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Supplementary Information

Selective Targeting Tumor Cells and Tumor Associated Macrophages Separately by Twin-like Core-shell Nanoparticles for Enhanced Tumor-localized Chemoimmunotherapy

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Experimental section

Materials: Injectable soya lecithin (phosphatidylcholine accounts for 95%, pH 5.0–7.0), DOTAP and DOPE were provided by A.V.T. pharmaceutical Co., Ltd. (Shanghai, China). SF was provided by Shanghai Biochempartner Co., Ltd. (Shanghai, China). IMD 0354 was purchased from Selleck. Carboxymethyl chitosan (average Mw = 50,000 Da) was obtained from Hebei Qiansheng biological technology Co., Ltd. MPITC was purchased from SIGMA Co., Ltd. (USA). The coumarin 6 was purchased from Aladdin Co., Ltd. (Shanghai, China). All reagents for HPLC analysis were of HPLC grade. All the other chemicals and reagents used were of analytical grade or higher, obtained commercially.

Animals: The female C57BL/6 mice (weight: 18–22 g, age: 6–8 weeks) were supplied by the Medical Animal Test Center of Shandong University (Jinan, China). All experiments were carried out in compliance with the Animal Management Rules of

the Ministry of Health of the People's Republic of China (document number 55, 2001) and the Animal Experiment Ethics Review of Shandong University.

Cell lines: Human hepatocellular carcinoma cell lines (HepG2), mouse hepatocarcinoma cell lines (Hepa1-6) and RAW247.4 were cultured in the DMEM medium supplemented with 10% fetal bovine serum (FBS). All cells were cultured in a 37 °C incubator with 5% CO_2.

Synthesis and characterization of MPITC-DOPE: The synthesis route of M-DOPE was shown as Figure S1A. MPITC (5.1 mg) was dissolved in MeOH (3 mL), and DOPE (10 mg) was dissolved in CH_3Cl_2 (200 μL). The solution of MPITC and DOPE were mixed in a balloon flask (20 mL) under stirring, and triethylamine (4.47 μL) was added. The reaction solution was stirred for 24 h at room temperature. The reactive solvent was removed by rotary evaporation, and final product was re-dissolved in CH_2Cl_2. Unreacted triethylamine was neutralized by HCl (0.1 M), and the water phase was extracted with CH_2Cl_2. The organic layer was washed with saturated sodium chloride solution, dried over Na_2SO_4, filtered, and was concentrated under reduced pressure. The chemical structure was confirmed by fourier transform infrared spectroscopy (FTIR) and ^1H nuclear magnetic resonance (^1H NMR) spectroscopy (AvanceTM DPX-300, Bruker BioSpin GmbH, Rheinstetten, Germany) in CD_3OD.

Stability of TCN. Physical stability of TCN was evaluated at 4 °C ± 2 °C. The changed particle size and drug loading efficiency were recorded by a Malvern Zetasizer Nano ZS instrument (Malvern Nano ZS-90, Malvern United Kingdom) and HPLC, respectively over a period of 7 days (n=3).

Normalization of tumor vasculature: After tumor suppression study, the tumor tissues from different groups were fixed in 4% formaldehyde and embedded in paraffin for analysis. Paraffin-embedded 10 mm tumor sections were prepared for immunohistochemical analysis. Then the sections of each group were staining with DAPI, CD31 and αSMA. Then the sections were imaged by a confocal laser scanning microscope (LSM 780, Carl Zeiss, Germany).

Assessment of cytokines: After tumor suppression study, blood were sampled from mice eyes and left for 30 mins. Then serum was collected by centrifugation at 3000 rpm for 15 mins. The analysis of cytokines TGF-β, IL-10, IL-12 and IFN-γ were performed on serum samples using ELASA kits (Dakewe Biotech Co., China).

The histological analysis: Major organs (heart, liver, spleen, lung, and kidney) of mice that were treated with different formulations were fixed in 4% PBS buffered paraformaldehyde and embedded in paraffin wax. The paraffin-embedded 5-mm sections were stained with hematoxylin and eosin (H&E) to assess histology.

