Supplementary Material

iRGD-mediated liposomal nanoplatform for improving hepatocellular carcinoma targeted combination immunotherapy and monitoring tumor response via IVIM-MRI

*Jiamin Li[a, b], Ruili Wei[a, b], Wang Yao[a, b], Xinrui Pang[a, b], Nianhua Wang[c], Shengsheng Lai[d], Xinhua Wei[b], Youyong Yuan[e, *], Xinqing Jiang[a, b, *], Ruimeng Yang[a, b, *]*

[a] School of Medicine, South China University of Technology, Guangzhou, 510006, China

[b] Department of Radiology, the Second Affiliated Hospital, School of Medicine, South China University of Technology, Guangzhou, 510180, China

[c] Department of Radiology, The First Affiliated Hospital of Guangzhou Medical University, 151 Yanjiangxi Road, Guangzhou, 510120, China.

[d] School of Medical Equipment, Guangdong Food and Drug Vocational College, Guangzhou, Guangdong, 510520, China

[e] School of Biomedical Sciences and Engineering, South China University of Technology, Guangzhou International Campus, Guangzhou, 511442, China

* Corresponding authors.

E-mail addresses: yuanyy@scut.edu.cn (Youyong Yuan), eyjiangxq@scut.edu.cn (Xinqing Jiang), eyruimengyang@scut.edu.cn (Ruimeng Yang),

2. Experimental Section

2.1. Materials

BMS-202 (Synonyms: PD-1/PD-L1 inhibitor 2) was purchased from Shenzhen Wenle Biotechnology Co., Ltd. Lenvatinib (Len) was purchased from Guangzhou Lide Biotechnology Co., Ltd. 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), Cholesteryl hemisuccinate (CHEMS), DSPE-mPEG, and DSPE-mPEG-iRGD were purchased from A. V.T. (Shanghai) Pharmaceutical Co., Ltd., Shanghai, P. R. China. Cy5 was purchased from Shanghai Chutai Biotechnology Co., Ltd. STAT1 antibody, PD-L1 antibody, and B2M antibody antibody were purchased from Abcam (Cambridge, UK). Mouse ELISA Kits were purchased from Wuhan Fine Biotech Co., Ltd. CD31, α-SMA, HIF-1α, Ki67, CD8, PD-L1 and Foxp3 primary antibodies were purchased from Servicebio (Wuhan, China). Dulbecco's modified eagle's medium (DMEM), trypsin-EDTA, and penicillin−streptomycin were obtained from Gibco BRL (Eggenstein, Germany). Fetal bovine serum (FBS) was obtained from ExCell Biology, Inc (Shanghai, China). Hoechst 33342 were purchased from Life Technologies. Flow cytometric antibodies for immune cell labeling were purchased from Biolegend (San Diego, CA, USA). All animal operations were in conformity to Guidelines for Care and Use of Laboratory Animals of South China University of Technology (SCUT) and approved by animal ethics of SCUT.

2.2. Colony forming assay

Colony formation experiments were used to further evaluate the effects of the dual-loaded liposomes of Len and BMS-202 on the proliferation of Hepa1-6 cells.

Hepa1-6 cells were seeded in a 6-well plate at a density of 1×10^3 cells per well and incubated for 24 h. Then, cells were cultured with fresh medium containing different concentrations of Len or iRGD-lip@Len/BMS-202 (Len 8, 16, and 30 μg/mL) for 7 days. Afterward, the medium was slowly removed, and the cells were fixed with 4% paraformaldehyde (PFA) for 20 min and stained with 1% crystal violet for 30 min. The clones were photographed using a mobile phone and the formation of clones was calculated using ImageJ software.

2.3. *In vitro* live assay

Hepa1-6 cells were seeded into 35mm-glass bottom dishes with a density of 1×10^5 per well incubated overnight. The cells were treated with PBS, iRGD-lip@BMS-202, iRGD-lip@Len, Len/BMS-202, or iRGD-lip@Len/BMS-202 for 24 h. The Live kit (Best Bio Science, China) was used for imaging cell viability. The fluorescent dye calcein-acetoxymethylester (Calcein-AM) was utilized to label live cells to assess therapy effect. The fluorescence of Calcein-AM dye was observed using confocal laser scanning microscopy (CLSM).

2.4. Wound healing assay

The wound healing assay was conducted to determine the effects of the nanodrug on the migration of HUVEC cells and Hepa1-6 cells. When HUVEC cell density reached 100%, wounds were created in the central area using a 200 μl pipette tip and the cells were washed with PBS buffer and treated with fresh medium containing Len, iRGD-lip@Len, and iRGD-lip@Len/BMS-202 for 24 h. Photos of the scrape line were taken at 0 h and 24 h. Each experiment was repeated thrice. Cell migration was

expressed as the percentage of wound closure in the wound area. The healing area was quantified by ImageJ software.

Hepa1-6 cells (2×10^5 cells /well) were seeded in 6 well plate and incubated for 24 h. Then, we used the tip of a 200 μl pipette to draw straight lines in the plates, and the cells were washed with PBS buffer and treated with fresh medium containing PBS, iRGD-lip@BMS-202, iRGD-lip@Len, Len/BMS-202, and iRGD-lip@Len/BMS-202 (with Len at a concentration of 8 μg/mL and BMS-202 at a concentration of 2.7 μg/mL) for 24 h and 48 h. The healing area was quantified using ImageJ software.

2.5. Western blot analysis

Briefly, after incubation with drugs for 24 h, the cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer on ice for 30 min. The total proteins were separated using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes. Firstly, the membranes were sealed with 5 mL 5% skim milk for 2 h, then, the membranes were incubated with anti-PD-L1 (1:1000), and anti-GAPDH (1:1000) primary antibodies at 4℃ overnight, respectively. After this incubation, the membranes were washed with Tris-buffered saline with Tween 20 (TBST) three times for 10 min each, and the membranes were incubated with HRP-conjugated secondary antibodies for 1.5 h. Finally, the immunoreactive bands were visualized using an Efficient Chemiluminescence Kit and photographed under a ChemiDoc XRS System (Bio-Rad). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control for western blotting.

