Simultaneous blocking of CD47 and PD-L1 increases innate and adaptive cancer immune responses and cytokine release

Shu Lian a, Ruizhi Xie a, Yuying Ye b, Xiaodong Xie a, Shuhui Li a, Yusheng Lu a,c, Bifei Li a, Yunlong Cheng a, Vladimir L. Katanaev d,e, Lee Jia a,c,e

a Cancer Metastasis Alert and Prevention Center, College of Chemistry, Fujian Provincial Key Laboratory of Cancer Metastasis Chemoprevention and Chemotherapy, Fuzhou University, Fuzhou, China
b Fujian Provincial People’s Hospital Affiliated to Fujian University of Traditional Chinese Medicine, Fuzhou 350004, China
c Marine Drug R&D Center, Institute of Oceanography, Minjiang University, Fuzhou 350108, China
d Translational Research Center in Oncohaematology, Department of Cell Physiology and Metabolism, Faculty of Medicine, University of Geneva, Switzerland
e Head of the Natural Products Drug Discovery Laboratory, School of Biomedicine, Far Eastern Federal University, Vladivostok, Russia

Article history:
Received 12 December 2018
Received in revised form 6 March 2019
Accepted 7 March 2019
Available online 14 March 2019

Keywords:
CD47/SIRP-α
PD-L1/PD-1
siRNA
Immune therapy
Liposome
Gene therapy
EpCAM targeted

ABSTRACT

Background: Treatment multiple tumors by immune therapy can be achieved by mobilizing both innate and adaptive immunity. The programmed death ligand 1 (PD-L1; or CD274, B7-H1) is a critical “don’t find me” signal to the adaptive immune system. Equally CD47 is a critical “don’t eat me” signal to the innate immune system and a regulator of the adaptive immune response.

Method: Both of CD47 and PD-L1 are overexpressed on the surface of cancer cells to enable to escape immune-surveillance. We designed EpCAM (epithelial cell adhesion molecule)-targeted cationic liposome (LPP-P4-Ep) containing si-CD47 and si-PD-L1 could target high-EpCAM cancer cells and knockdown both CD47 and PD-L1 proteins.

Findings: Efficient silencing of CD47 and PD-L1 versus single gene silencing in vivo by systemic administration of LPP-P4-Ep could significantly inhibited the growth of solid tumors in subcutaneous and reduced lung metastasis in lung metastasis model. Target delivery of the complexes LPP-P4-Ep increased anti-tumor T cell and NK cell response, and release various cytokines including IFN-γ and IL-6 in vivo and in vitro.

Interpretation: This multi-nanoparticles showed significantly high-EpCAM tumor targeting and lower toxicity, and enhanced immune therapeutic efficacy. Our data indicated that dual-blockade tumor cell-specific innate and adaptive checkpoints represents an improved strategy for tumor immunotherapy.

Fund: This research supported by the Ministry of Science and Technology of the People’s Republic of China (grant number 2015CB931804); the National Natural Science Foundation of China (NSFC, grant numbers 81703555, U1505225 and 81773063), and the China Postdoctoral Science Foundation (grant number 2015M620268).

© 2019 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Cancer immunotherapy is now considered a pillar of cancer treatment, alongside surgery, chemotherapy, and radiation. The use of immunotherapy for cancer has become widespread in recent decades and is used to treat both solid and hematological malignances [1]. Recently emerging research targets include co-inhibitory and co-stimulatory markers of the innate and adaptive immune system [2]. Both the innate and adaptive immune system are critical to the efficacy of cytotoxic immune therapy [3,4].

PD-L1 (programmed death-ligand 1) is a member protein that is highly expressed on many tumor cells [5,6]. The programmed cell death protein 1 (PD-1), are receptors expressed on the surface of cytotoxic T-cells that interact with PD-L1 on APCs (antigen presenting cell), which helps the cancer cell evade T-cell-mediated death, and dampens anti-tumor adaptive immune responses. Immune checkpoint inhibitors prevent the receptors PD-1 and ligand PD-L1 from binding to each other, thereby disrupting signaling [7–9].

