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Supplementary Materials for

A bi-adjuvant nanovaccine that potentiates immunogenicity of neoantigen for combination immunotherapy of colorectal cancer

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This PDF file includes:

Supplementary Materials and Methods

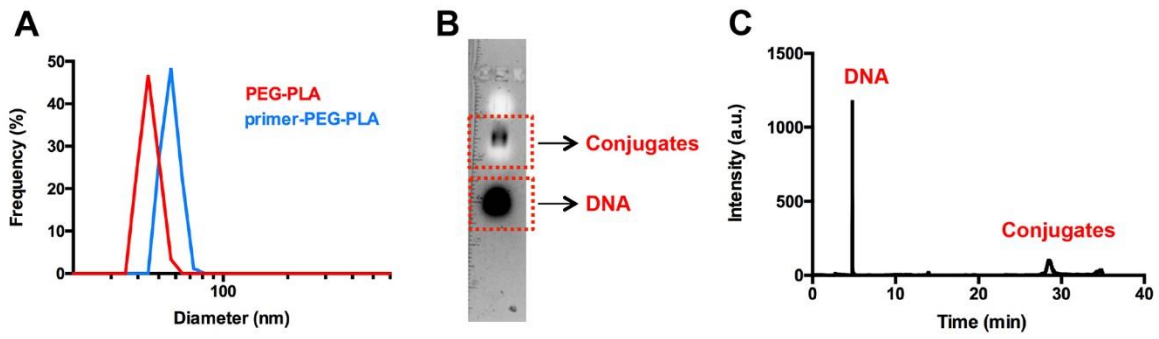

Fig. S1. Characteristics of primer-PEG-PLA conjugates. (**A**) A DLS graph of PEG-PLA micelles and primer-PEG-PLA nanoparticles. (**B**) A gel electrophoresis image and (**C**) reverse phase HPLC chromatograms of DNA-PLA-PEG conjugates.

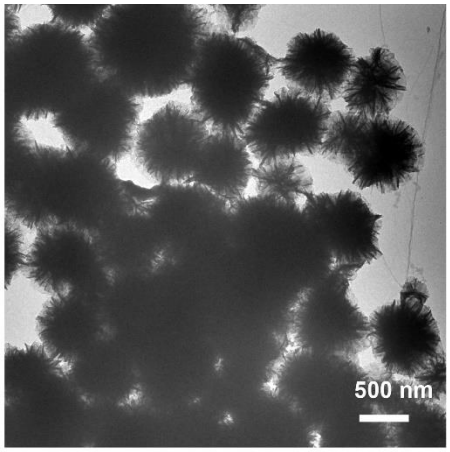

Fig. S2. TEM images of CpG MPs.

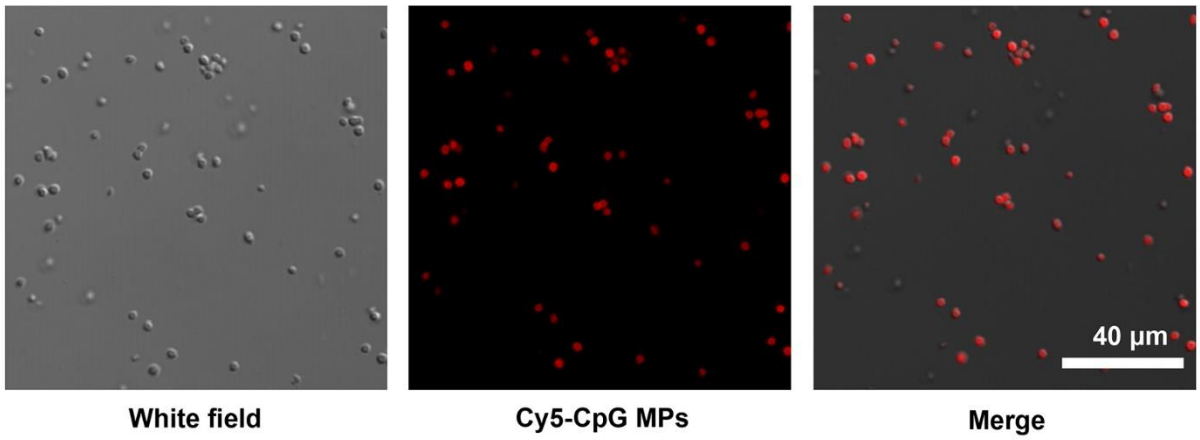

Fig. S3. Fluorescence confocal microscopy images of CpG MPs labeled with Cy5 *via* Cy5-dUTP.