2.6. Establishment of subcutaneous tumor model

To develop tumors in C57BL/6J mice, 2×10^6 Hepa1-6 cells suspended in 100 μL of PBS were subcutaneously implanted in the right thoracic wall of the mice, and the tumor sizes were monitored every two days. The tumor size of each mouse was allowed to grow to 70 mm^3 calculated using the following formula: tumor volume = 0.5 × (tumor width)^2 × (tumor length).

2.7. Establishment of orthotopic tumor model

Firstly, the abdominal hair of the mouse was shaved, and the abdominal area was disinfected with iodine. Then, a 1.5 cm skin incision was made on the mouse's upper abdomen, followed by a 1 cm peritoneal incision, and gentle pressure was applied to the chest cavity to expose the left lobe of the liver. Subsequently, 25 μL of Hepa1-6-luc cells suspension (approximately 2×10^6 cells, Hepa1-6-luc cells: matrix gel = 1:1) was slowly injected into the left liver lobe of each mouse. After injection, the incision and the skin were closed. One week after cell injection, the established mice were confirmed by *in vivo* fluorescence imaging system (UniNano NIR-II imaging system) after injection of D-luciferin (15 mg/kg, 100 μL) for 10 min.

2.8. Validation of tumor targeting ability of iRGD *in vivo*

For *in vivo* fluorescence imaging, we validated the tumor accumulation and biodistribution of iRGD-targeted liposomes in the orthotopic tumor model. The free Cy5 or lip@Cy5 or iRGD-lip@Cy5 was intravenously injected into the established mice and then sacrificed after 24 h. The major organs and tumors from liver tissues were collected and analyzed by IVIS Spectrum *in vivo* imaging system.

Additionally, mice bearing Hepa1-6 subcutaneous tumors were also intravenously injected with free Cy5, lip@Cy5, or iRGD-lip@Cy5. Then, fluorescence imaging experiments were performed on an imaging system at the scheduled time points. After 48 h, the tumor tissues and organs including the heart, spleen, lung, kidney, and liver were collected and then fluorescence intensity was analyzed to characterize drug distribution.

2.9. *In vivo* therapeutic effects evaluation

For the treatment of subcutaneous HCC model, mice were randomly divided into five groups and treated as follows:(G1) PBS, (G2) Len/BMS-202, (G3) iRGD-lip@BMS-202, (G4) iRGD-lip@Len, (G5) iRGD-lip@Len/BMS-202. The free Len was administered by gavage, while other drugs were administrated intravenously (Len: 12 mg/kg, BMS-202: 4 mg/kg). The drugs were administered every two days for a continuous 5 times. The weight of the mice and the size of the tumors were monitored every other day, and tumor volumes of subcutaneous tumors were calculated according to the aforementioned formula. After one day at the end of treatment, the mice were euthanized and the histological analysis was carried out after treatments.

For the treatment of the orthotopic HCC model, mice were randomly divided into five groups and treated as follows: (G1) PBS, (G2) Len/BMS-202, (G3) iRGD-lip@BMS-202, (G4) iRGD-lip@Len, (G5) iRGD-lip@Len/BMS-202. The therapeutic strategy was the same as for subcutaneous tumors. 9.4-T MRI was performed to monitor tumor development in the liver on the 6th and 12th day of treatment. The livers of the mice that was sacrificed at the end point were removed and fixed with 4% PFA,

sectioned, and stained with hematoxylin-eosin (H&E) stain.

2.10. Flow cytometric analysis

The subcutaneous tumors from therapeutic experiments of the mentioned mice were used for the analysis and evaluation of immune cells. The infiltrating lymphocytes in tumors (TILs) of each group were analyzed by flow cytometry. The collected TILs were incubated with anti-CD45-BV510, anti-CD3-FITC, anti-CD8-BV421, anti-CD4-BV650 antibodies to determine the content of $CD4^+$ T cells and $CD8^+$ T cells. The Tregs infiltrating in the tumors were stained by anti-CD45-BV510, anti-CD4-BV650, anti-CD25-APC, and anti-Foxp3-PE antibodies. In addition, the MDSCs ($CD45^+CD11b^+Ly6C^+$) in the tumor of each group were stained by anti-CD45-BV510, anti-CD11b-FITC, and anti-Ly6C-BV421 antibodies and subjected to flow cytometric analysis.

2.11. Enzyme-linked immunosorbent assay (ELISA)

To evaluate the cytokines after receiving different treatments at the 13th day, The mouse blood was collected via removing the mouse's eyeball and transferred to 1.5 mL EP tube. The blood was left to stand for 30 min, then centrifuged at 4 ℃ and 1000 g for 15 min. The plasma was collected in a new EP tube. Then the plasma was analyzed by ELISA Kits to detect the IFN-γ, tumor necrosis factor-alpha (TNF-α), interleukin-2 (IL-2), transforming growth factor-beta (TGF-β), arginase-1 (Arg-1), and CXCL10 according to the manufacturer’s instructions.

2.12. Tumor H&E, immunohistochemical, and immunofluorescence staining

After treatments, mice bearing Hepa1-6 subcutaneous tumors were sacrificed,

with their tumor tissues harvested, weighed, and repeatedly washed with PBS. Tumor tissues were fixed using 4% PFA. The slices of tumor were stained with H&E, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL), and Ki67 to evaluate the therapeutic effect. To assess the normalization of blood vessels within the tumor, double immunofluorescence staining for $CD31^+$ and α-SMA^+ was also performed, and the degree of vascular normalization was measured by calculating the percentage of blood vessels covered by pericytes, which is the ratio of the co-localization area of α-SMA^+ and $CD31^+$ to the $CD31^+$ localization area. Subsequently, the impact of vascular normalization on tumor hypoxia was evaluated using hypoxia-inducible factor 1-alpha ($HIF\text{-}1\alpha^+$) immunofluorescence staining to assess the level of tumor hypoxia. Additionally, the expression of $Foxp3^+$, $CD8^+$ T lymphocytes, and PD-L1 in each tumor section was analyzed using the immunohistochemical staining method to evaluate the tumor immune microenvironment.