Determination of M1- and M2-associated gene expression level by Real-time PCR in cell: For further prove the re-polarization of M2-type TAM ability of CMCS/M-IMD-CLN, M1-type TAM signs including iNOS, TNFα, IL-12p40 and CD86 and M2-type TAM signs including Arginase I, CD206, IL-10 gene expression was measured by quantitative Real-time PCR experiment. In brief, RAW264.7 were seeded into 12-well plates at a density of 6×10^6 per dish and incubated overnight at 37 °C. IL-4 (15 ng/mL) was added and incubated for 12 h to promote macrophages polarizing to M2-type. First, CMCS/M-IMD-CLN (15 μg/mL) was incubated with medium at pH 6.5

for 1 h. Then fresh media contained CMCS/M-IMD-CLN (post-incubated), M-IMD-CLN, IMD-CLN and IMD solution were added and incubated with polarized M2-type TAMs for 24 h. Then, the medium was replaced with fresh medium and incubated for another 24 h. Finally, the cellular total mRNA in each well extracted using different reagents as described below.

Total mRNA was extracted with Trizol (Invitrogen, Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol and then quantified using a Nanodrop (Thermo Fisher Scientific, Waltham, MA). The complementary DNA (cDNA) was synthesized based on the isolated mRNA using first-strand cDNA Synthesis Kit (Beyotime, Haimen, Zhejiang, People's Republic of China) and then used for RT-PCR kit with Taq (Beyotime, Haimen, Zhejiang, People's Republic of China). The PCR was performed at 95 °C for 1 min. 50 cycles at 94 °C for 15 s. 60 °C for 45 s. 72 °C for 15 s. The relative M1- and M2-associated gene expression levels were determined by the comparative Ct method.

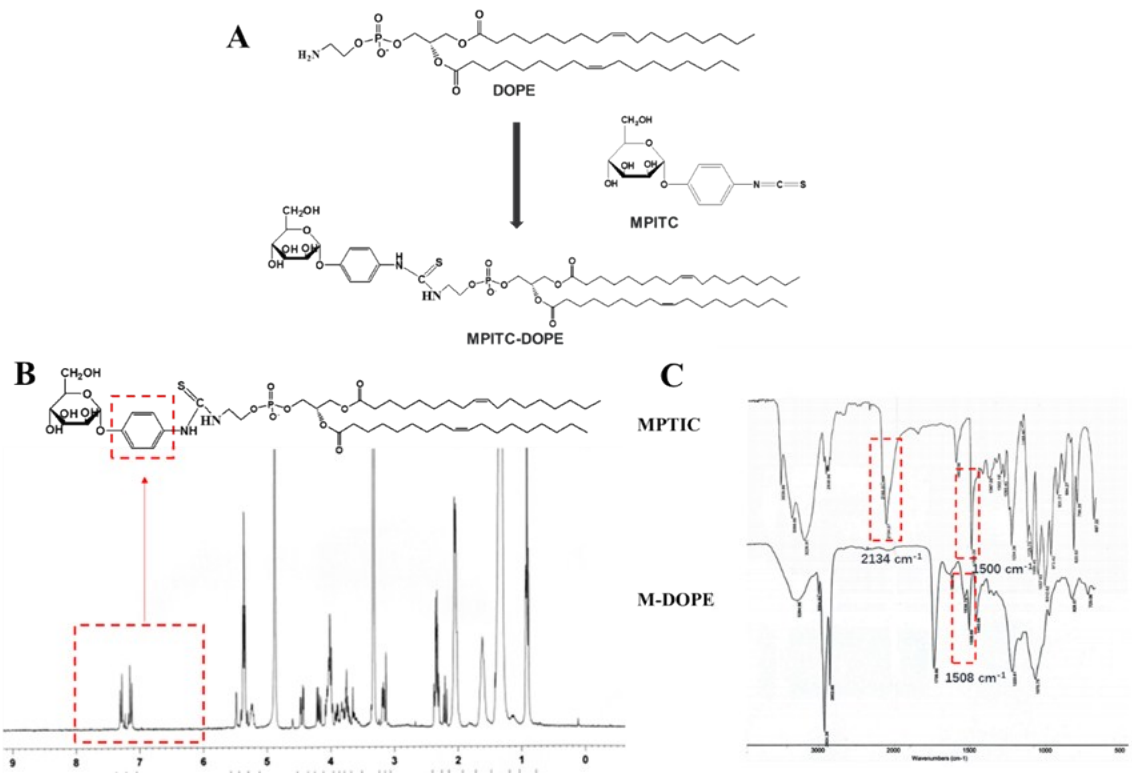

Figure S1 Synthesis and characterization of M-DOPE. (A) synthesis route of M-DOPE (B) ^{1}H NMR spectrum of M-DOPE (C) FTIR Spectrophotometry of MPTIC and M-DOPE.

