Science Advances

MAAAS

Supplementary Materials for

D-lactate modulates M2 tumor-associated macrophages and remodels immunosuppressive tumor microenvironment for hepatocellular carcinoma

Shulan Han *et al.*

Corresponding author: Jianfeng Guo, jguo@jlu.edu.cn; Zhuo Yu, zhuoyu@shutcm.edu.cn

This PDF file includes:

Figs. S1 to S19
Tables S1 to S3
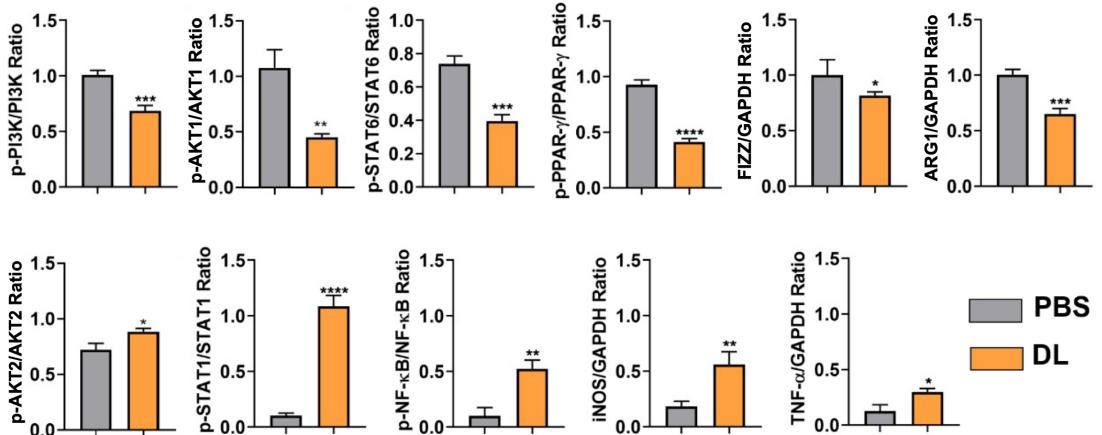
Figure S1. Qualification of protein bands in Figure 3D. Data were expressed as the mean ± SEM. ($n = 3$; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and $****P < 0.0001$).
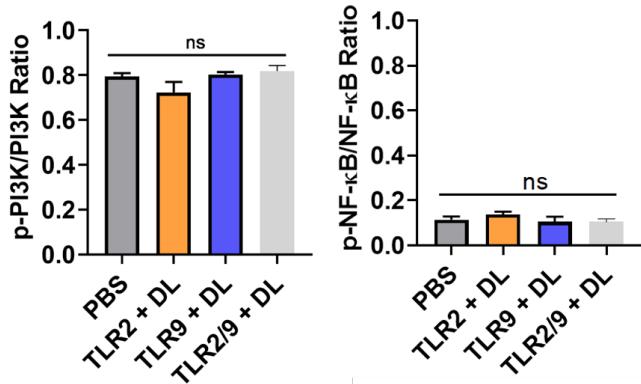

Figure S2. Qualification of protein bands in Figure 3F. Data were expressed as the mean ± SEM ($n = 3$; ns = no significance).
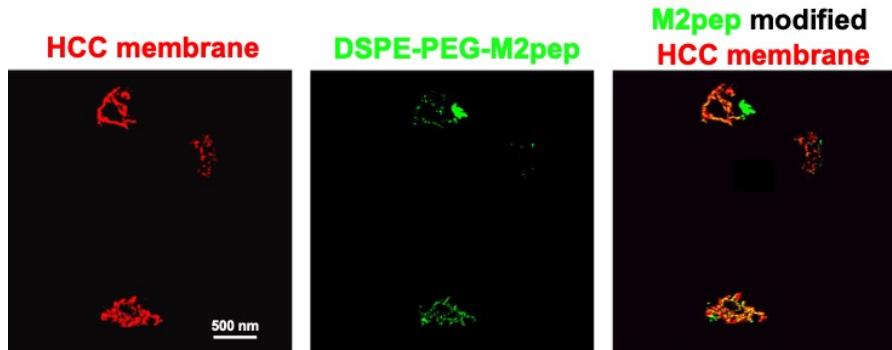

Figure S3. The DSPE-PEG-M2pep modified HCC membrane was characterized using CLSM. The DSPE-PEG-M2pep was labeled with FITC (ThermoFisher), and the HCC membrane was labeled with DiD (ThermoFisher). Subsequently, 100 µg of DSPE-PEG-M2pep were added with 1 mg of HCC membrane for 30 min at 4 °C, and the content was passed once through the extruder equipped by a 0.2 µm polycarbonate porous membrane, forming the DSPE-PEG-M2pep modified HCC membrane.
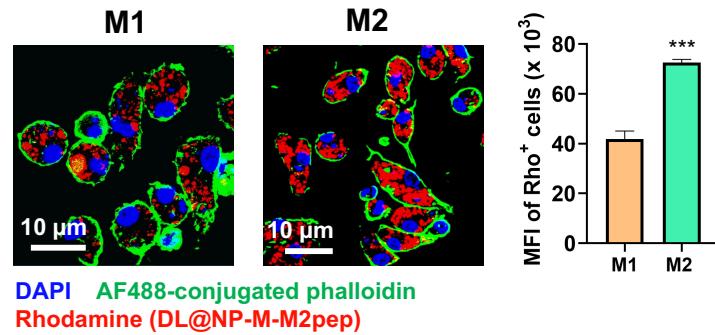
Figure S4. Cellular uptake of DL@NP-M-M2pep in M1 and M2 macrophages. The M1 macrophages (IFN-γ and LPS co-stimulated BMDMs) or M2 macrophages (IL-4 stimulated BMDMs) (2×10^5 per well) were seeded in 6-well plates overnight and incubated with rhodamine (0.05 wt%)-loaded DL@NP-M-M2pep ([c] of DL = 50 mmol/L) for 6 h. Subsequently, cells were treated DAPI (cell nucleus) and Alexa Fluor® 488-conjugated phalloidin (cyto-membrane) for 20 min before the CLSM analysis ($n = 3$; ***$P < 0.001$).
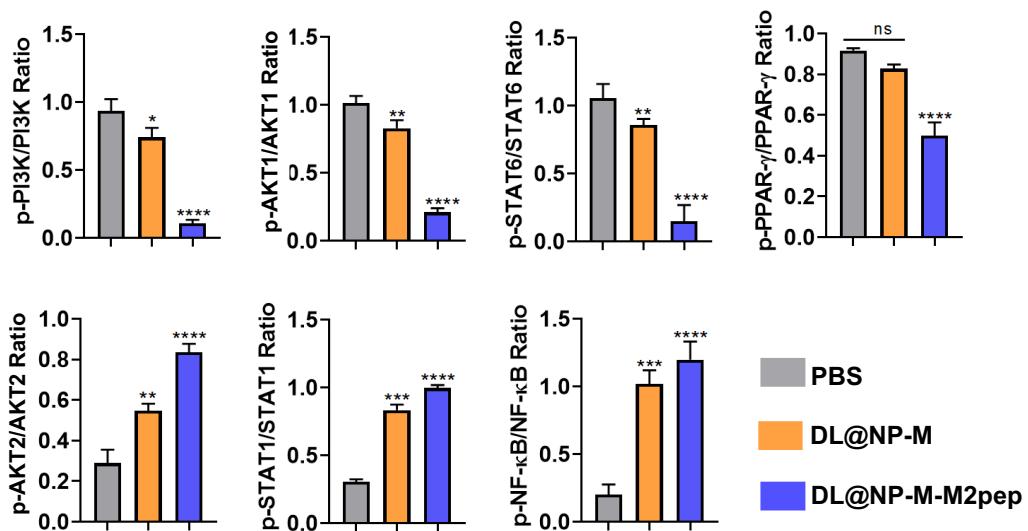