2.13. IVIM-MRI image acquisition

IVIM-MRI examinations were performed on a 3.0-T MR system (Verio, Siemens, Erlangen, Germany) using a knee coil on days 0, 6, and 12. All mice were anesthetized by intraperitoneal injection of 1.5% sodium pentobarbital (50 mg/kg). The protocols for MRI consisted of a three-plane localizer, axial T2-weighted imaging (T2WI), and IVIM-MRI. The turbo spin echo (TSE) T2WI scan parameters were as follows: repetition time/echo time (TR/ TE) = 1500/69 ms, bandwidth = 203 Hz/Px, matrix size = 64×64, field of view (FOV) = 64 mm, slice thickness = 2 mm, scan time = 4 min and 27 s. IVIM was performed using the following sequences: TR/TE = 1900/75 ms,

bandwidth = 930 Hz/Px, FOV=98 mm, slice thickness = 2 mm, diffusion gradients applied in three orthogonal directions with 9 b values: 0, 50, 100, 150, 200, 400, 600, 800, 1000 s/mm^2, scan time = 6 min and 33 s.

2.14. IVIM-MRI image post-processing

Post-processing of IVIM data were performed using third-party software (MITK-Diffusion). We identified the tumor border by delineating regions of interest (ROIs) on the largest cross section of the tumor. The biexponential model equation was expressed as $SI/SI_0 = (1-f)\cdot\exp(-bD) + f\cdot\exp(-bD*)$. Here, SI_0 represents the mean signal intensity of the ROI at a b value of 0, while SI refers to the signal intensity at higher b values. The b value indicates the diffusion sensitivity coefficient. The D values represent the pure diffusion coefficient, indicating the diffusion movement of water molecules. The f values represent the perfusion fraction, indicating the volumetric ratio of the perfusion effect caused by local microcirculation within the ROI to the overall diffusion effect. D* values represent the pseudo-diffusion coefficient generated by blood microcirculation.

2.15. *In vivo* anti-metastasis efficacy

To examine the efficacy of immunotherapy against metastatic HCC, a lung metastasis mouse model was established. Initially, an Hepa1-6 tumor bearing model was established by injecting Hepa1-6 cells into the left liver lobe of mice. After 7 days, Hepa1-6 cells (at a density of 2×10^6) were injected via the tail vein. Subsequently, the animals were randomly divided into 5 groups (n = 3): (G1) PBS, (G2) Len/BMS-202, (G3) iRGD-lip@BMS-202, (G4) iRGD-lip@Len, (G5) iRGD-lip@Len/BMS-202. At

the end of the monitoring period, mice were euthanized, the lung tissues were collected, fixed in 4% PFA, embedded in paraffin, sliced and stained with H&E stain.

2.16. *In vivo* biosafety analysis

Male C57BL/6J mice were randomly divided into five groups (n = 3), followed by treatment with the abovementioned therapeutic regimen. On day 13, whole blood samples were collected from each mouse serum separation. The blood biochemical indicators such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), serum creatinine (CR), lactate dehydrogenase (LDH), and creatine kinase (CK) were measured to assess the biocompatibility of the fabricated nanoplatforms. Tissues from various mouse organs, including the heart, liver, spleen, lung, and kidney of the mice in five groups were excised, fixed in 4% PFA, embedded in paraffin, sliced and stained with H&E stain.

Supplementary Figures

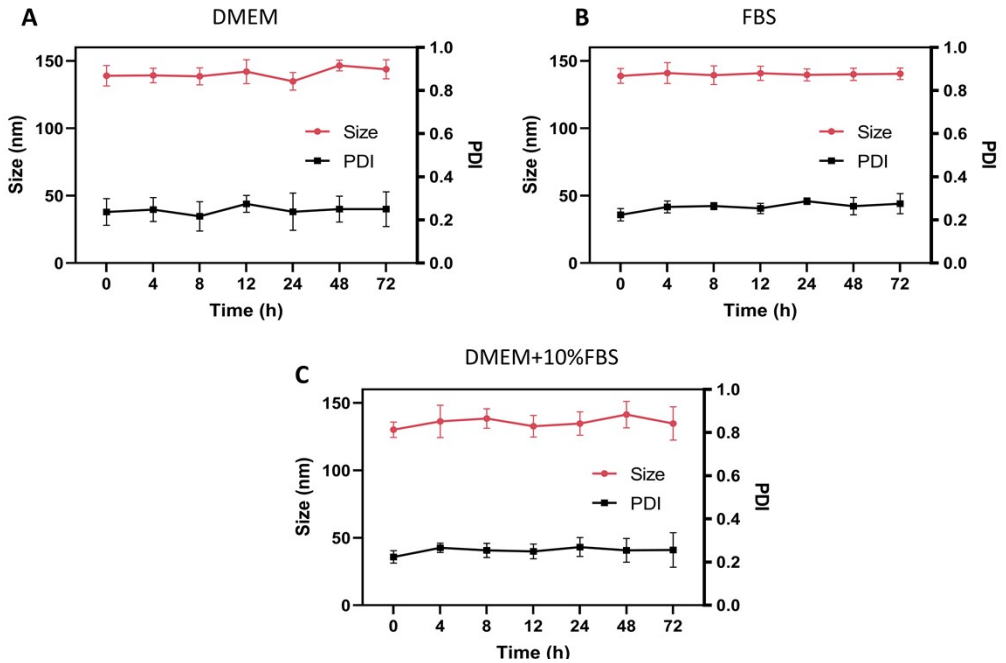


Fig. S1. The size and PDI changes of iRGD-lip@Len/BMS-202 after incubation in (A) DMEM, (B) FBS, (C) DMEM with 10% FBS for 72 h.