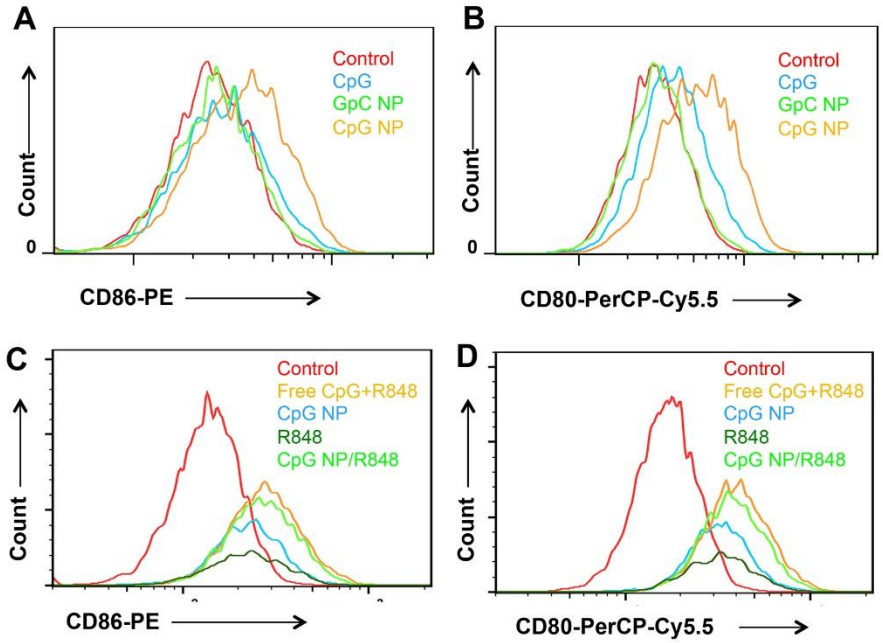

Fig. S4. Co-stimulatory factors expression on DCs. (**A**, **B**) Flow cytometry results of CD86 and CD80 expression on DC2.4 cells treated with CpG ODN, CpG NPs (100 nM CpG equivalents), and (**C**, **D**) free R848 (500 nM) mixed with CpG ODN (100 nM), CpG NP or R848 alone, and CpG NP encapsulated with R848.