CD47, first identified as Integrin-Associated Protein (IAP) [10–13], is another cell-surface immunoglobulin that negatively regulates anti-tumor immunity through suppression of phagocytosis. CD47 transmits an inhibitory “don’t eat me” signal upon ligation with its receptor signal regulatory protein α (SIRPα), which is expressed primarily on phagocytic cells, including monocytes, macrophages, dendritic cells and neutrophils [14,15]. CD47-SIRPα axis has been explored its essential role...
2 Materials and methods

2.1 Materials

DOPE (Dioleoylphosphatidylethanolamine) was obtained from A.V.T (Shanghai) pharmaceutical Co, Ltd. DC-Chol (3β-[N-(N', N′-Dimethylaminoethane) carbamoyl] cholesterol) was purchased from abin bioscience Inc (Shanghai). MAL-PEG-COOH (Maleimide-(ethylene glycol)-carboxyl, average MW 3400 Da) was obtained from Shanghai Ponsure Bio, Inc. The high performance liquid chromatography (HPLC)-purified anti-EPgDNA aptamer (Ep, sequence: HS-5′-CAC TAC AGA GGA TGC TGT CCC ACC TTA TGG CCG GGT GGC CTG-3′, 48 bp, MW = 15 k Da) with or without Cy5 modification was synthesized by sangon Biotech Co., Ltd,(Shanghai, China). All siRNAs were dissolved or diluted to a concentration of 20 μM in diethyl pyrocarbonate (DEPC) water (Invitrogen) and listed in Table 1.

Anti-mouse CD45-FITC, anti-mouse CD3e-PE, anti-mouse CD8a-PerCP-Cy5.5, anti-mouse CD49b (Integrin alpha 2)-PE-Cy7, mouse anti-human CD74-FITC, mouse anti-human PD-L1-APC, anti-mouse CD47-FITC, anti-mouse PD-L1-PE and anti-mouse EpCAM-PE were obtained from eBioscience. Mouse anti-human CD47 antibody and rabbit anti-human PD-L1 antibody were obtained from Abcam. Human cytokerin ELISA kit IL-6, human cytokine ELISA kit IFN-γ, mouse cytokerin ELISA kit IL-6 and mouse cytokine ELISA kit IFN-γ were purchased from Lianke Biological Co., Ltd. All other unlabeled materials are of analytical grade. Anti-mouse PD-L1 antibody was obtained from Wanlei Biotechnology, Inc. Anti-mouse CD47 antibody was obtained from Abcam. Anti-α-defactin antibody was purchased from Beijing Dingguo Biotechnology, Inc.

2.2 Synthesis of MAL-PEG-DOPe

Synthesis of MAL-PEG-DOPe refers to previously published articles [22,23]. The conjugation of carboxyl groups of MAL-PEG-COOH to the amine groups of DOPE was accomplished using the EDC/NHS technique. The process was carried out as follows: 30 mg carboxyl-modified PEG was dissolved in dichloromethane and mixed with EDC (5 mg) and NHS (4 mg). The solution was stirred continuously for 2 h at room temperature. Subsequently, 8 mg DOPE (MAL-PEG-COOH: DOPE = 1:1, molar ratio) was added, and the reaction proceeded overnight under nitrogen. The reaction product was dried out most dichloromethane in rotary evaporator and then added to cold acetonitrile. The unreacted DOPE was centrifuged at 2414g for 10 min which was insoluble in cold acetonitrile. And the supernatant was dried to thin lipid in rotary evaporator. The film was hydrated with DD water. The reaction product was enclosed in dialysis bag (MW = 8 k Da) and transferred into 50 mL of DD water solution to separate free EDC/ NHS/ MAL-PEG-COOH at room temperature for 48 h. The final product DOPE-PEG-MAL was subsequently freeze-dried by lyophilizer. To confirm the DOPE-PG-MAL conjugation, the samples were examined by nuclear magnetic resonance spectroscopy.