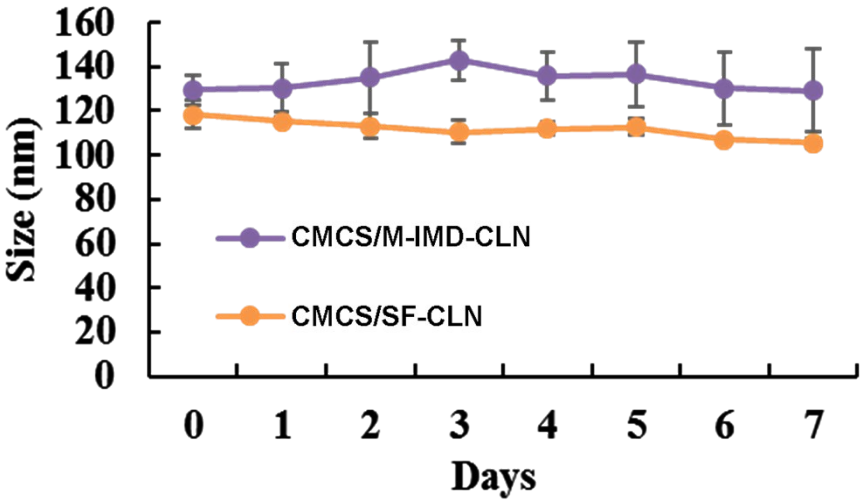

Figure S2 Preliminary study of the CMCS/M-IMD-CLN and CMCS/SF-CLN stability at 4 °C ± 2 °C during 7 days. (n=3)

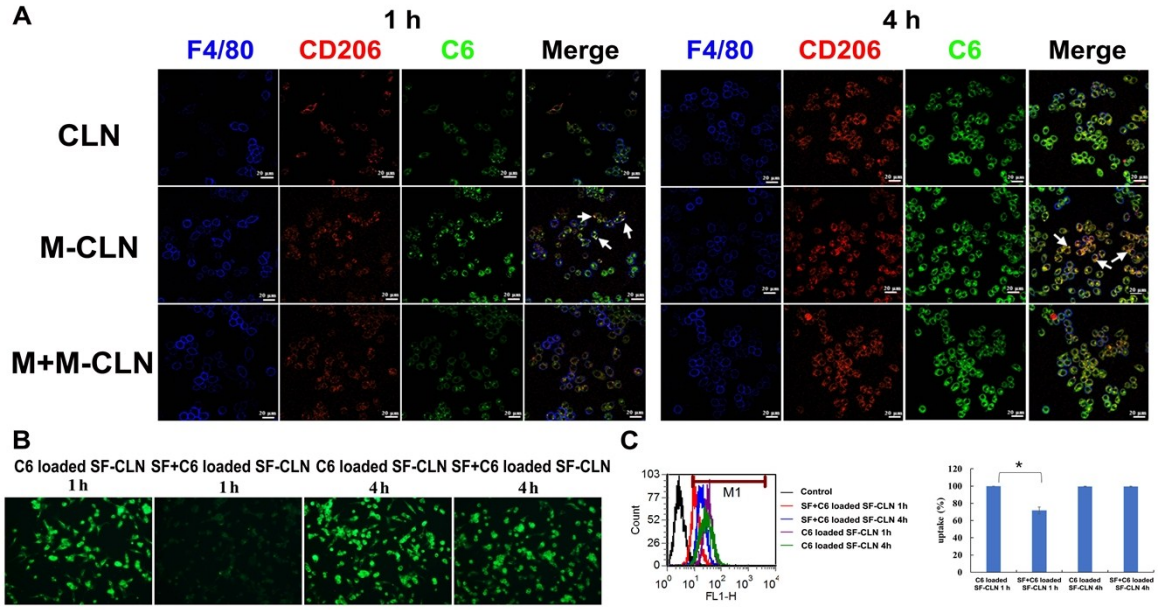

Figure S3 Targeted ability of TCN in vitro. (A) Confocal laser scanning microscopy images of competitive inhibition of M-CLN on RAW264.7 (Scan bar: 20 μm). (B) Fluorescence micrographs of competitive inhibition of SF-CLN on Hepa1-6 (200×). (C) Flow cytometric analysis of competitive inhibition of SF-CLN.

