stirred for 2 h. The reaction was quenched by addition of saturated aqueous $NaHCO_3$ solution (30 mL), and extracted with DCM (30 mL* 3 times). The organic phase dried over anhydrous Na_2SO_4. The solution was filtered and the solvent was removed under reduced pressure. The residue was purified by silica gel chromatography (0%–30% [mixture of 3% NH_4OH, 22% MeOH in dichloromethane] in dichloromethane). 173 mg of compound **RAL2** was obtained as a yellow oil, yield 82%. ^1H NMR (300 MHz, $CDCl_3$) δ 8.15 (dd, *J* = 8.4, 1.4 Hz, 1H), 7.81 (dd, *J* = 8.4, 1.2 Hz, 1H), 7.50 (ddd, *J* = 8.4, 6.9, 1.5 Hz, 1H), 7.34 – 7.27 (m, 1H), 5.57 (s, 2H), 4.93 (s, 2H), 4.81 (p, *J* = 6.3 Hz, 3H), 3.56 (q, *J* = 7.1 Hz, 2H), 2.60 – 2.32 (m, 12H), 2.28 (t, *J* = 7.5 Hz, 6H), 1.87 (t, *J* = 7.5 Hz, 2H), 1.59 – 1.39 (m, 21H), 1.36 – 1.19 (m, 60H), 1.13 – 1.04 (m, 2H), 0.90 – 0.83 (m, 18H). HRMS (*m/z*): $[M+H]^+$ calcd. For $C_{77}H_{137}N_6O_9$, 1290.0442; found: 1290.0438.

CD40 mRNA sequence

The coding sequence for mouse CD40 mRNA was confirmed on multiple databases, including Uniprot, Genbank, and Ensemble. The sequence used in this study has the highest annotation score and is most referenced on all three databases.