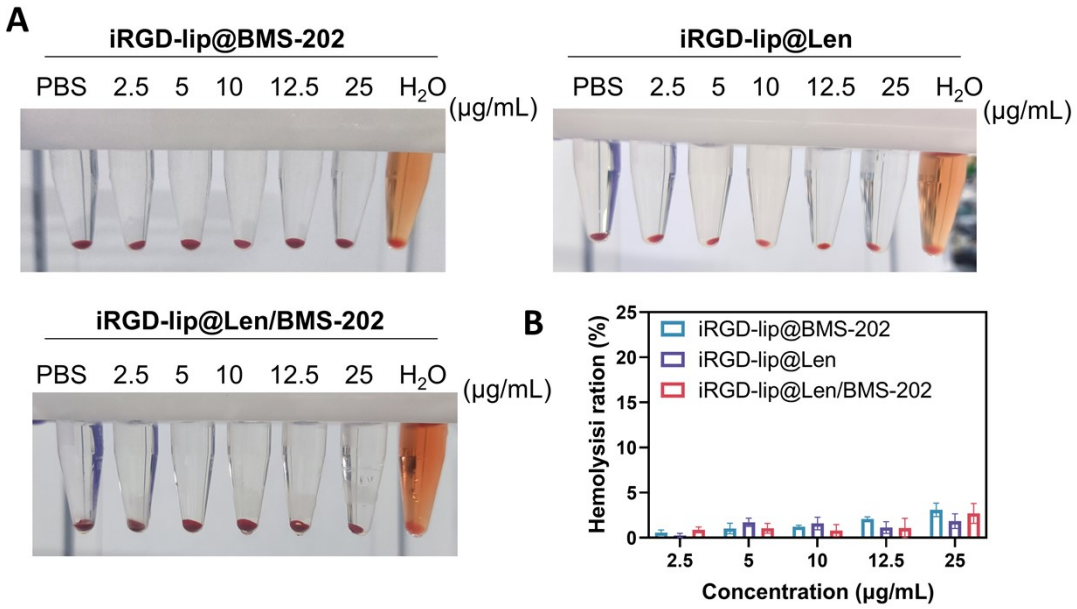


Fig. S2. (A) Hemolysis photos and (B) hemolysis assays of iRGD-lip@BMS-202, iRGD-lip@Len and iRGD-lip@Len/BMS-202 at different concentrations. The positive control was water, while the negative control was PBS.

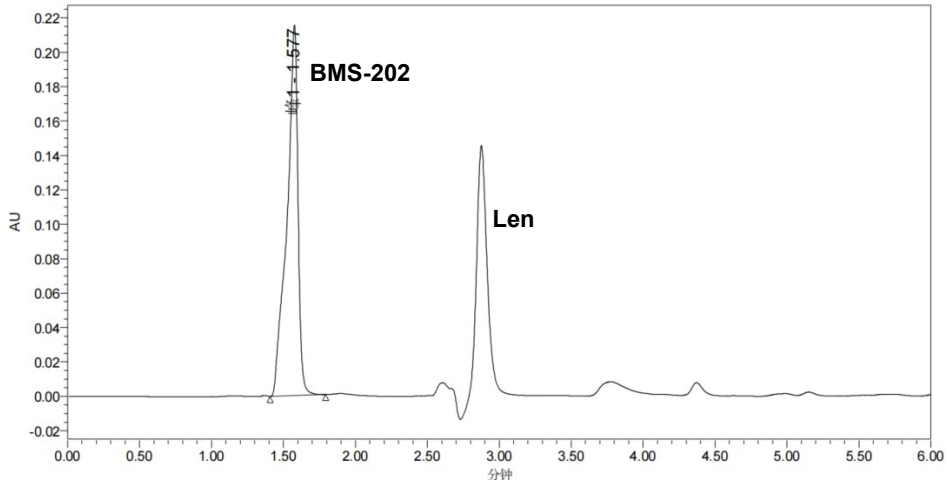


Fig. S3. HPLC results of Len and BMS-202 in iRGD-lip@Len/BMS-202 liposomes.

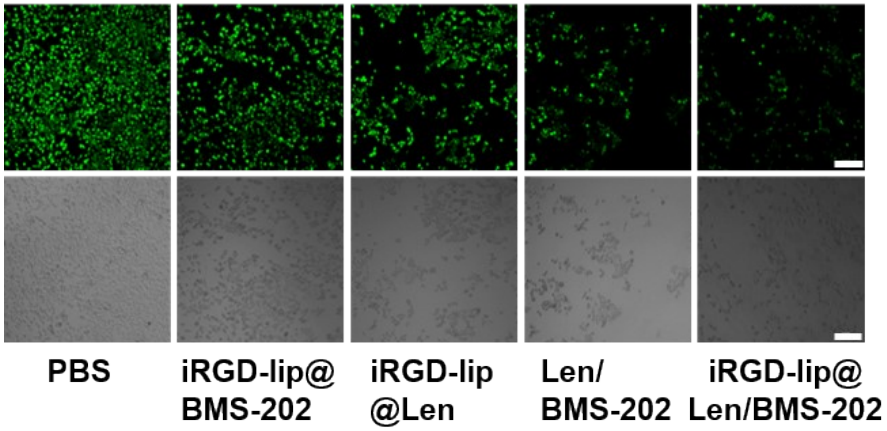


Fig. S4. Fluorescence image of living Hepa1-6 cancer cells after treating with PBS, iRGD-lip@BMS-202, iRGD-lip@Len, Len/BMS-202, or iRGD-lip@Len/BMS-202 for 24 hours. The treated cells were then stained with Calcein-AM (scale bar: 100 μm).