Figure S5. Qualification of protein bands in Figure 5B. Data were expressed as the mean ± SEM ($n = 3$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ and ns = no significance to PBS).
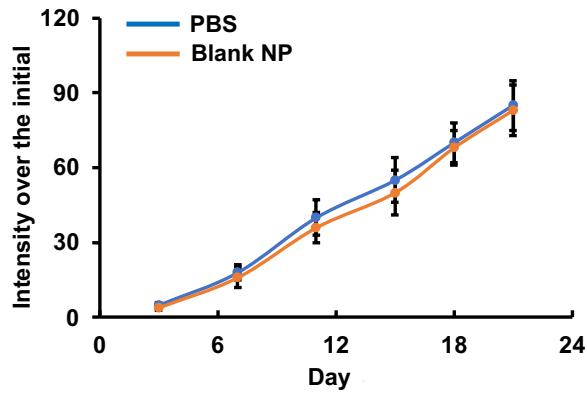
Figure S6. Blank NP (DL@NP-M-M2pep without DL) achieved no antitumor effect in Hepa1-6-luc-derived orthotopic HCC mice. Tumor inoculation and treatment scheme (PBS and blank NP) were same as those in Figure 7A (blue line = PBS and brown line = blank NP). The HCC development over a 21-day period ($n = 3$).
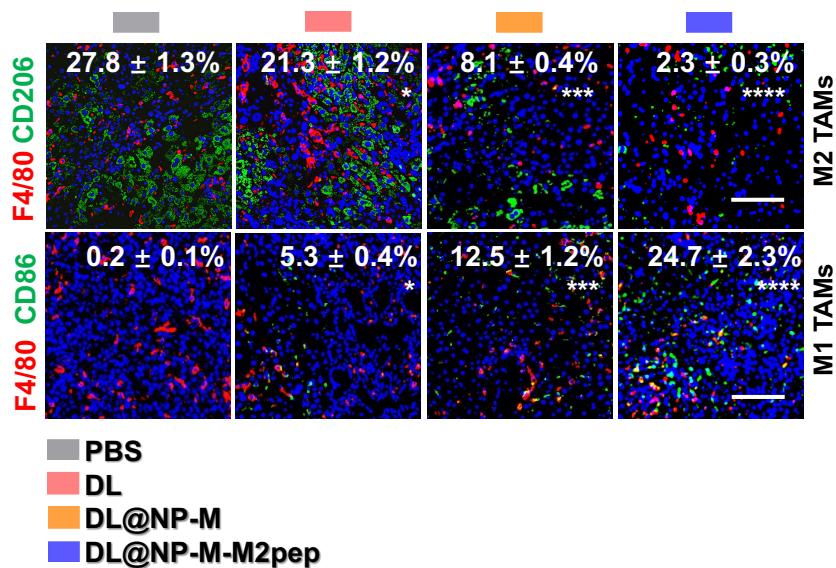