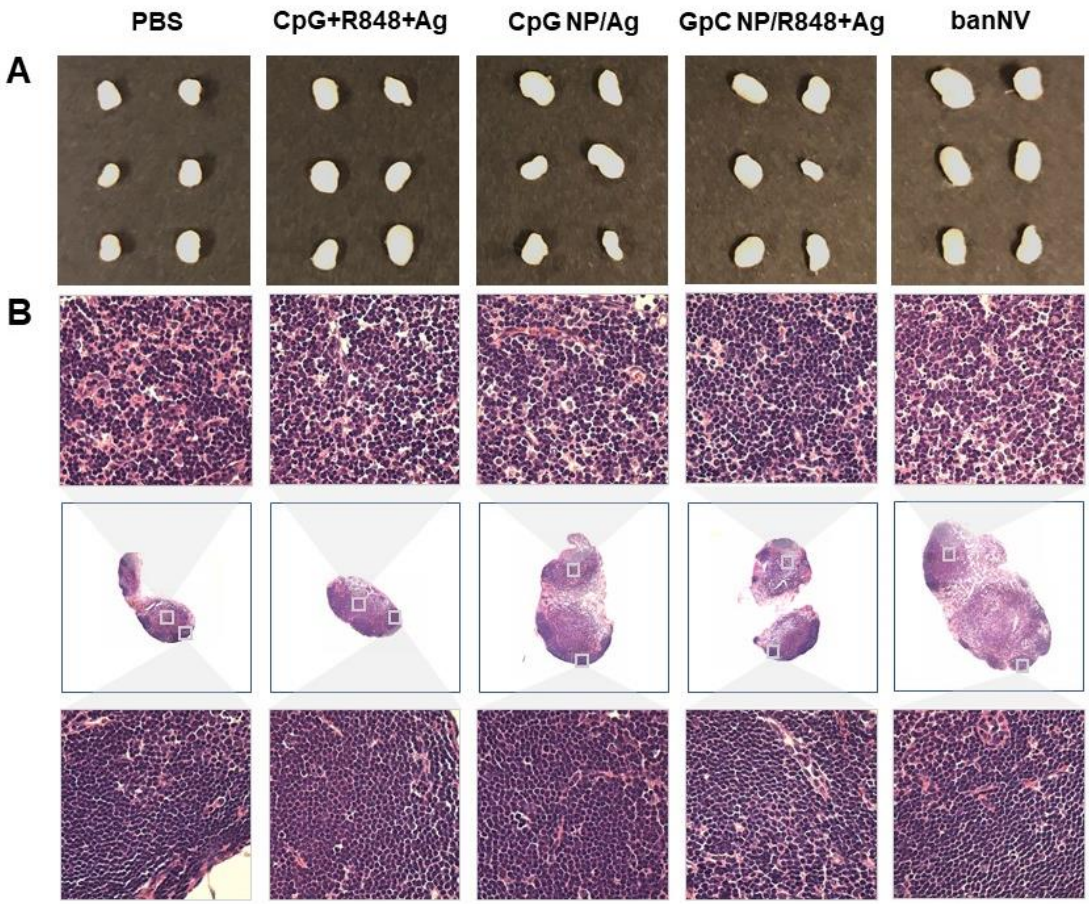

Fig. S5. Photographs and H&E staining slice images of inguinal lymph nodes. (**A**) Photographs and (**B**) H&E staining of inguinal lymph nodes and sections (40×) collected on day 6 from C57BL/6 mice treated with PBS, soluble forms of CpG+R848+Ag (Adpgk), CpG NP encapsulated with Ag (Adpgk), GpC NP encapsulated with R848 and Ag(Adpgk), and banNVs (CpG: 2 nmol, R848: 8μg per mouse, Adpgk: 20 μg) on day 0, 2, and day 4 (n = 3 mice per group). The definition of abbreviation is listed on Table S2.

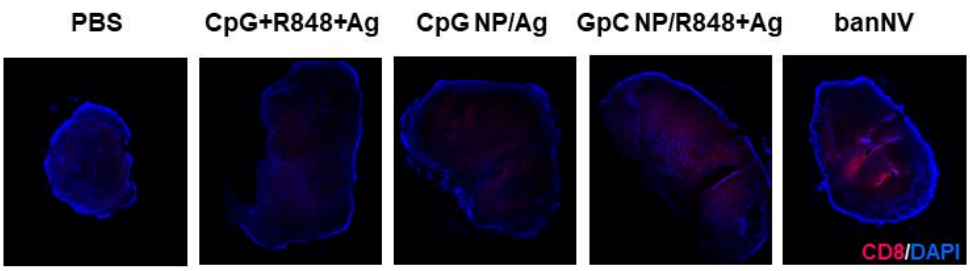

Fig. S6. Immunofluorescence images of LNs (10×) collected on day 6 from C57BL/6 mice treated with PBS, soluble forms of CpG + R848 + Ag (Adpgk), CpG NP encapsulated with Ag (Adpgk), GpC NP encapsulated with R848 and Ag (Adpgk), and banNVs (CpG: 2 nmol, R848: 8 μg per mouse, Adpgk: 20 μg) on days 0, 2, and 4 (*n* = 3 mice per group). The definition of abbreviation is listed on Table S2.