| Cells            | CD74 | PD-L1 | EpCAM |
|------------------|------|-------|-------|
| Lung cancer cells| 99.5%| 84.3% | 99.5% |
| A549             | 99.9%| 99.3% | 10.6% |
| H1975            | 99.7%| 99.9% | 79.0% |
| He1f             | 5.7% | 3.83% | 1.18% |
| H1299            | 99.7%| 63.5% | 14.0% |
| SPAC-1           | 94.8%| 60.5% | 99.8% |
| Breast cancer    |      |       |       |
| MCF-7            | 99.6%| 9.47% | 81.6% |
| MDA-MB-231       | 38.2%| 9.37% | 99.3% |
| MDA-MB-435       | 98.5%| 14.5% | 99.8% |
| Mice cancer cells|      |       |       |
| 4 T1             | 99.7%| 78.2% | 98.8% |
| B16F10           | 99.7%| 92.4% | 43.7% |
| LLC              | 99.3%| 74.8% | 12%   |
| CT26             | 99.8%| 91.9% | 21.6% |

Note: positive rate ≤10% is not expressed; 10% ≤ positive rate ≤30% for low expression; 30% < positive rate ≤ 60% for medium expression positive rate >60% for high expression.
2.3. Preparation of black liposomes

The steps for the synthesis of liposomes were based on published articles [24], and with minor modifications [25,26]. Briefly, MAL-PEG-Dope, DOPE and DC-Chol at a molar ratio of 0.1: 1:1 (about 8 µmol total lipids) were dissolved in 10 mL dichloromethane and then the lysate were dried into thin lipid film in a rotary evaporator. The film was hydrated using DD water (LPP), After that, si-CD47 or/and si-PD-L1 and LPP complexes were gently mixed to form LPP-4 /LPP-P /LPP-P4 complexes. The LPP-4 /LPP-P /LPP-P4 complexes were formed by electrostatic interaction between positive (liposomes) and negative charges (siRNA). DC-Chol and DOPE were used to prepare liposome complexes (LP) with the similar process, except the MAL-PEG-Dope was not added. All liposomes are stored at 4 °C before use.

EpCam was combined with LPP using the method published by Wu [27]. Eight micromoles of liposomes with MAL-activated PEG-DOPE on the surface were incubated with HS-EpCam at a ratio of 10:1 for 24 h at 4 °C in darkness. Ultrafiltration was used to remove small molecular weight residues in LPP-Ep solution (50 k MWCO, Millipore, USA), and then the solution was resuspended in DD water. Cy5 modified LPP-Ep (LPP-Ep-Cy5) was prepared with the same process.

The uniform naming of synthetic materials: LPP-P4-Ep for liposome-PEG-EpCAM contained si-PD-L1 and si-CD47, LPP-Ep for liposome-PEG-EpCAM contained si-PD-L1, LPP-4-Ep for liposome-PEG-Ep-Cam contained si-CD47, LPP-Ep for liposome-PEG-Ep-Cam, LPP for liposome-PEG without aptamer, LP for liposome without any aptamer or PEG.

2.4. Characterization of LPP-Ep liposome

To confirm EP-CAM conjugation, LPP-Ep or EP-CAM free was analyzed by agarose electrophoresis refer to Cheng [30]. Samples loaded were as follows: 1, LPP-Ep; 2, EpCAM; 3, LPP; 4, mixture of EP-CAM and LPP; 5, 10 bp marker. The electrophoresis was managed for 30 min at 80 V, and then the gel was stained with Syber Gold for 30 min at room temperature in the darkness.

To calculate EP-CAM conjugation amount, the standard solutions of Ep-cy5 were analyzed by fluorescence spectrophotometer (F-7000, Hitachi, Varian Ltd., USA). The binding efficiency of EP-CAM-Cy5 with liposomes was detected by collecting the unbounded EP-CAM-Cy5 after ultrafiltration and calculating their conjugation concentration. Binding concentration = adding total concentration - unbounded concentration under ultrafiltration. (Cy5 under λ ex = 647 nm and λ em = 670 nm).