ATGGTGTCTTTGCCTCGGCTGTGCGCGCTATGGGGCTGCTTGTTGACAGCGGTCCAT
CTAGGGCAGTGTGTTACGTGCAGTGACAAACAGTACCTCCACGATGGCCAGTGCTGT
GATTTGTGCCAGCCAGGAAGCCGACTGACAAGCCACTGCACAGCTCTTGAGAAGAC
CCAATGCCACCCATGTGACTCAGGCGAATTCTCAGCCCAGTGGAACAGGGAGATTC
GCTGTCACCAGCACAGACACTGTGAACCCAATCAAGGGCTTCGGGTTAAGAAGGAG
GGCACCGCAGAATCAGACACTGTCTGTACCTGTAAGGAAGGACAACACTGCACCAG
CAAGGATTGCGAGGCATGTGCTCAGCACACGCCCTGTATCCCTGGCTTTGGAGTTAT
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GAACTTGGAGGTCCTACAGAAAGGAACGAGTCAGACTAATGTCATCTGTGGTTTAA
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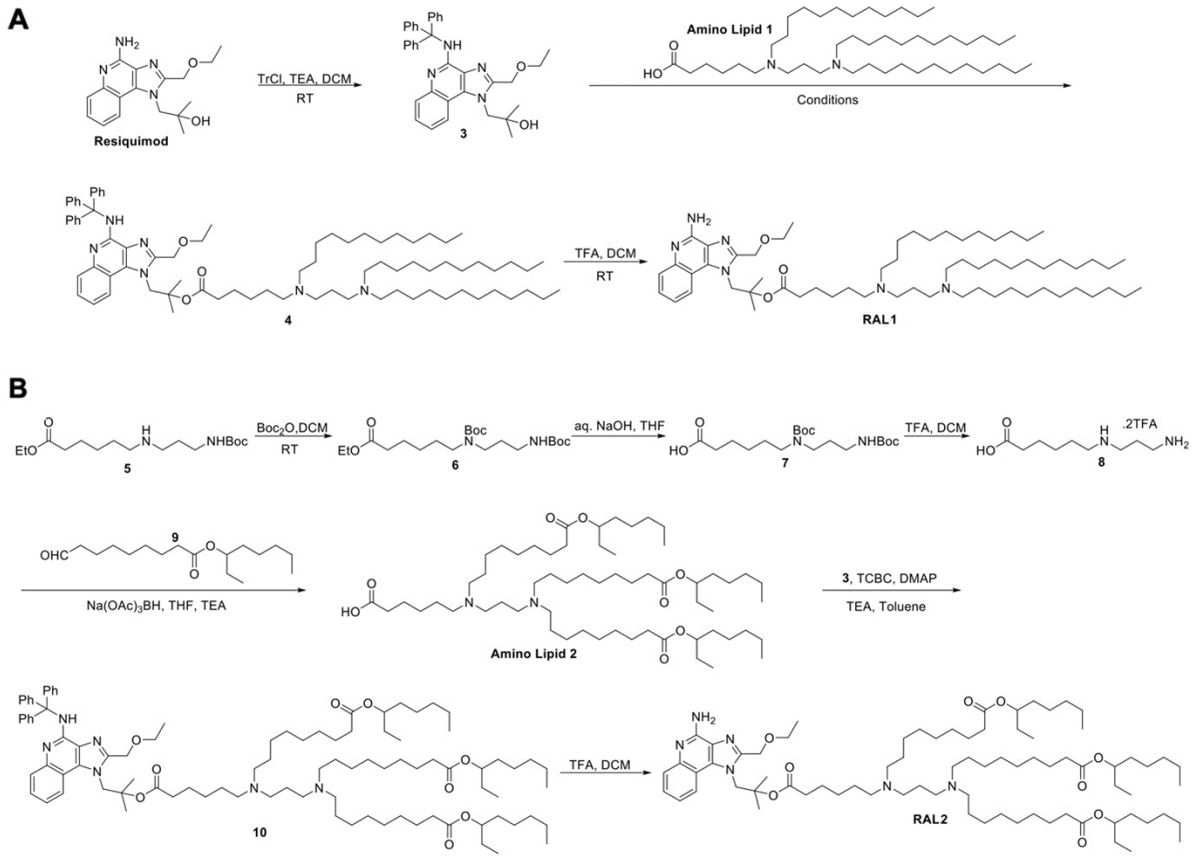
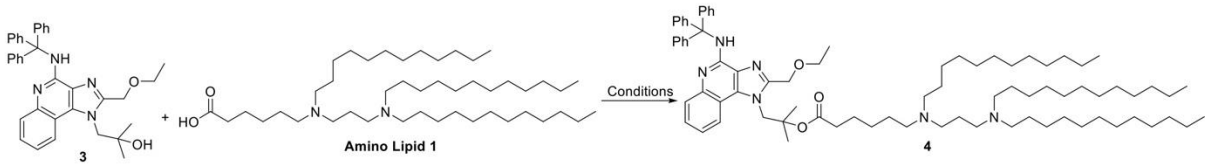


Figure S1. The synthetic route to Resiquimod Amino Lipids. (A) Resiquimod amino lipid 1, RAL1 and (B) Resiquimod amino lipid 2, RAL2.



Entry	Conditions	Results
1	DCC, DMAP, DCM, rt to 50 °C	No reaction
2	EDC, DMAP, DCM, rt to 50 °C	No reaction
3	CDI, DCM, rt to 40 °C	No reaction
4	TCBC, TEA, DMAP, **DCM**, rt to 40 °C	Trace
5	TCBC, TEA, DMAP,**THF**, rt to 40 °C	No reaction
6	TCBC, TEA, DMAP,**THF/Toluene**(1:2), rt to 40 °C	Trace
7	TCBC, TEA, DMAP, **Toluene**, rt to 40 °C	Yield 35% (28 mg scale)

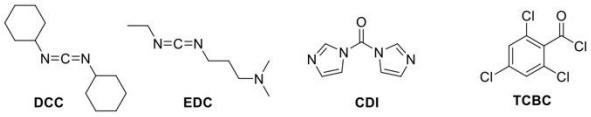


Figure S2. The optimization of the esterification reaction.