**Figure S7.** The M2 and M1 TAMs were detected using immunofluorescent staining assay (bar = 100 μm; $n = 4$; *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$ to PBS).
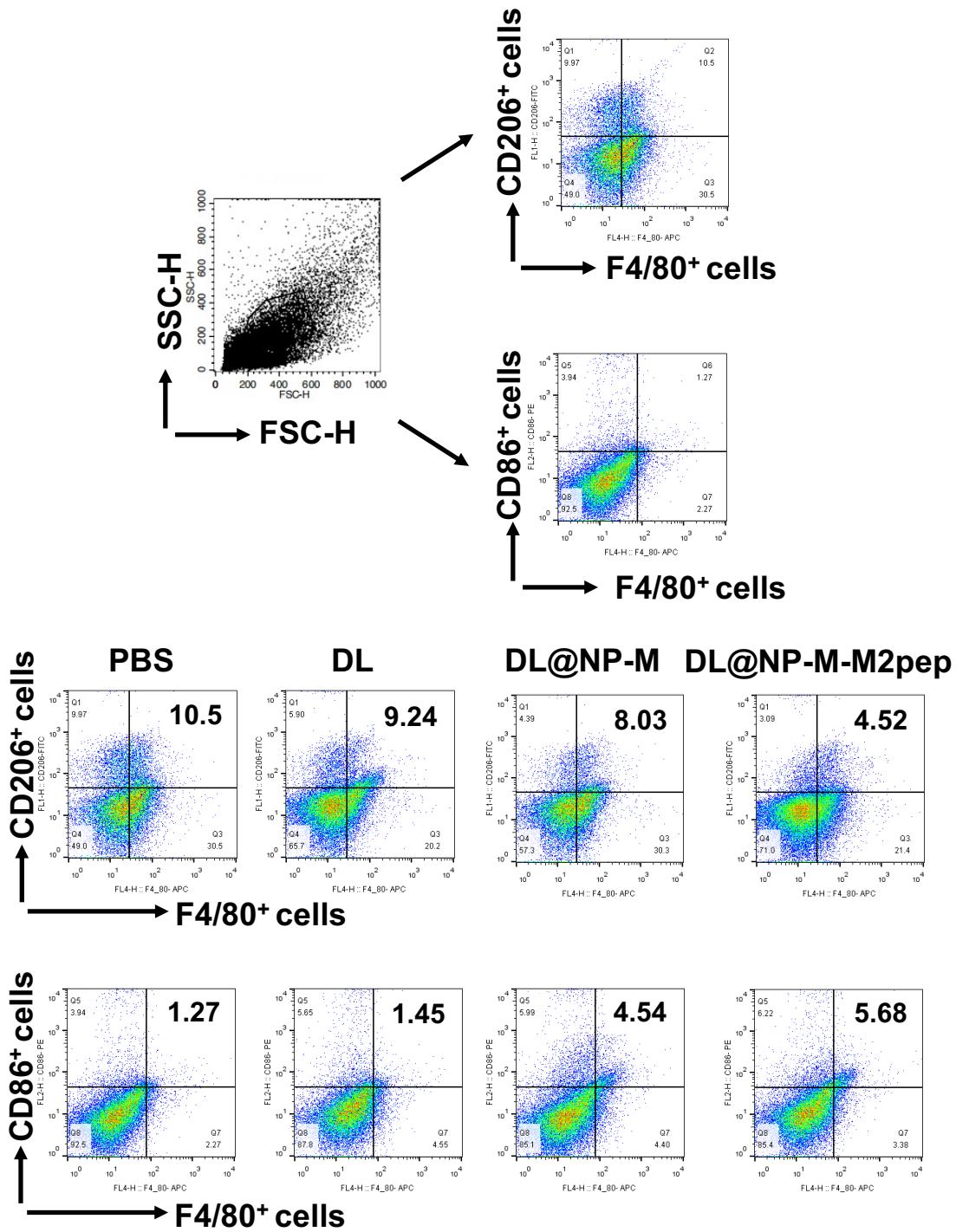
Figure S8. The M2 TAMs (F4/80$^+$ CD206$^+$) and M1 TAMs (F4/80$^+$ CD86$^+$) in tumor tissue (Figure 7F).
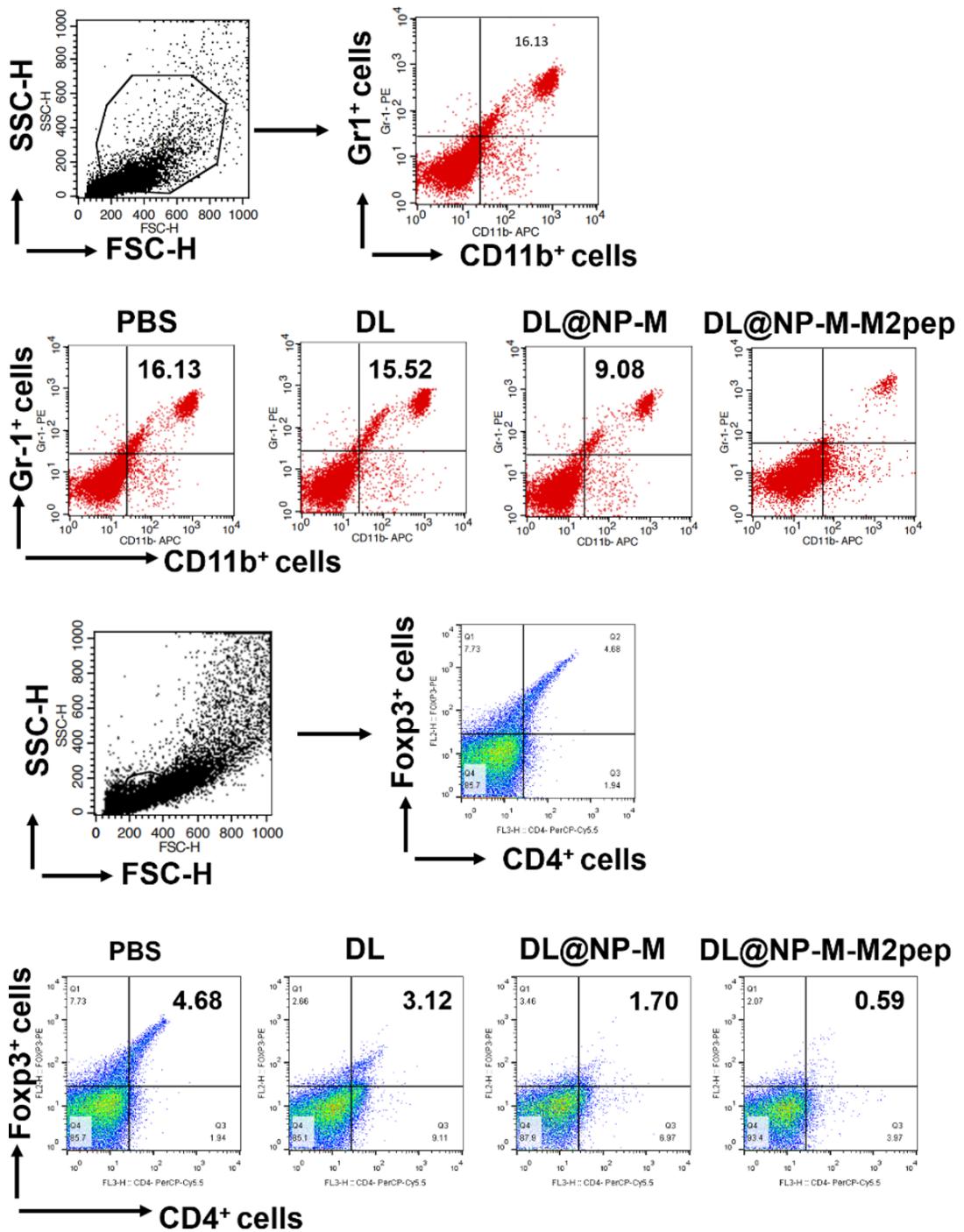
Figure S9. The MDSCs (CD11b$^+$ Gr-1$^+$) and Tregs (CD4$^+$ Foxp3$^+$) in tumor tissue (Figure 8A).
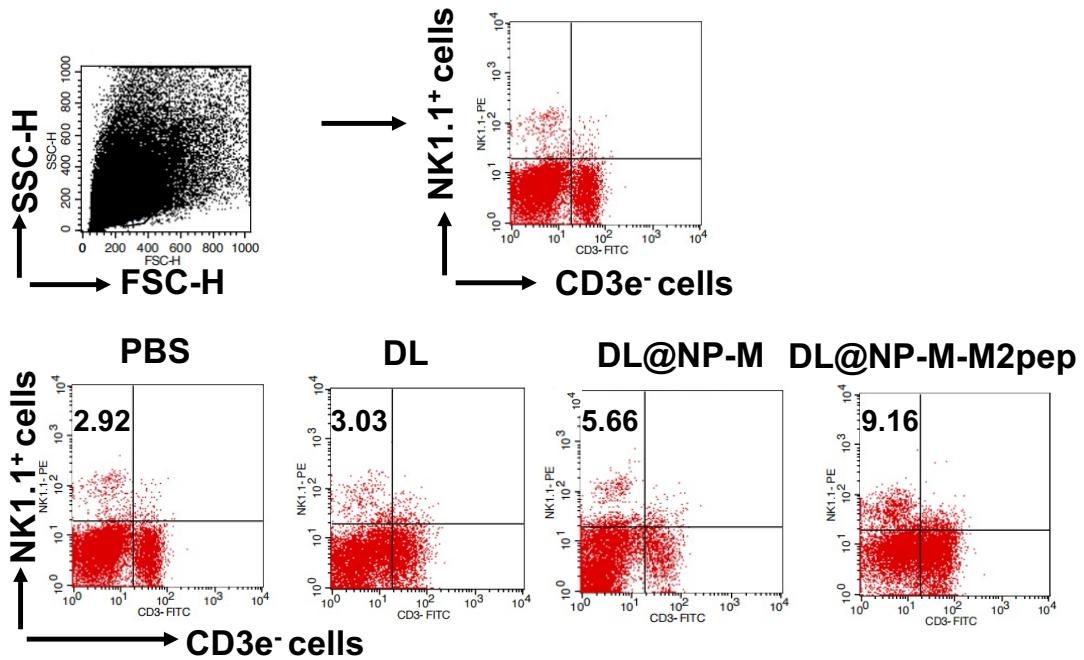
Figure S10. The NK cells (NK1.1$^+$ CD3e$^-$) in tumor tissue (Figure 8A).
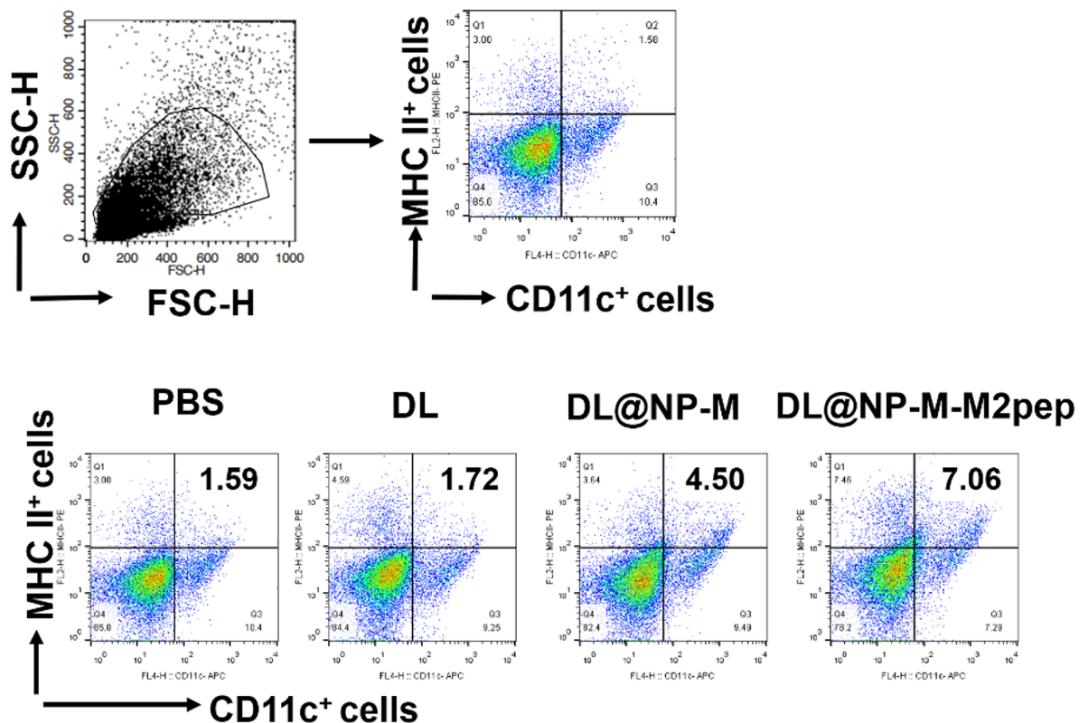
Figure S11. The activated DCs (CD11c$^+$ MHC II$^+$) in tumor tissue (Figure 8A).