To evaluate the characteristics of the liposomal formulations, the mean particle size and zeta potential of a series of liposome complexes were measured using a Malvern Instruments Zetasizer HS III (Malvern UK) at room temperature. Each batch was analyzed in triplicate. Scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM) system (Bruker, USA) were used to detect the morphology of LPP-Ep.

Agarose gel electrophoresis was used to detect si-CD47 or si-PD-L1 loading ability of LPP-Ep. The 50 nmol si-CD47 or si-PD-L1/liposome complexes were added to agarose gel in TAE buffer. The N/P ratio of LPP-Ep/siRNA was 1, 2, 3, 5, 10, 20, 30. The electrophoresis was managed for 80 V for 30 min, and then the gel was stained with Syber Gold for 30 min at room temperature in the darkness.

To test the stability of siRNA, LPP-Ep containing siRNA was co-incubated with 1640, PBS and FBS. After different time, LPP-Ep was lysed using 2% heparin sodium. The stability ability of siRNA was tested by agarose gel.

In vitro si-CD47-FAM and si-PD-L1-Cy5 release experiments with LPP-P4 and LPP-Ep were performed in 1 mL PBS solution with 5.6 and 7.4 pH values (FAM under λ ex = 493 nm and λ em, = 520 nm; Cy5 under λ ex = 647 nm and λ em = 670 nm). The method was as following: 1 mL of LPP-P4 or LPP-P4-Ep solution was placed in a dialysis bag (MW = 50 k Da) and then immersed in 5.6 or 7.4 PBS solution. The process reaction was kept at 37 °C, 200 µl of each sample was collected at different time points and the total siRNA released from LPP/LPP-Ep complex was detected using fluorescence spectrophotometer at 493 nm and 647 nm and calculated the concentration through the standard curve. The release result was repeated three times and the results were expressed as mean ± SEM.

2.5. In vitro cell assays

The HELF, A549, PC-9, H1975, H1299, SPICA-1, MCF-7, MDA-MB-231, MDA-MB-435, 4 T1, B16F10, LLC, and CT26 cells were purchased from the Cell Resource Center of Shanghai Institute for Biological Sciences (Chinese Academy of Sciences, Shanghai, China). The PC-9, H1299, SPICA-1, MCF-7, 4 T1, B16F10, LLC, and A549 cells were cultured in 1640 RPMI medium containing 10% FBS and 1% penicill-streptomycin (v/v). The HELF, MDA-MB-231, MDA-MB-435 and H1975 cells were grown in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin (v/v). The cells were maintained in a humidified cell incubator at 37 °C with 5% CO2.

HELF, A549, PC-9, H1975, H1299, SPICA-1, MCF-7, MDA-MB-231 and MDA-MB-435 cells were respectively stained with mouse anti-human CD47 (FITC-labelled), mouse anti-human PD-L1 (CD274) (APC-labelled) and mouse anti-human CD326 (EpCAM) (PE-labelled) antibody and incubated at 4 °C for 30 min in the dark. B16F10, 4 T1, LLC, and CT26 cells were respectively stained with mouse anti-CD47 (FITC-labelled), anti-mouse PD-L1 (PE-labelled) and anti-mouse EpCAM (PE-labelled) antibody and incubated at 4 °C for 30 min in the dark. Background staining was performed by staining cells with isotype-matched control. After staining, cells were washed with the staining buffer (2% FBS, 2 mM EDTA in PBS) and resuspended in 500 µL of the staining buffer. Flow cytometric analysis was carried out on the BD FACSariaII (BD Biosciences), and the obtained data were analyzed with Flowjo software.

To analysis the apt-EP-CAM-mediated targeting, confocal cytometry and flow cytometry were used to detect a binding specificity assay on A549, PC-9 and HELF cells. EP-CAM-negative cells HELF were used as control. HELF, PC-9 and A549 cells were seeded in 6-well plates with 10% FBS medium in flow cytometry analysis. After 24 h, cells were incubated with 1640 medium containing 1, 10, 25, 50, 100 nM Cy5-Ep-CAM for 4 h or fresh medium containing 50 nM Cy5-Ep-CAM for 1, 2, 4, 6, 8 h. After washing with PBS, cells were digested and then detected by flow cytometry.