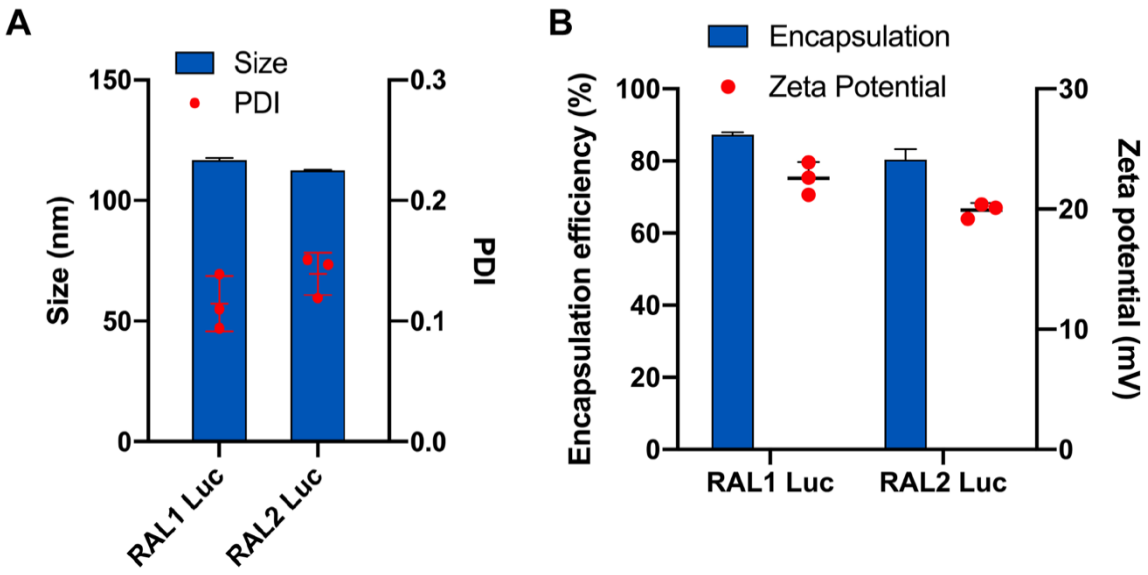


Figure S3. Characterization of RAL1 Luc-LNPs and RAL2 Luc-LNPs. (A) Size and PDI of RAL1-LNPs and RAL2-LNPs carrying Luc mRNAs prepared by pipetting. (B) Encapsulation efficiency and zeta potential of RAL1 Luc-LNPs and RAL2 Luc-LNPs. Data in A and B are presented as the mean ± S.D. (n=3).