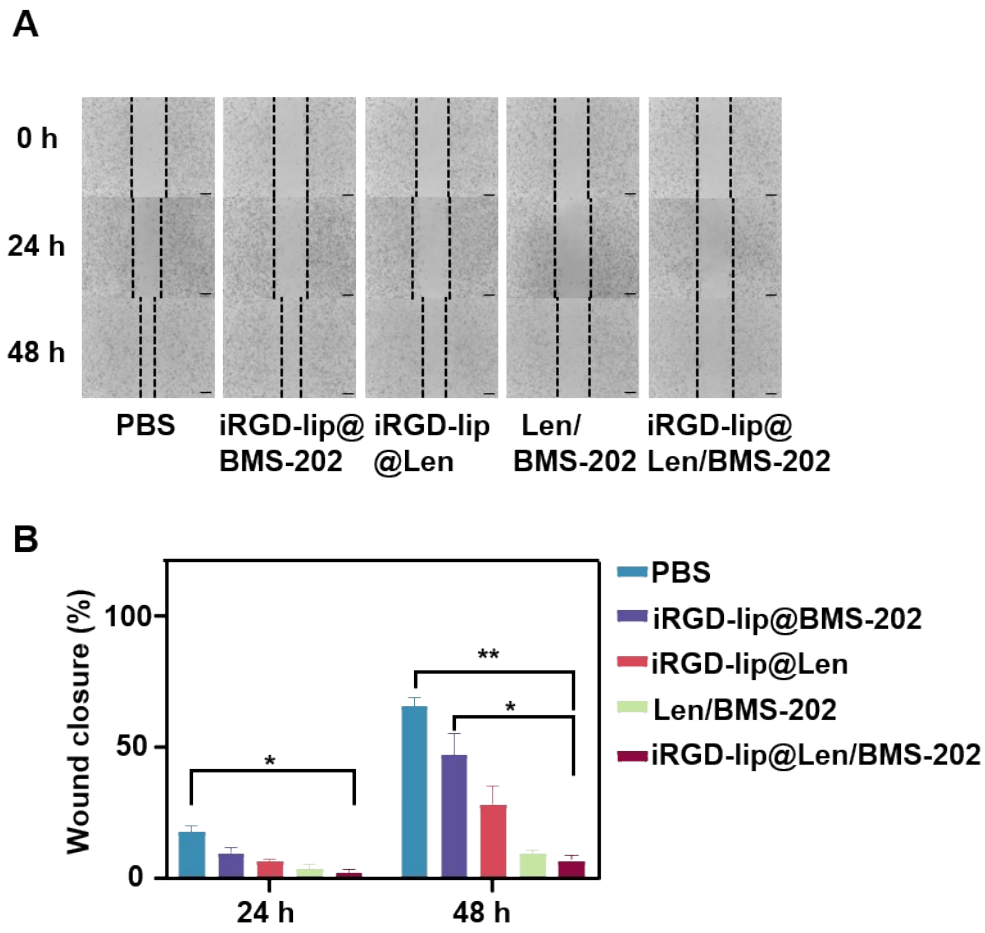


Fig. S5. (A) Scratch assay of Hepa1-6 cancer cells after treating with PBS, iRGD-lip@BMS-202, iRGD-lip@Len, Len/BMS-202, or iRGD-lip@Len/BMS-202 for different times, respectively (scale bar: 50 μm). (B) Images from Scratch assays were quantified with Image J software (n = 3).

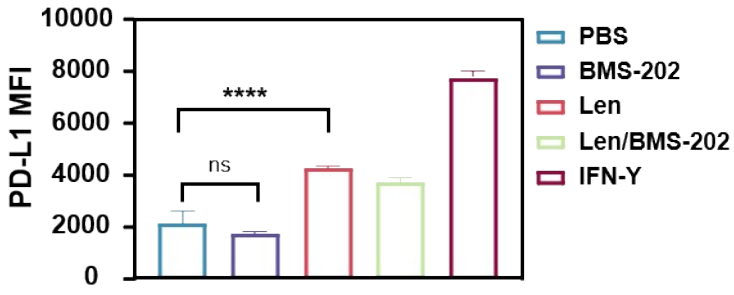


Fig. S6. Flow cytometric analysis of PD-L1 expression of Hepa1-6 cells after different treatments.

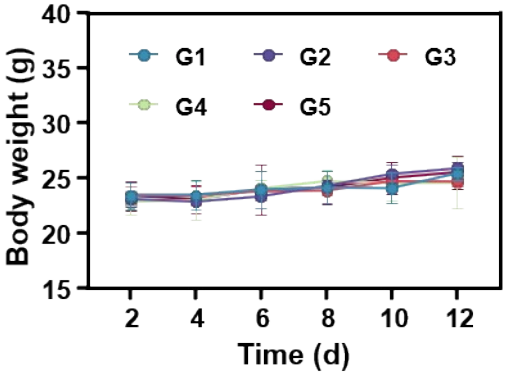


Fig. S7. Body weights of tumor-bearing mice receiving various treatments (n = 5).

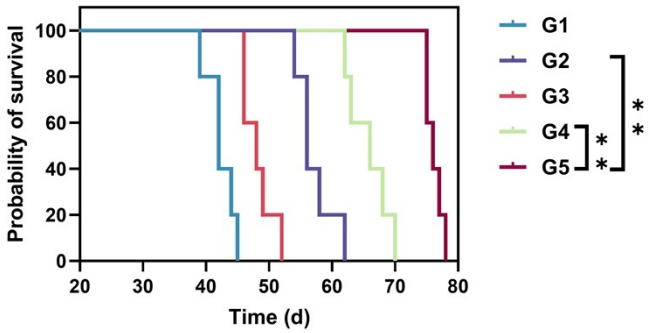


Fig. S8. The survival curve Hepa1-6 subcutaneous- bearing tumor mice during survival evaluation, (n = 5).

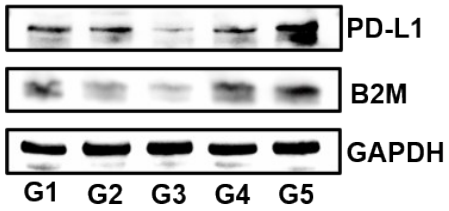


Fig. S9. Western bolt of the PD-L1 and B2M expression of Hepa1-6 subcutaneous tumor after different treatments.