For quantitative comparison of siRNA uptake efficiency, FAM-siCD47 and Cy5-siPD-L1 mean on PC-9, A549, HELF and 4 T1 cells were measured by confocal imaging and flow cytometry. Briefly, 5 × 10⁴ cells were seeded into 6 well plates and incubated for 24 h. Each cells were incubated with 1640 medium containing 1, 10, 25, 50, 100 nM Cy5-Ep-CAM for 4 h or fresh medium containing 50 nM Cy5-Ep-CAM for 1, 2, 4, 6, 8 h. After washing with PBS, cells were digested and then detected by flow cytometry.

To prove the silencing efficiency of CD47 and PD-L1 proteins in PC-9 and A549, HELF and 4 T1 cells were transfected with different formulation, including LPP-NC-Ep, LPP-4-Ep, LPP-P-Ep and LPP-P4-Ep with PBS free medium at different dose of siRNA for 6 h. And then cells were harvested with normal medium for another 48 h or 72 h and washed three times with PBS. Detection step refers to step 2.5.2. The inhibition of EP-CAM was also detected by flow cytometry. Detection step refers to step 2.5.2. PC-9 cells were also incubation with free aptamer EP-CAM at different concentrations for 24 h, the expression of EP-CAM was detected by flow cytometry.
Cytotoxicity of LP, LPP, LPP-Ep was assayed by MTT assay in HELF, A549, PC-9 and 4 T1 cell lines. The 10,000 cells were seeded into 96-well plates for per well. After 24 h, medium with LP, LPP, LPP-Ep complexes at 50 nM concentration siRNA was added. After continued incubation for 48 h, the cytotoxicity effect for different complexes was assessed by the MTT assay. Meanwhile, PC-9 cells were incubated with LPP-Ep, LPP-4-Ep, LPP-P-Ep and LPP-P4-Ep complexes at a dose of 50 nM siRNA concentration without changing culture medium. The absorbance at 490 nm was determined by a microplate reader. Normal cultured cells served as control group. All samples were repeated 5 times.

A flow cytometric, target-based cytotoxicity assay was conducted according to methods previously published [28,29]. Peripheral blood mononuclear cells (PBMCs) from anonymous healthy donors were obtained from Fujian Provincial People’s Hospital Affiliated to Fujian University of Traditional Chinese Medicine (Fuzhou, China). Monocytes were isolated from Ficoll-Isoopaque density centrifugation. The samples were stored at 4 °C and processed within 6 h after their collection. Target cells PC-9 were labelled with 3 μM CFSE (from a 1 mM stock solution in dimethyl sulfoxide) in phosphate-buffered saline (PBS) for 15 min at 37 °C in a volume of 1 mL and then washed twice with PBS. Labelled cells were immediately seeded in 6-well culture plates at a density of 3 × 10^5 cells/well for 24 h. After transfection by PBS free medium with LPP-Ep, LPP-NC-Ep, LPP-4-Ep, LPP-P-Ep and LPP-P4-Ep liposomes complexes (50 nM), the CFSE labelled-PC-9 were mixed with effect cells monocytes at T: E ratios 1:10. In parallel, PC-9 cells incubated monocytes alone was measure as basal apoptosis. Cells were incubated for 36 h at 37 °C in a humidified atmosphere of 5% CO₂ and then harvested with 0.25% trypsin (GenView). Cells were washed in PBS-1% FBS and incubated with 500 μL of PI (1 μg/mL) for 15 min at 37 °C in the dark before determined. CFSE-labelled cells can distinguish the target cells and effect cells. While PI (Propidium iodide) was added to determine the ratio of cell death. Flow cytometric analysis data were collected on the BD FACSAria III, and the obtained data were analyzed with FlowJo software.