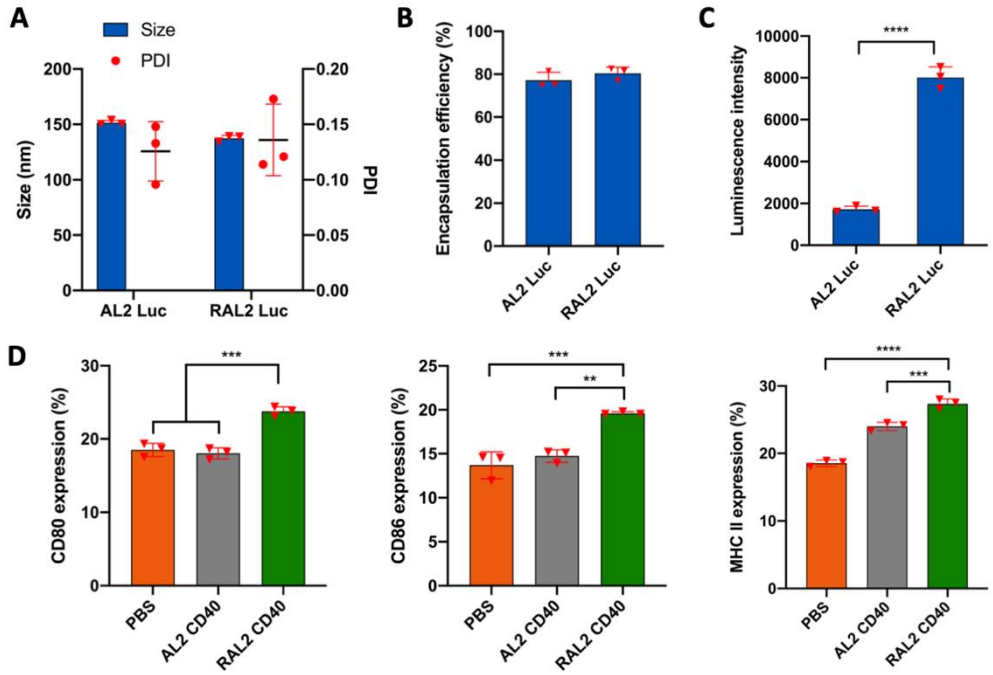


Figure S4. RAL2-LNPs mediated mRNA delivery *in vitro* and DC maturation in BMDCs *ex vivo*. (A) Size and PDI of RAL2-LNPs with/without R848 conjugation carrying Luc mRNAs. (B) Encapsulation efficiency of Luc mRNAs using RAL2-LNPs with/without resiquimod conjugation. (C) Delivery of Luc mRNA in JAWS II cells. (D) Expression of DC activation markers, CD80, CD86 and MHC II on BMDCs treated with PBS, Amino lipid 2 CD40-LNPs, RAL2 CD40-LNPs. Data in A-D are presented as the mean ± S.D. (n=3). Statistical significance in C and D is analyzed by unpaired, two-tailed Student's *t* tests. **P < 0.01; ***P < 0.001; ****P < 0.0001.