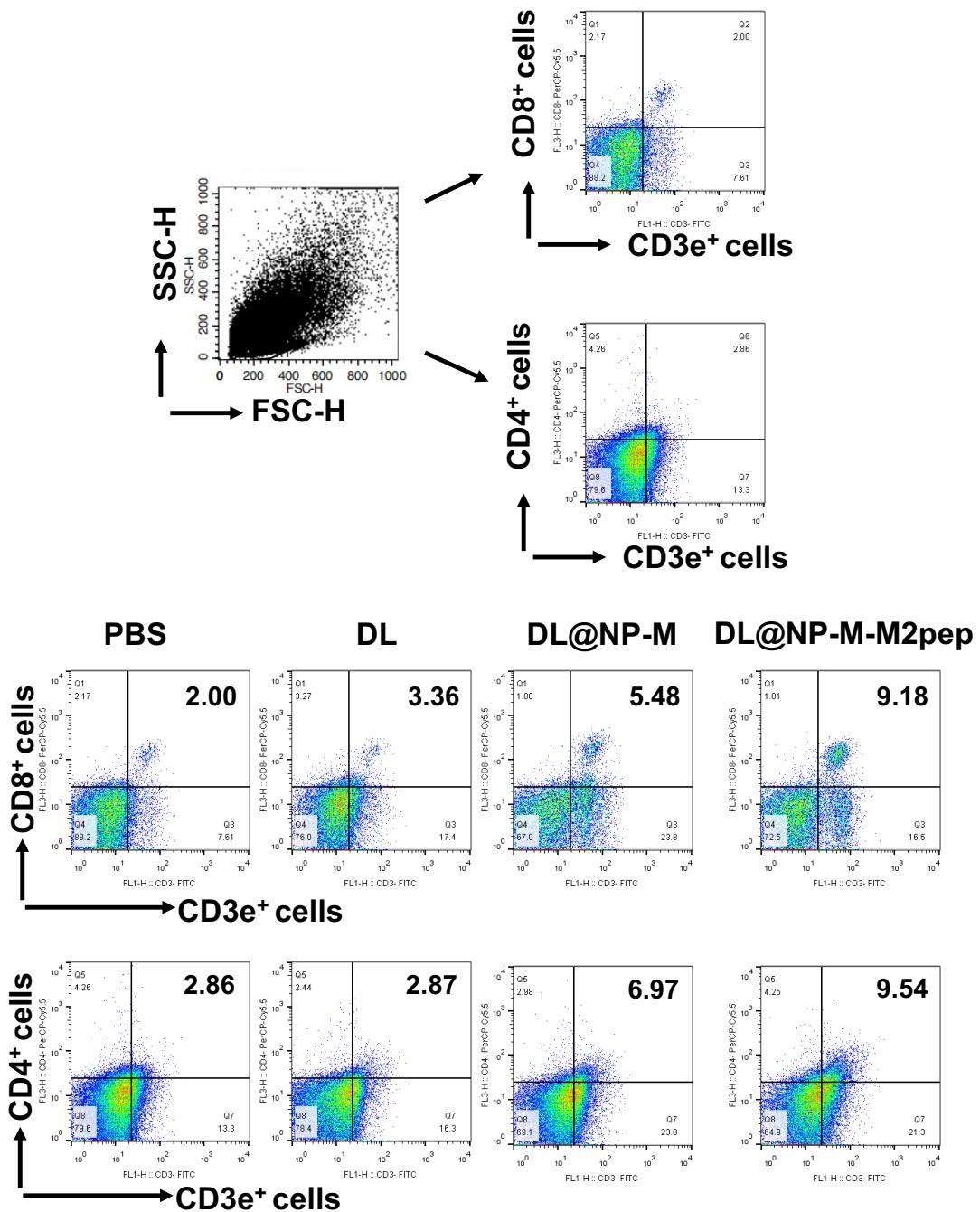
Figure S12. The CD8$^+$ cytotoxic T cells (CD3e$^+$ CD8$^+$) and CD4$^+$ helper T cells (CD3e$^+$ CD4$^+$) in tumor tissue (Figure 8A).
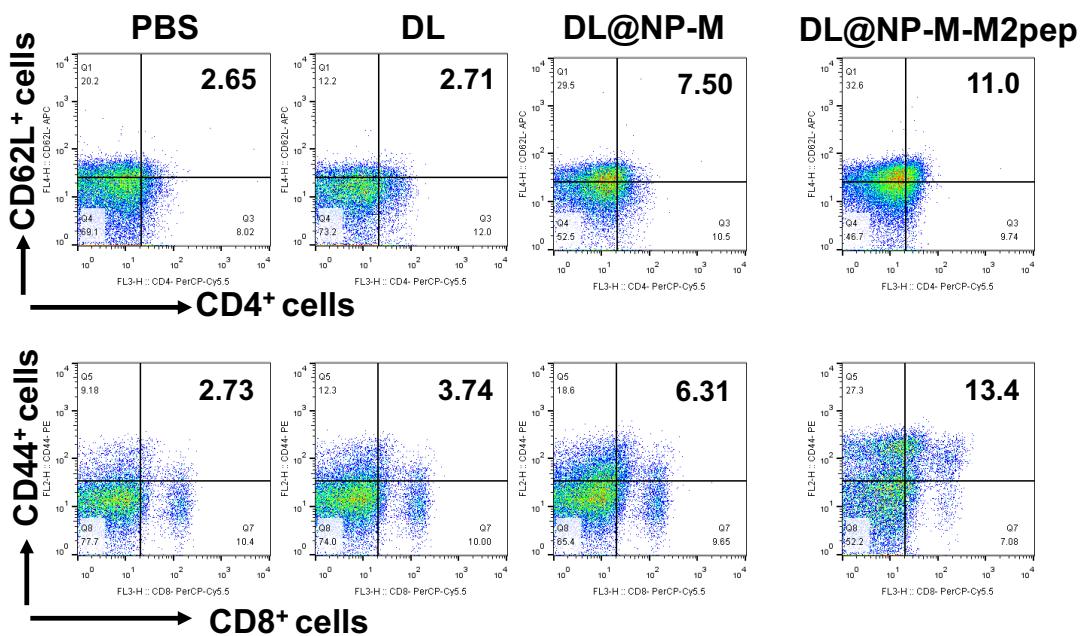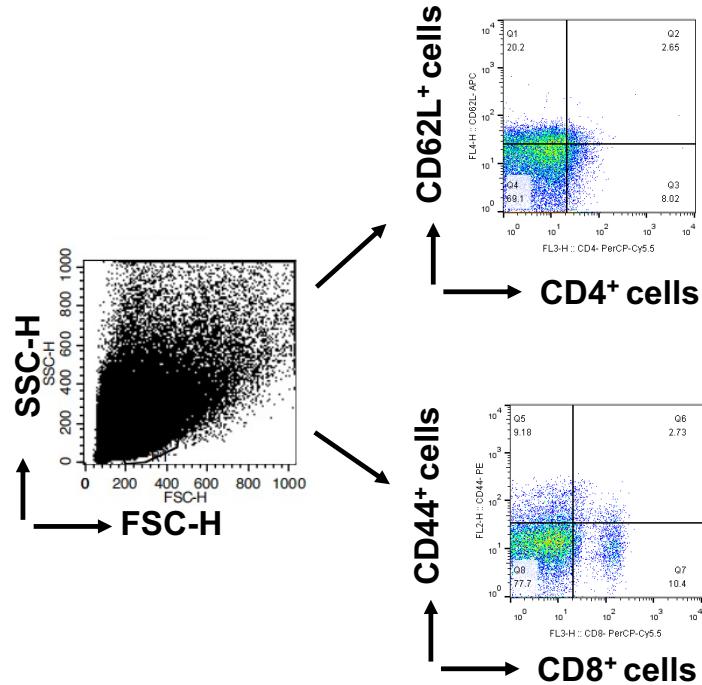
Figure S13. The memory CD4$^+$ T cells (CD62L$^+$ CD4$^+$) and memory CD8$^+$ T cells (CD44$^+$ CD8$^+$) in tumor tissue (Figure 8A).
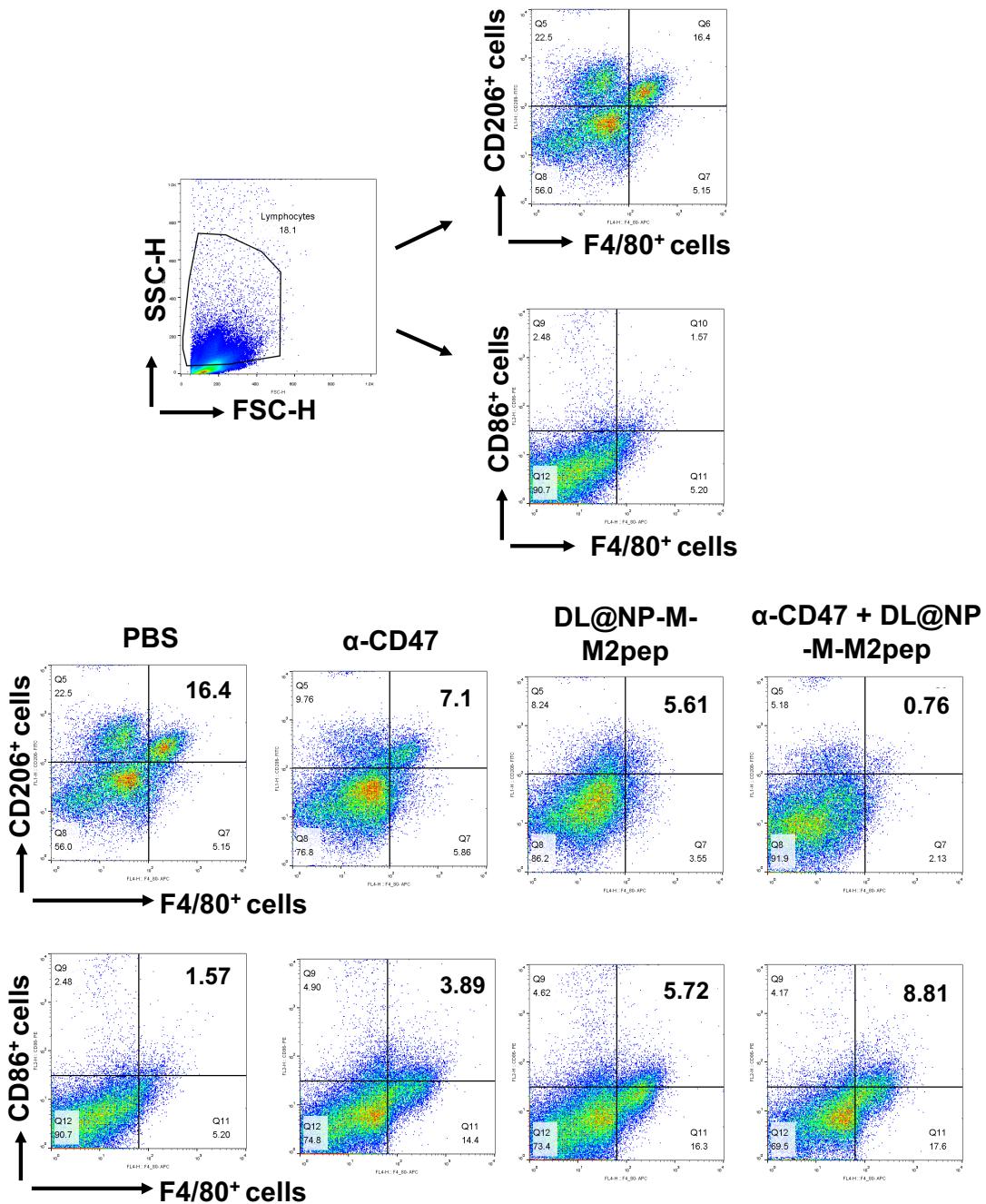
Figure S14. The M2 TAMs (F4/80$^+$ CD206$^+$) and M1 TAMs (F4/80$^+$ CD86$^+$) in tumor tissue (Figure 9E).
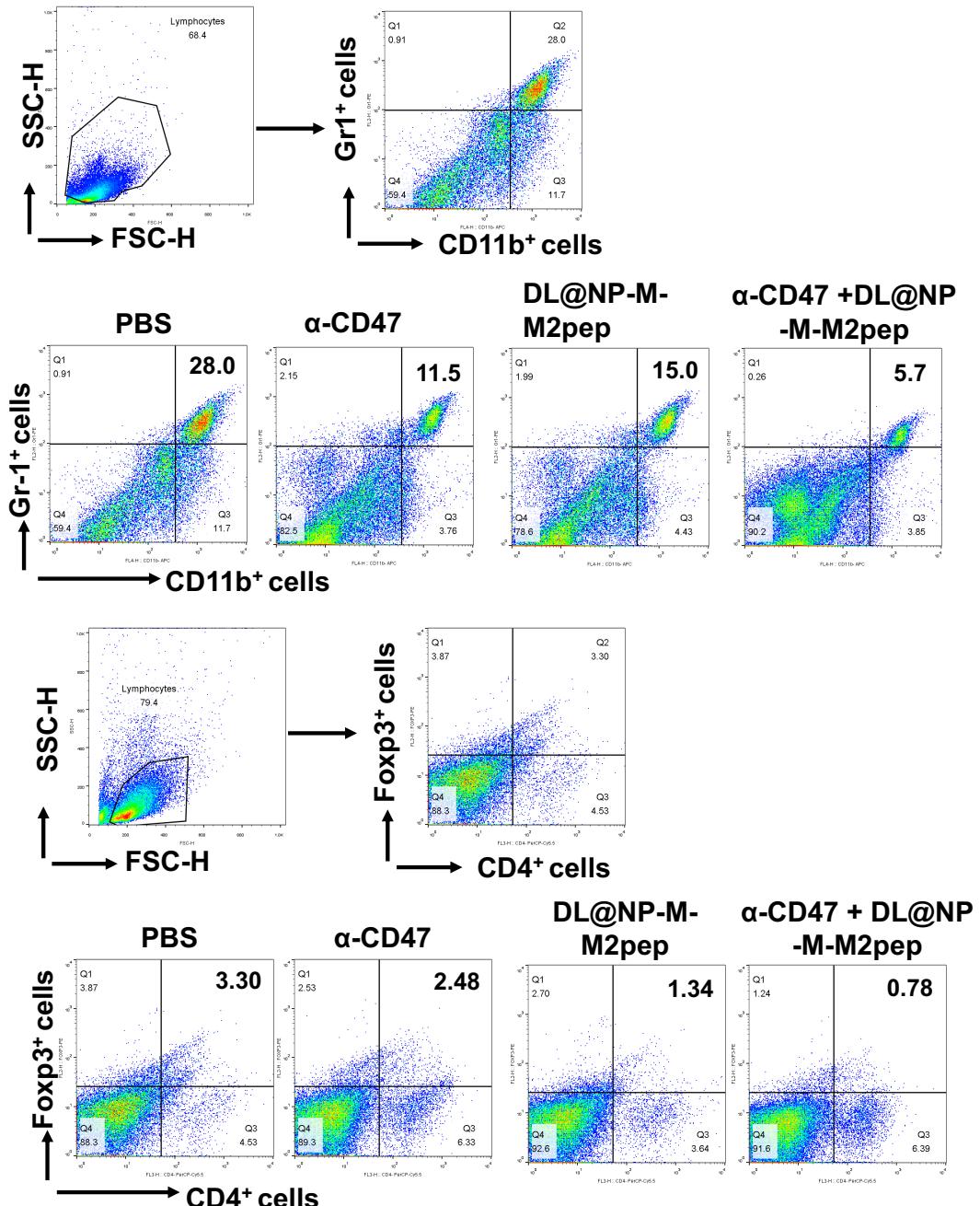
Figure S15. The MDSCs ($CD11b^+$ $Gr-1^+$) and Tregs ($CD4^+$ $Foxp3^+$) in tumor issue (Figure 9E).