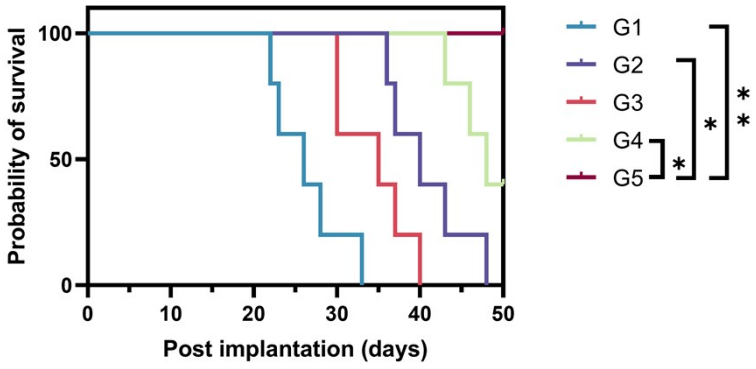


Fig. S10. The survival curves of orthotopic HCC-bearing mice during survival evaluation, n = 5.

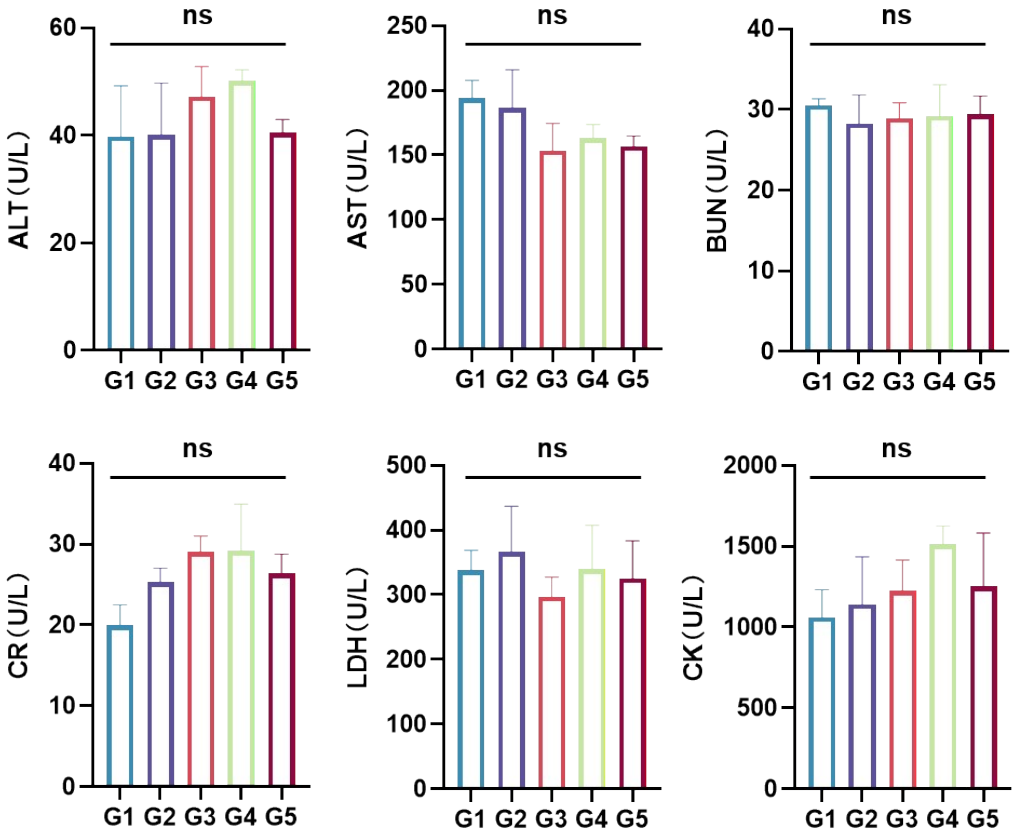


Fig. S11. Biochemical analysis after different treatments. Data are presented as mean ± SD (n=3). The statistical analysis was performed with ANOVA analysis, ns, not significant.

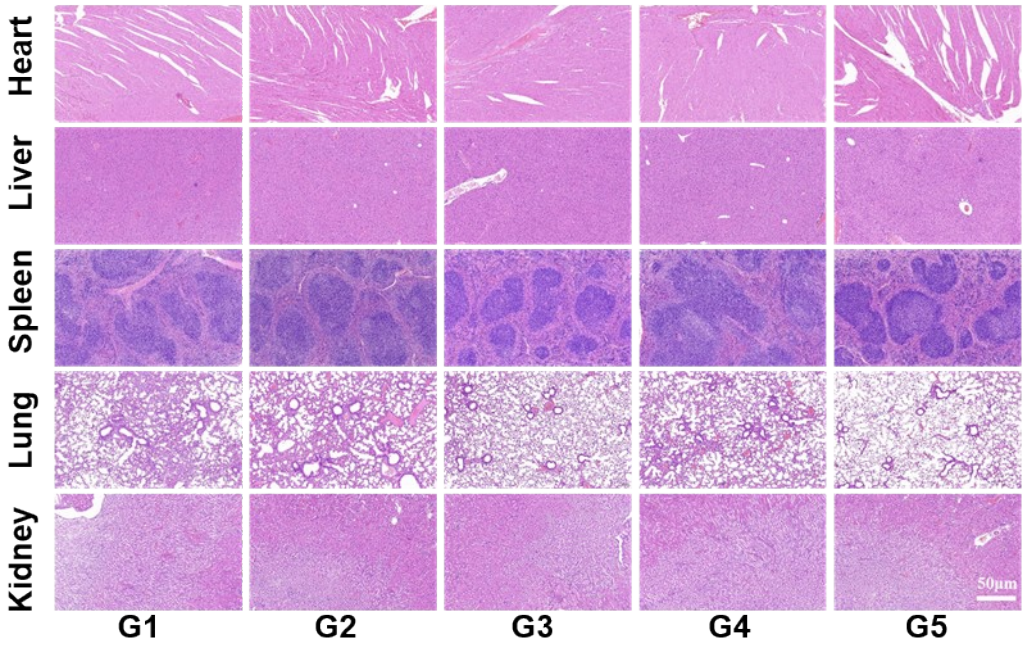


Fig. S12 Representative H&E images of major organs after different treatments.