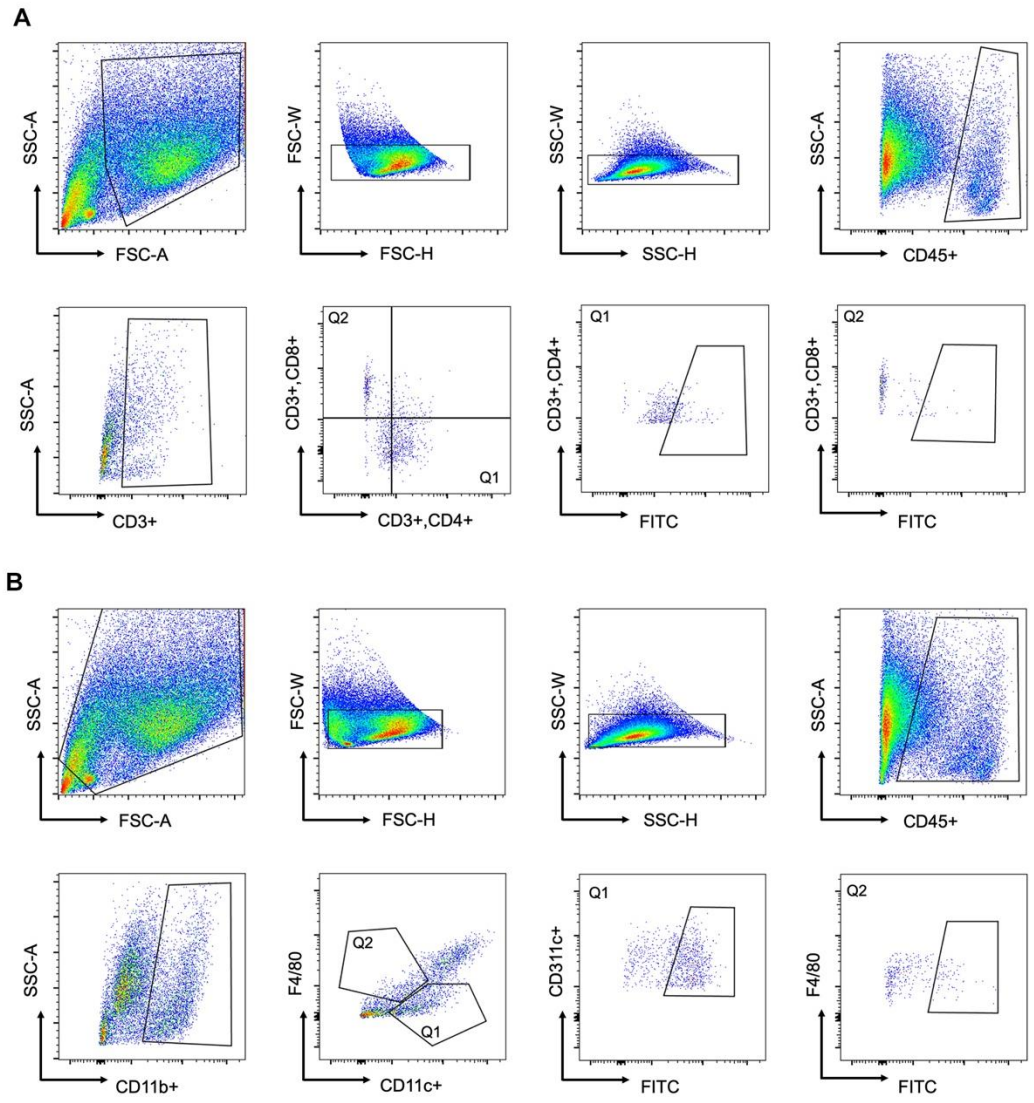


Figure S5. Flow cytometry gating. Cells were first gated on FSC/SSC to define single cells. Then, gate CD45 positive cells, CD3 positive cells, CD4/CD8 positive cells and OX40/GFP positive cells. Also, gate CD45 positive cells, CD11b positive cells, CD11c/F4/80 positive cells and OX40/GFP positive cells.