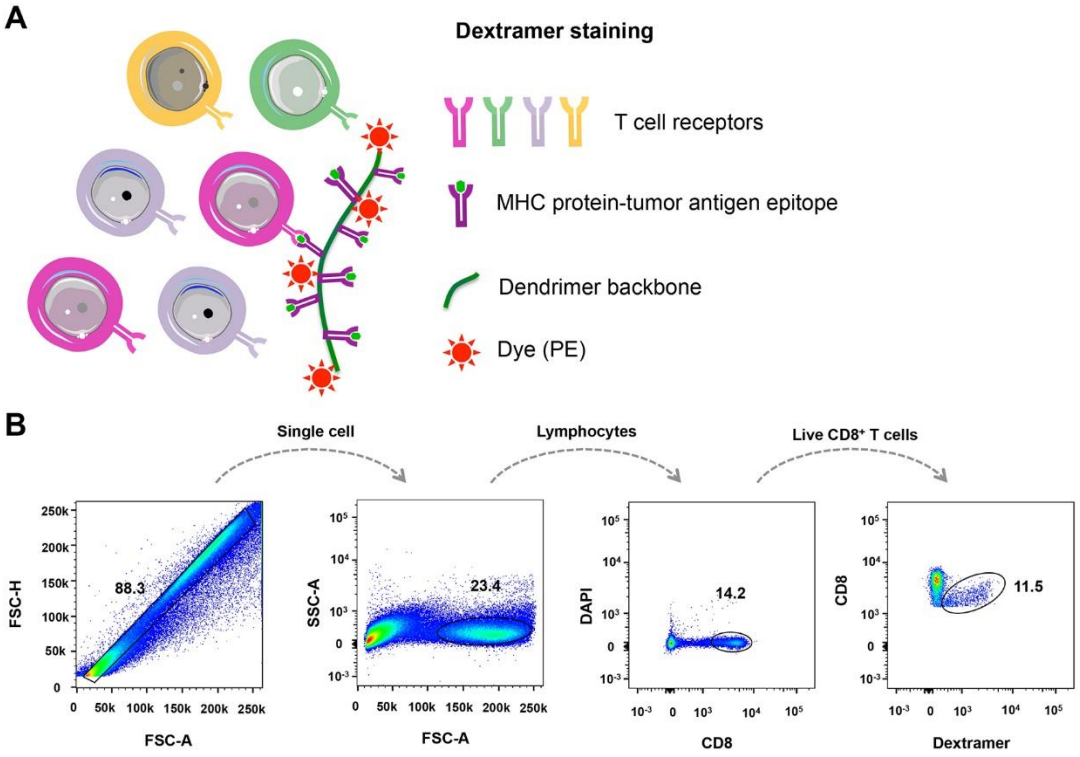

Fig. S7. Scheme of dextramer staining and gating tree flow cytometric analysis. (**A**) Schematic description of dextramer staining of MC38 tumor related neoantigen-specific T cells. (**B**) Gating tree used for flow cytometric analysis of neoantigen specific $CD8^+$ T cells using a dextramer staining assay.

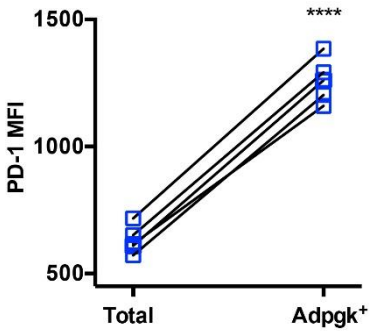

Fig. S8. PD-1 median intensity of fluorescence gated from PD-1^+ $CD8^+$ peripheral T cells.