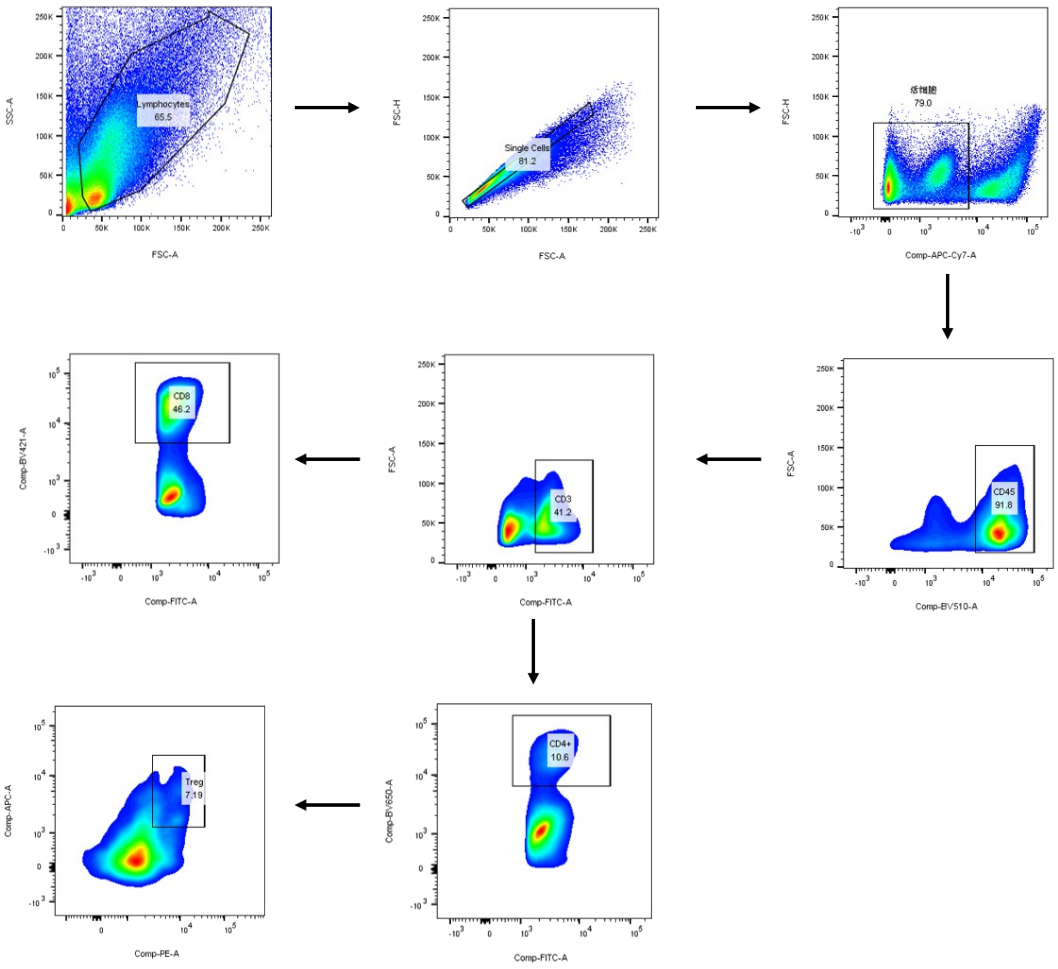


Fig. S13 Gating strategy to identify $CD45^+CD3^+CD4^+$ T cells, $CD45^+CD3^+CD8^+$ T

cells, and $CD45^+CD3^+CD4^+CD25^+$ T cells in tumor tissues. Cell populations were gated sequentially following arrows.