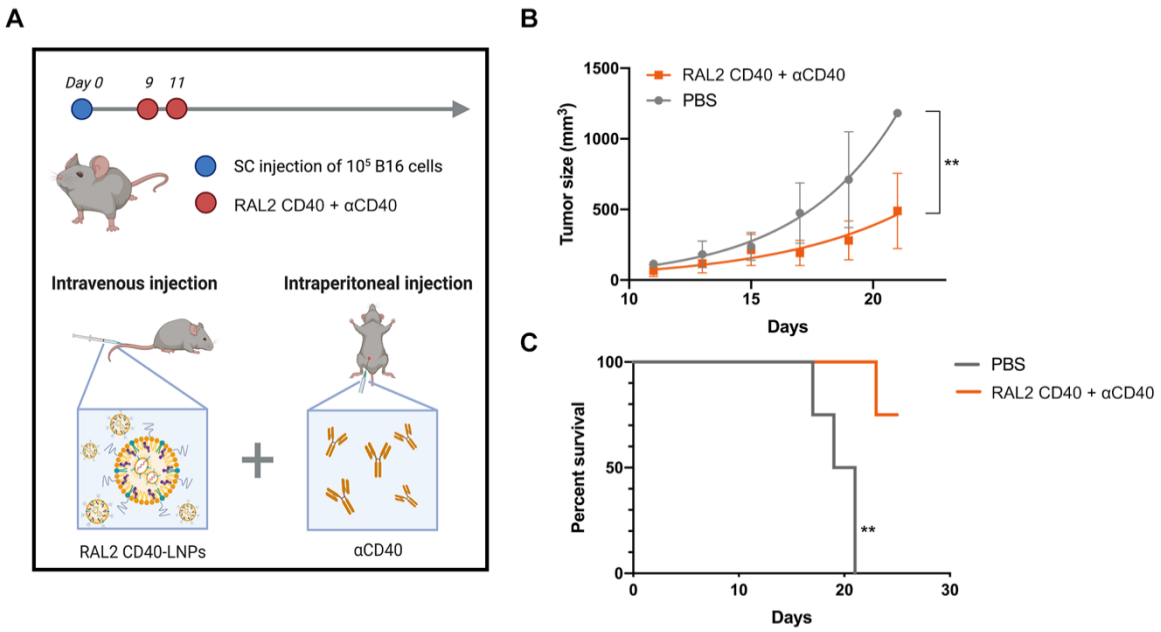


Figure S6. Systematic anti-tumor effect of RAL2 CD40-LNPs in subcutaneous B16 tumor-bearing mice. (A) Experiment setup. RAL2-LNPs were administered through intravenous (i.v.) injection and anti-CD40 Abs were administered through intraperitoneal (i.p.) injection. (B) Growth of B16 tumor after tumor implantation on day 0 (n=4-5). (C) Mice survival after the initial tumor implantation on day 0 (n=4-5). Data in B is presented as the mean ± S.E.M. Data in B is analyzed by unpaired two-tailed student's *t* test. Survival analysis in C is performed with the log-rank test. **P < 0.005.

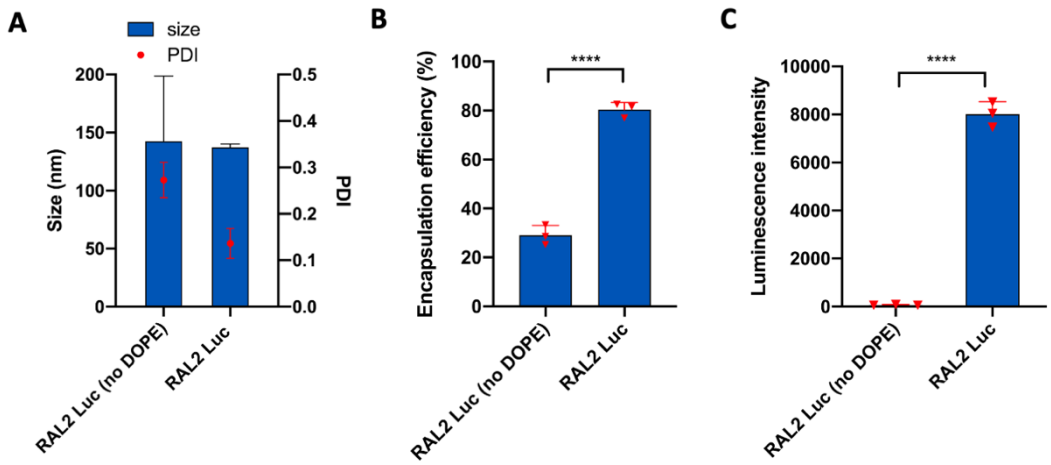


Figure S7. Characterization of RAL2-LNPs with or without DOPE. (A) Size and PDI of RAL2-LNPs with or without DOPE carrying Luc mRNAs. (B) Encapsulation efficiency of Luc mRNAs using RAL2-LNPs with or without DOPE. (C) Delivery of Luc mRNA in JAWS II cells. Data in A, B, and C are presented as the mean ± S.D. (n=3). Statistical significance in B and C is analyzed by unpaired, two-tailed Student's *t* tests. ****P < 0.0001.

Supplemental Reference

[1] C. Zhang, X. Zhang, W. Zhao, C. Zeng, W. Li, B. Li, X. Luo, J. Li, J. Jiang, B. Deng, D. W. McComb, Y. Dong, *Nano Res.* **2019**, *12*, 855.

[2] X. Zhang, B. Li, X. Luo, W. Zhao, J. Jiang, C. Zhang, M. Gao, X. Chen, Y. Dong, *ACS Appl. Mater. Interfaces* **2017**, *9*, 25481.