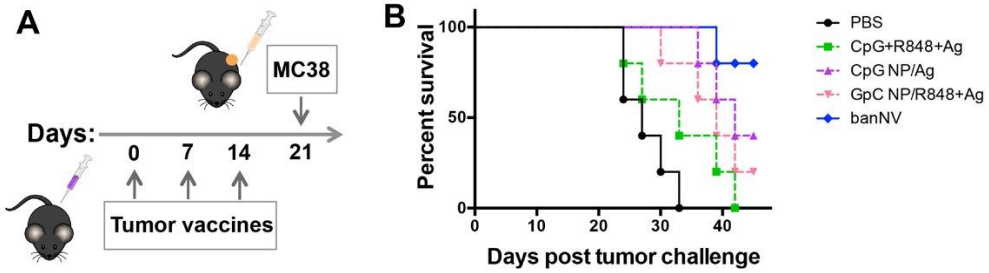

Fig. S9. Tumor challenge of C57BL6 mice after immunization of banNVs. (**A**) Experimental design for tumor challenge in immunized C57BL/6 mice with MC38 cells on day 21 after the first immunization (CpG: 2 nmol, R848: 8μg, Adpgk: 20 μg). (**B**) Mouse survival after s.c. challenging C57BL/6 mice with MC38 cells (0.3×10^6) (n = 5 mice per group). The definition of abbreviation is listed on Table S2.

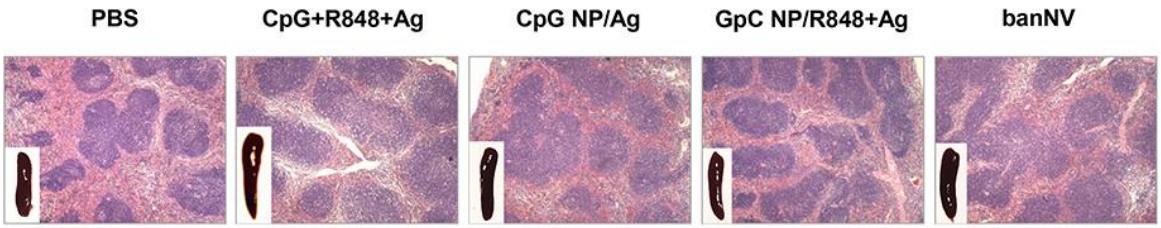

Fig. S10. Hematoxylin and eosin (H&E) staining of the spleen sections (10×) on day 6 from C57BL/6 mice (with photographs of spleens inserted) treated with PBS, soluble forms of CpG + R848 + Ag (Adpgk), CpG NP encapsulated with Ag (Adpgk), GpC NP encapsulated with R848 and Ag (Adpgk), and banNVs (CpG: 2 nmol, R848: 8 μg per mouse, Adpgk: 20 μg) on days 0, 2, and 4. The definition of abbreviation is listed on Table S2.

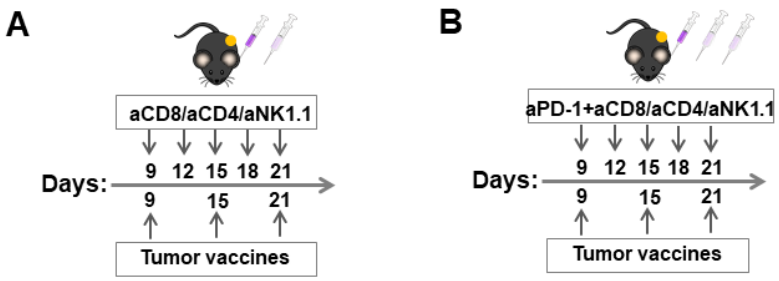

Fig. S11. Experimental design of immune depletion study. Experimental design for immune depletion in C57BL/6 mice treated with indicated formulations of vaccines (CpG: 2 nmol, R848: 8 μg, Adpgk: 20 μg) and immune depletion antibodies (aCD8: 100 ug, aCD4: 100 μg, aNK1.1: 100 μg) (**A**) with or (**B**) without aPD-1 (200 μg) treatment (*n* = 5 mice per group).

Table S1. DNA sequences.