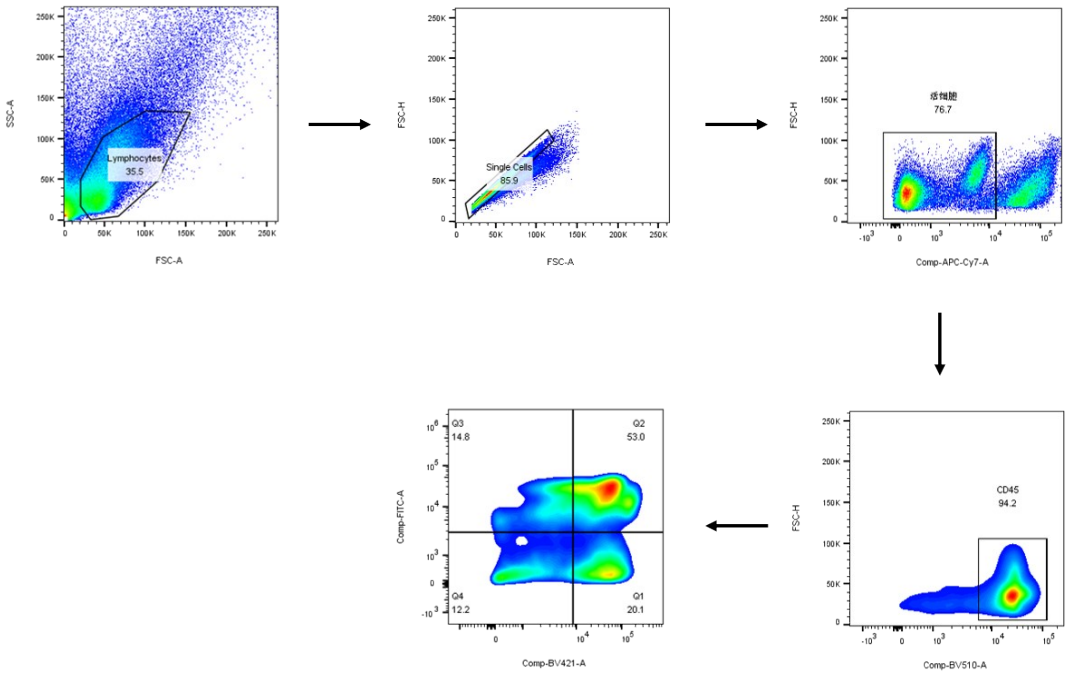
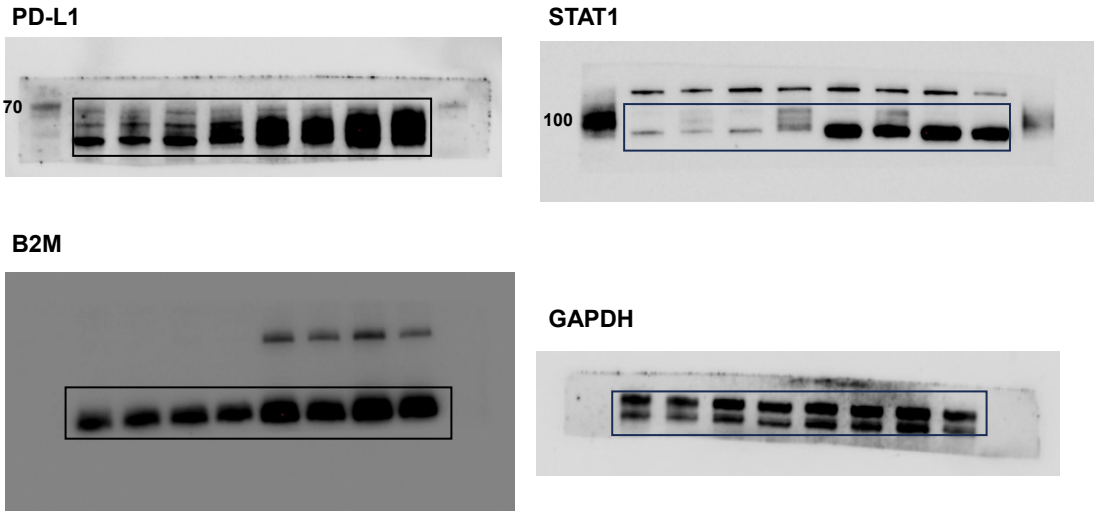
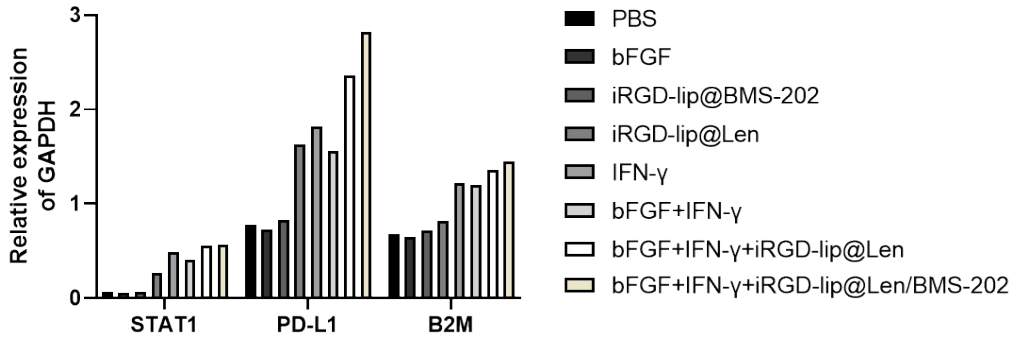


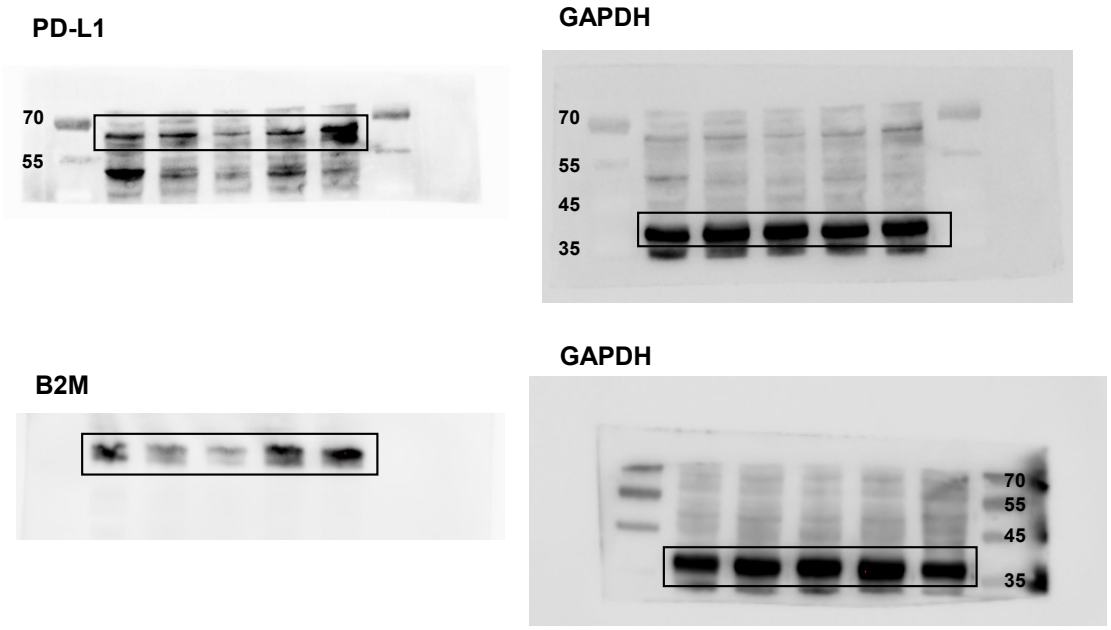
Fig. S14 Gating Strategy to identify $CD45^+Ly6C^+CD11b^+$ cells in tumor tissues. Cell populations were gated sequentially following arrows.

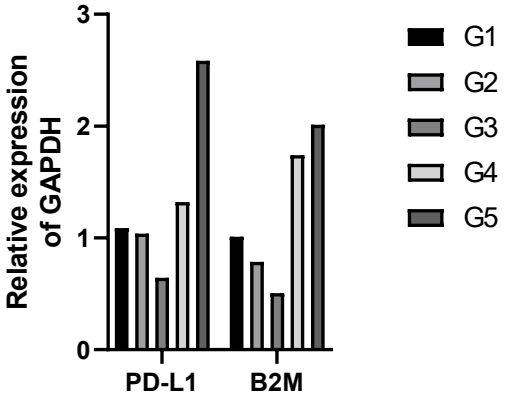
Raw data of Fig. 2I





Raw data of Fig. S9





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