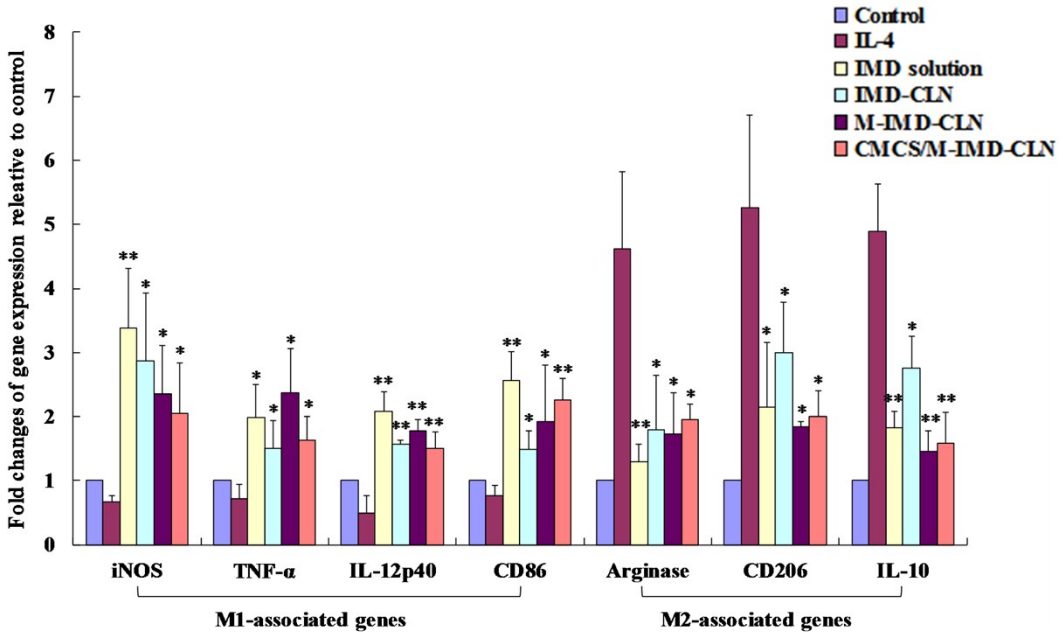

Figure S4 Re-polarization of M2-type TAMs ability of CMCS/M-IMD-CLN. M1-type TAM signs including iNOS, TNFα, IL-12p40 and CD86 and M2-type TAM signs including Arginase I, CD206, IL-10 gene expression was measured by quantitative RT-PCR (qRT-PCR). All data are representative of at least three (n = 3) independent experiments for each experimental group and are displayed as mean ± standard deviation. *$p < 0.05$, **$p < 0.01$ compared to IL-4 group, indicates statistically significant difference (Student's t-test).