	Sequences (5'-3')
CpG	TCCATGACGTTCCTGACGTT
Primer (CpG)	5'-Thiol-ACGTTCCTGACGTTTTTCAGCGTGACTTTTCCATGACGTTCC-3'
Primer (GpC)	5'-Thiol-AGCTTCCTGAGCTTTTTCAGCGTGACTTTTCCATGAGCTTCC-3'
CpG template	5'-phosphate-CGCTGAAAAACGTCAGGAACGTCATGGAAAAAAAACGTCAGGAACGTCATGGAAAAAAAACGTCAGGAACGTCATGGAAAAGTCA-3'
GpC template	5'-phosphate-CGCTGAAAAAGCTCAGGAAGCTCATGGAAAAAAAAGCTCAGGAAGCTCATGGAAAAAAAAGCTCAGGAAGCTCATGGAAAAGTCA-3'

Table S2. Definition of abbreviations used in the manuscript.

Abbreviation	Detailed definition
CpG MP (Fig. 2D, I, J; Fig. S2-S4)	CpG templates assembled microparticle
CpG NP (Fig. 2I, J; Fig. 3A, B, C; Fig. S4)	CpG nanoparticle: CpG MPs condensed by polypeptide
CpG ODN (Fig. 2J)	CpG templates
GpC NP (Fig. 3A, B, C)	GpC nanoparticle: GpC MPs condensed by polypeptide
CpG+R848 (Fig. 3A, B, C)	Soluble mixture of CpG and R848
CpG NP/R848 (Fig. 3A, B, C)	R848 encapsulated into CpG NP
CpG+CSIINFEK$_{(FITC)}$L (Fig. 3D)	Soluble mixture of CpG and CSIINFEKL peptide modified with FITC
CpG NP/CSIINFEK(FITC)L (Fig. 3D)	CSIINFEK$_{(FITC)}$L encapsulated in CpG NP
CpG+R848+SIINFEKL (Fig. 3E)	Soluble mixture of CpG, R848 and SIINFEKL peptide
banNV (Fig. 3E)	R848 and SIINFEKL peptide encapsulated in CpG NP
CpG+ CSIINFEK$_{(FITC)}$L (Fig. 4A)	Soluble mixture of CpG and CSIINFEK(FITC)L
CpG NP/CSIINFEK$_{(FITC)}$L (Fig. 4A)	CSIINFEK(FITC)L encapsulated in CpG NP
CpG+R848+Ag (Fig. 5A, B, E, F; Fig. 6A, C; Fig. S5-7, S9)	Soluble mixture of CpG, R848 and Adpgk (neoantigen)
CpG NP/Ag (Fig. 5A, B, E, F; Fig. 6A, C; Fig. S5-7, S9)	Adpgk encapsulated in CpG NP
GpC NP/(R848+Ag) (Fig. 5A, B, E, F; Fig. 6A, C; Fig. S5-7, S9)	R848 and Adpgk encapsulated in GpC NP
banNV (Fig. 5A, B, E, F; Fig. 6A to E; Fig. S5-7, S9)	R848 and Adpgk encapsulated in CpG NP
CpG+R848+aPD-1 (Fig. 6B, C)	Soluble mixture and R848 followed by aPD-1 treatment

Table S3. Tumor progression rate and regression rate.

Tumor Challenge	Progression Rate[§] (median [IQR[ϕ]])	Regression Rate[φ] (median [IQR])
PBS	21.31 [17.46, 23.34]	NA
CpG+R848+Ag	8.17 [7.06, 26.94]	NA
CpG NP/Ag	14.14 [-1, 11.08]	NA
GpC NP/R848+Ag	4.18 [3.75, 6.87]	NA
banNV	3.65 [-1, 6.64]	NA
Tumor Treatment		
PBS	60.61 [35.14, 73.09]	NA
CpG+R848+Ag	25.41 [7.40, 32.33]	NA
CpG NP/Ag	10.41 [6.80, 14.13]	NA
GpC NP/R848+Ag	9.80 [5.46, 15.23]	NA
banNV	9.95 [-1, 17.88]	NA
aPD-1	20.30 [13.21, 37.70]	NA
CpG+R848+aPD-1	8.32 [6.81, 12.69]	NA
banNV+aPD-1	NA	1 [-4.93, 1]

[§] Progression Rate: (Final tumor size – Initial tumor size)/Initial tumor size
[φ] Regression Rate: (Initial tumor size – Final tumor size)/Initial tumor size
[ϕ] IRQ: interquartile range