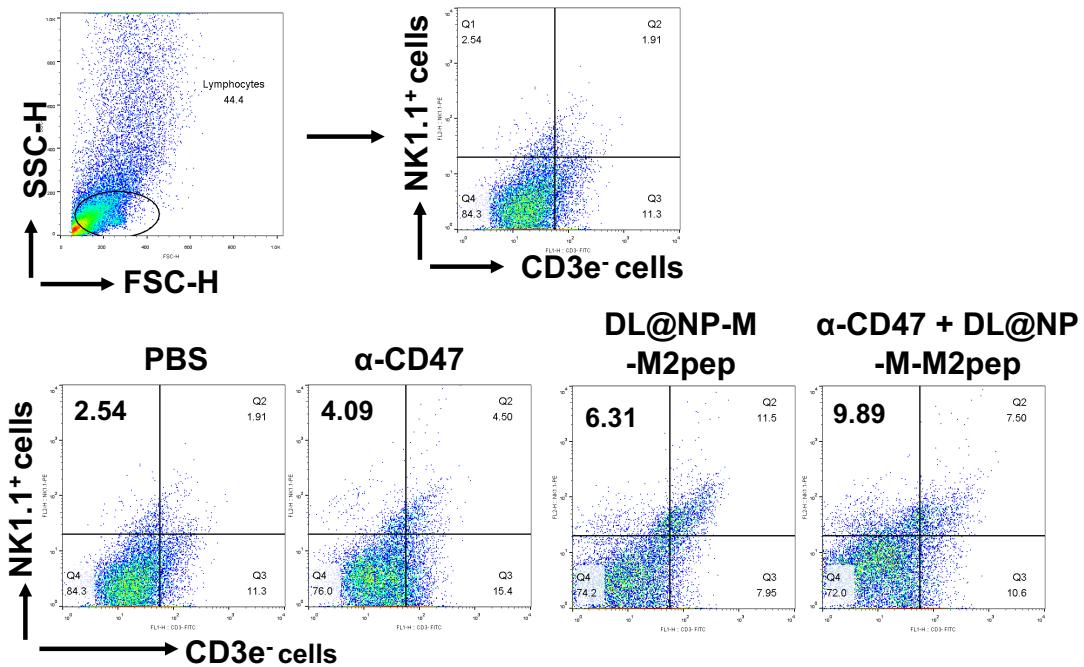
Figure S16. The NK cells (NK1.1$^+$ CD3e$^-$) in tumor tissue (Figure 9E).
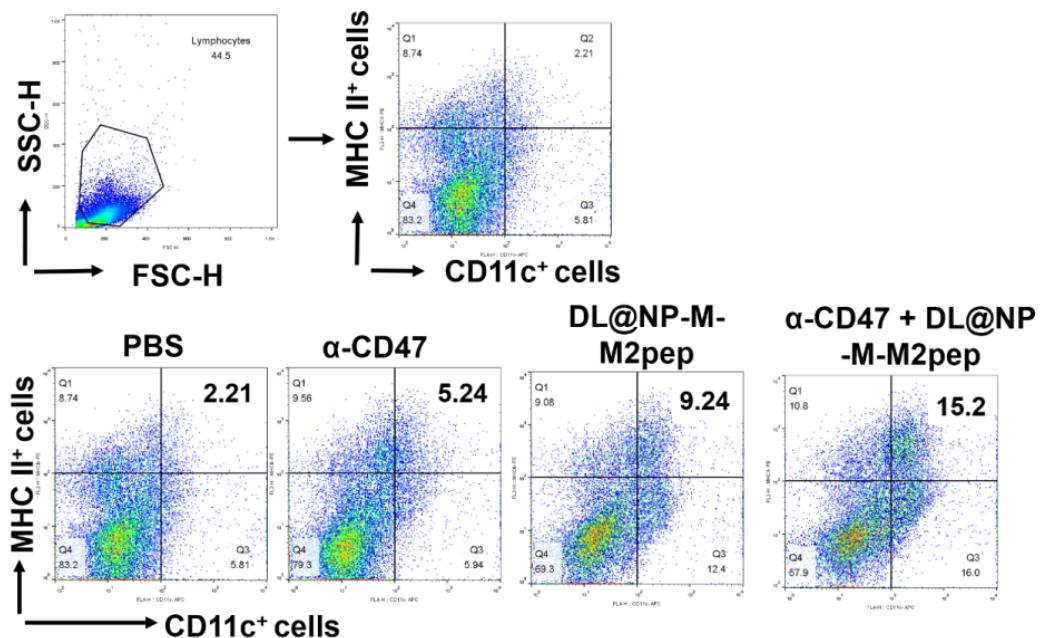
Figure S17. The activated DCs (CD11c$^+$ MHC II$^+$) in tumor tissue (Figure 9E).
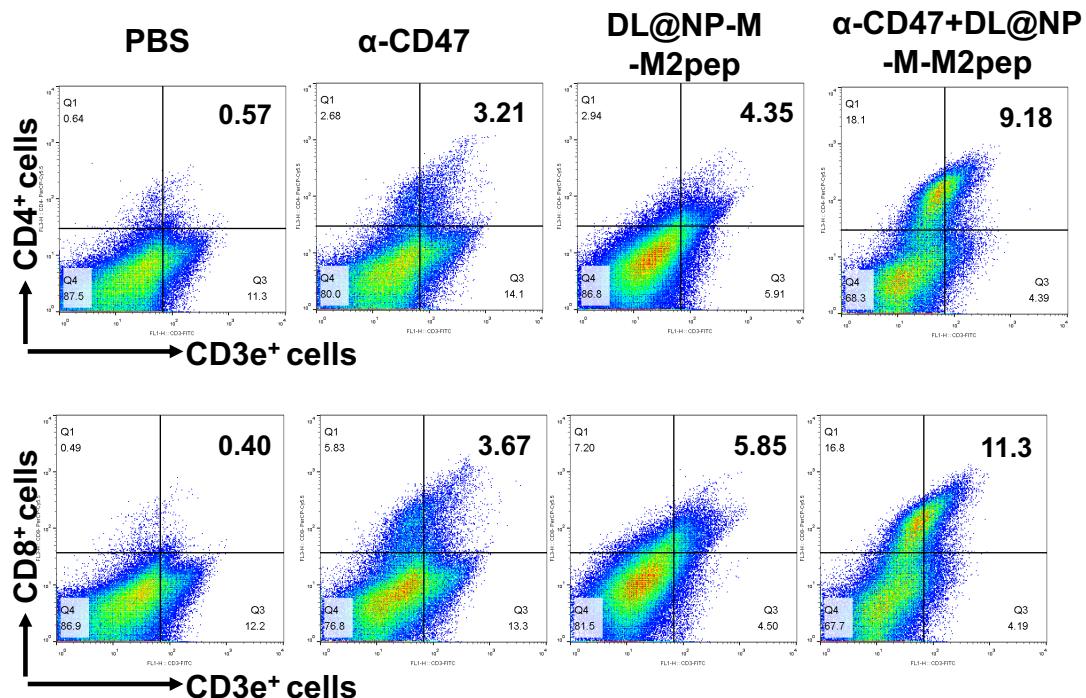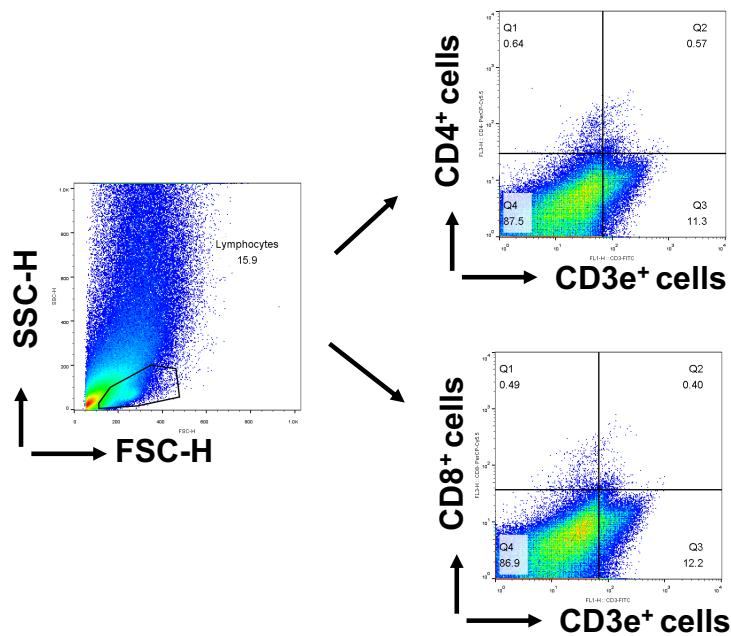
Figure S18. The CD4$^+$ helper T cells (CD3e$^+$ CD4$^+$) and CD8$^+$ cytotoxic T cells (CD3e$^+$ CD8$^+$) in tumor tissue (Figure 9E).
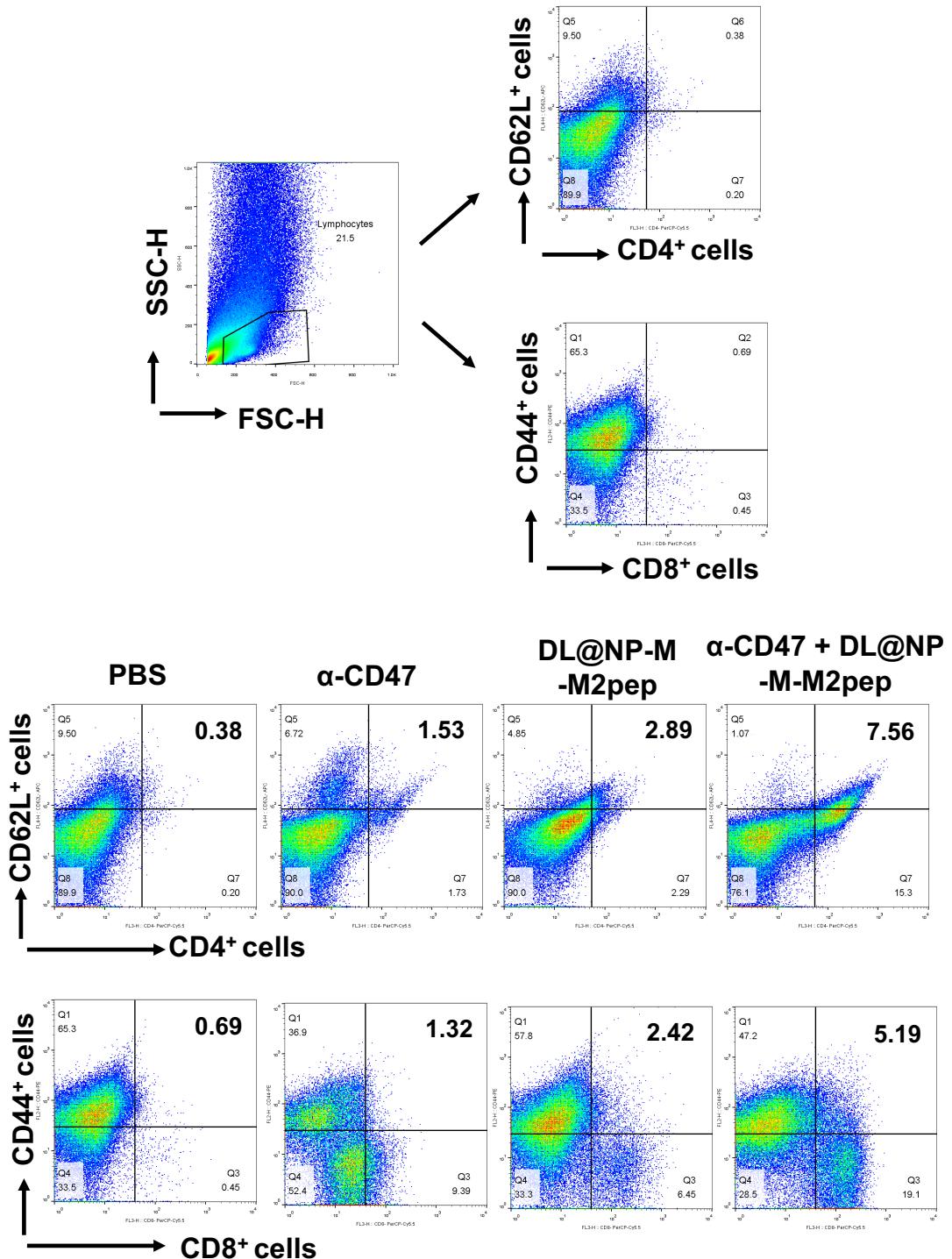
Figure S19. The memory CD4$^+$ T cells (CD62L$^+$ CD4$^+$) and memory CD8$^+$ T cells (CD44$^+$ CD8$^+$) in tumor tissue (Figure 9E).