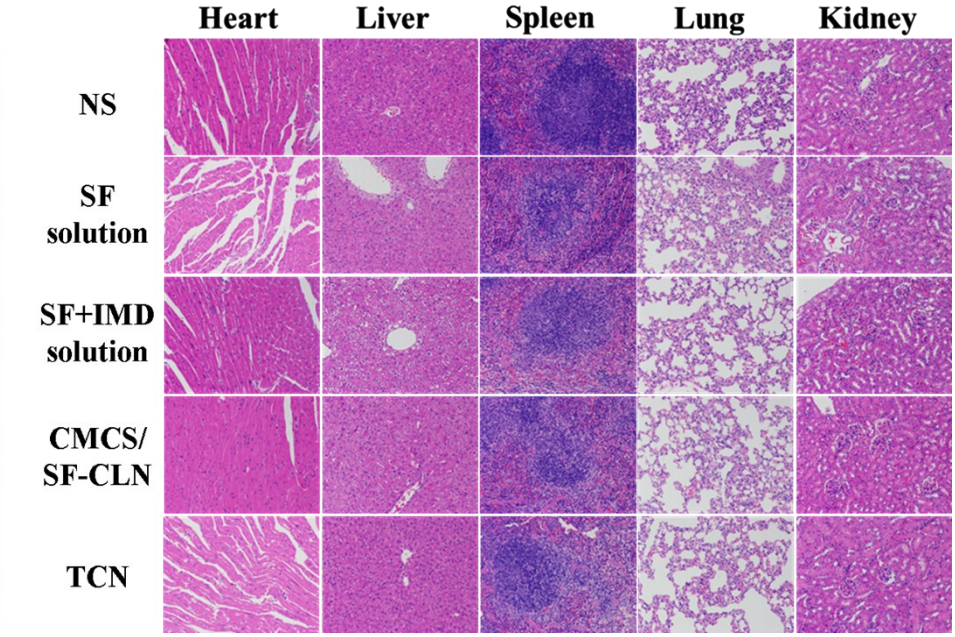

Figure S5 Histological assays. Representative microscopy images of H&E stained histological sections on Hepa1-6 bearing female C57BL/6 mice after treatment with different formulations.

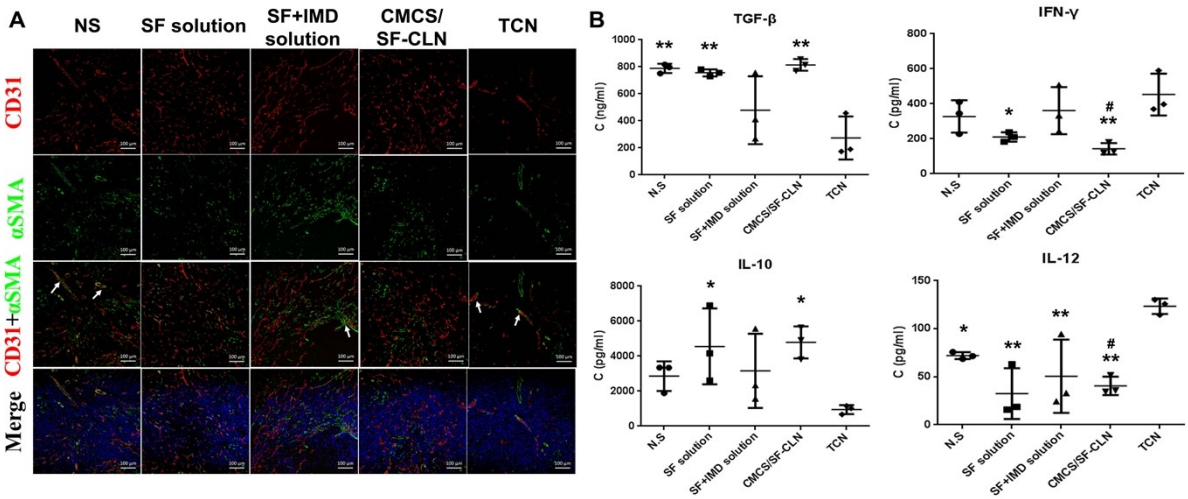

Figure S6 Tumor vascular and cytokines evaluation in vivo. (A) Vascular normalization study. Confocal laser scanning microscopy images of tumor tissues were stained by DAPI (blue), CD31 (red) and α-SMA (green) after treatment with different formulations. (B) Histograms of cytokines in peripheral blood from Hepa1-6 bearing female C57BL/6 mice after treatment with different formulations (n = 3), *$p < 0.05$, **$p < 0.01$ statistically significant difference compared with TCN NPs group; #$p < 0.05$, statistically significant difference compared with NS group.

Table S1. Summary of average sizes, polydispersity index and zeta potentials of SF-CLN, M-IMD-CLN, CMCS/SF-CLN, CMCS/M-IMD-CLN

	SF-CLN	**M-IMD-CLN**	**CMCS/SF-CLN**	**CMCS/M-IMD-CLN**
Size (nm)	152.6±9.5	169.9±6.1	117.9±3.4	129.4±6.8
PDI	0.248±0.009	0.279±0.001	0.277±0.010	0.291±0.003
Zeta (mV)	26.7±2.4	28.4±3.2	-21.1±2.5	-25.5±0.8
DL	7.24%±0.32%	7.21%±0.71%	5.22%±0.25%	7.43%±0.51

Table S2. IC50 of SF solution, CMCS/SF-CLN and SF-CLN on Hepa1-6 cells after 48 h incubation (n=3).

Groups	SF solution	CMCS/SF-CLN	SF-CLN
IC50	7.71±0.48	5.90±0.53*	3.32±0.32**

*$p < 0.05$, **$p < 0.01$, compared with SF solution, Student's t-test (two-tailed).