Table S1. Antibody list in the study.

Antibody	Company	Catalog No.	Assay	Dilution
Anti-PI3K (Mw of PI3K = 85 kDa)	Affinity	AF6241	WB	1:2000
Anti-p-PI3K (Mw of p-PI3K = 80 k Da)	Affinity	AF3241	WB	1:2000
Anti-AKT1 (Mw of AKT1 = 55 kDa)	Affinity	AF0836	WB	1:2000
Anti-p-AKT1 (Mw of p-AKT1 = 60 kDa)	Affinity	AF8355	WB	1:2000
Anti-AKT2 (Mw of AKT2 = 60 kDa)	Affinity	AF6264	WB	1:2000
Anti-p-AKT2 (Mw of p-AKT2= 60 kDa)	Affinity	AF3264	WB	1:2000
Anti-STAT6 (Mw of STAT6 = 94 kDa)	Affinity	AF6302	WB	1:2000
Anti-p-STAT6 (Mw of p-STAT6 = 94 kDa)	Affinity	AF3301	WB	1:2000
Anti-STAT1 (Mw of STAT1 = 84 kDa)	Affinity	AF6300	WB	1:2000
Anti-p-STAT1 (Mw of p-STAT1 = 84 kDa)	Affinity	AF3300	WB	1:2000
Anti-PPAR-γ (Mw of PPAR-γ = 57 kDa)	Affinity	AF6284	WB	1:1000
Anti-p-PPAR-γ (Mw of p-PPAR-γ = 57 kDa)	Affinity	AF3284	WB	1:1000
Anti-TNF-α (Mw of TNF-α = 17 kDa)	Affinity	AF7014	WB	1:500
Anti-FIZZ (Mw of FIZZ = 11 kDa)	Affinity	DF8288	WB	1:1000
Anti-ARG1 (Mw of ARG1 = 35 kDa)	Affinity	DF6657	WB	1:2000
Anti-iNOS (Mw of iNOS = 130 kDa)	Affinity	AF0199	WB	1:500
Anti-NF-κB (Mw of NF-κB = 65 kDa)	Affinity	BF8005	WB	1:2000
Anti-p-NF-κB (Mw of p-NF-κB = 65 kDa)	Affinity	AF2006	WB	1:2000
Anti-GAPDH (Mw of GAPDH = 37 kDa)	Affinity	AF7021	WB	1:2000
Goat anti-rabbit IgG (H+L) HRP	TransGen Biotech	HS101-01	WB	1:4000

APC anti-mouse CD11c	Biolegend	117310	Flow	1:500
APC anti-mouse CD86	Biolegend	105012	Flow	1:500
PE anti-mouse I-A/I-E (MHC II)	Biolegend	107608	Flow	1:500
APC anti-mouse F4/80	Biolegend	123116	Flow	1:500
PE anti-mouse CD86	Biolegend	105008	Flow	1:500
Brilliant Violet 421™ anti-mouse CD206 (MMR)	Biolegend	141717	Flow	1:500
PE anti-mouse NK-1.1	Biolegend	108708	Flow	1:500
FITC anti-mouse CD3	Biolegend	100204	Flow	1:500
PerCP/Cyanine5.5 anti-mouse CD8a	Biolegend	100734	Flow	1:500
Alexa Fluor® 700 anti-mouse CD4	Biolegend	100430	Flow	1:500
PE anti-mouse/human CD44	Biolegend	103008	Flow	1:500
APC anti-mouse CD62L	Biolegend	104412	Flow	1:500
APC anti-mouse/human CD11b	Biolegend	101212	Flow	1:500
PE anti-mouse Ly-6G/Ly-6C (Gr-1)	Biolegend	108408	Flow	1:500
Brilliant Violet 421™ anti-mouse FoxP3	Biolegend	126419	Flow	1:500

Mw, molecular weight; PI3K, phosphatidylinositol-3-kinase; p-PI3K, phosphorylation of PI3K; AKT, serine/threonine protein kinases; p-AKT, phosphorylation of AKT; STAT, signal transducers and activators of transcription; PPAR, peroxisome proliferator-activated receptor; iNOS, inducible nitric oxide synthase; TNF-α: tumor necrosis factor-alpha; Arg-1, arginine-1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; NF-κB, nuclear factor-kappa B; PE, phycoerythrin; APC, allophycocyanin; MHC II, major histocompatibility complex II; Gr-1, granulocyte-differentiation antigen-1; FoxP3, forkhead box protein 3; WB, western blot; flow, flow cytometry

Table S2. ELISA kit in the study.

Cytokine ELISA kit	Company	Catalog No.
Mouse IL-10 Precoated ELISA Kit	Dayou	1211002
Mouse IL-4 Precoated ELISA Kit	Dayou	1210402
Mouse TGF-β Precoated ELISA Kit	Dayou	1217102
Mouse IL-12 Precoated ELISA Kit	Dayou	1211202
Mouse TNF-α Precoated ELISA Kit	Dayou	1217202
Mouse IFN-γ Precoated ELISA Kit	Dayou	1210002

ELISA: enzyme-linked immunosorbent assay; TGF-β: transforming growth factor-β; IL-10: interleukin-10; IL-4: interleukin-4; IL-12: interleukin-12; TNF-α: tumor necrosis factor-alpha; IFN-γ: interferon-gamma.

Table S3. Primer list for RT-PCR.

Primer	Company	Applied Biosystems/Ref.
IL-4	Thermo Fisher Scientific	Mm00445259_m1
IL-10	Thermo Fisher Scientific	Mm01288386_m1
TGF-β	Thermo Fisher Scientific	Mm01298616_m1
IL-12	Thermo Fisher Scientific	Mm00434169_m1
TNF-α	Thermo Fisher Scientific	Mm00443260_g1
IFN-γ	Thermo Fisher Scientific	Mm01168134_m1
GAPDH	Thermo Fisher Scientific	Mm99999915_g1
IL-1β	GENEWIZ	Forward:5'-3' GAAATGCCACCTTTTGACAGTG Reverse: 5'-3' GAAATGCCACCTTTTGACAGTG
Fizz1	GENEWIZ	Forward:5'-3' TCCAAGGGCTCTGACATCTG Reverse: 5'-3' TCTTAGGACAGTTGGCAGCA
Arg1	GENEWIZ	Forward: 5'-3' CTCCAAGCCAAAGTCCTTAGAG Reverse: 5'-3' GGAGCTGTCATTAGGGACATCA
iNOS	GENEWIZ	Forward: 5'-3' GTTCTCAGCCCAACAATACAAGA Reverse: 5'-3' GTGGACGGGTCGATGTCAC

IL-4: interleukin-4; IL-10: interleukin-10; TGF-β: transforming growth factor-β; IL-12: interleukin-12; TNF-α: tumor necrosis factor-alpha; IFN-γ: interferon-gamma GAPDH: glyceraldehyde-3-phosphate dehydrogenase; CD206: costimulatory molecule 206; Arg-1: arginase 1; iNOS: inducible nitric oxide synthase.