For cytokes detection, 10^5 cancer cells were plated in 6-well round-bottom microplates for per well and incubated overnight. Cells were treated by LPP-Ep, LPP-NC-Ep, LPP-4-Ep, LPP-P-Ep and LPP-P4-Ep liposomes complexes. The original medium was removed, and fresh medium or medium containing monocytes was added to the wells at an E/T of 10:1. The cancer cells and monocytes were incubated in an incubator for 24 h. ELISA was performed on the supernatants using a human cytokine ELISA kit (Lianke) to quantify IFN-γ and IL-6 secretion.

2.6. Tumor growth inhibition in vivo

Cells 4 T1 were harvested and suspended in PBS with concentration 5 × 10^6 cells/100 μL. Balb/C mice were inoculated with 4 T1 cells by subcutaneously injecting 5 × 10^5 4 T1 cells in 100 μL PBS on the hind legs. When the tumor volumes reached 100 mm^3, Balb/C mice were treated by LPP-P4-Ep complexes from 14th day after inoculation. LPP-Eps containing 8 μg CD47 or/and PD-1 or control siRNA (about 0.4 mg/kg) were subcutaneously injected every two day for a totally of 5 treatment. Digital caliper was used to calculate tumor size. The tumor volume formula was (1/2 × length × width [2]).

To detect biodistribution of LPP-Ep in 4 T1 model mice, Balb/C mice were injected with PBS (control) containing LPP linking Cy5-EpCAM (0.4 mg/kg) via subcutaneous injection. After 2, 4 and 12 h, mice were executed. The tissue samples (tumor, heart, liver, spleen, lung and kidney) were collected. Similarly, part of tissue samples were prepared by a freezing microtome in 7 μm. Nucleus was stained with Hoechst 33258 and florescence intensity of Cy5 was observed by confocal microscope.

The tissues including heart, liver, spleen, lung, kidney and tumor were executed. And then they were dehydrated, embedded in paraffin, and stained with hematoxylin and eosin (H&E). Histological observations were examined by a microscope (Zeiss, Germany). Before soaking with formaldehyde, the weight of the spleen s is weighed with an electronic balance. Spleen size was measured with digital caliper. After the sacrifice of the mice, six groups of tumors mRNA and proteins were collected. The method of RT-PCR referred to Cheng [30] to detect mRNA and the method of western blot referred to Lian [31] before to detect CD47, PD-L1 and β-actin proteins.

Immunofluorescence assay of CD47 and PD-L1 in tumor tissues was executed using paraffin sections. Tissues were sliced into 4.5 μm and blocked by 5% BSA for 2 h, and then incubated with anti-CD47/anti-PD-L1 antibodies (Abcam) overnight at 4 °C. After that, the slides were incubated with FITC-labelled goat anti-rabbit secondary antibody, and then washed with PBS and stained with Hoechst 33258.

Mice blood samples were collected from the mice eyes with the capillaries. Fifty microliter of mice blood was drawn from each mice and collected in 1.5 mL EP tube containing ethylenediaminetetraacetic acid. Cells were incubated with anti-mouse CD19a, anti-mouse CD3, anti- mouse CD45, anti- mouse CD8a and anti- mouse CD49b for 30 min at 4 °C in the dark. Ten-fold volume of red blood cell lysis buffer was then added to the blood to remove the red blood cells (RBCs). Cell suspensions were pooled and centrifuged 300 g for 5 min at room temperature. Cells were washed with PBS-1% FBS for twice before determined. Flow cytometric analysis data were collected on the BD FACSAria III (BD Biosciences), and the obtained data were analyzed with FlowJo software.

After the final administration for 2 days mice were executed and tumors were separated for ELISA analysis. The tumor was lysed and the solution was collected. ELISA was performed on the solution using a mouse cytokine ELISA kit (Lianke) to quantify IFN-γ and IL-6 secretion. The cytokines of the blood were determined by collecting supernatant before the mice were sacrificed. Mouse blood samples were collected from the mice eyes with the capillaries. All samples, kit controls and standards were analyzed in triplicate.

2.7. Metastasis inhibition assay invivo

PBS containing 1 × 10^5 4 T1 cells/100 μL was injected to Balb/C via tail vein to build metastasis model. LPP-Ep containing 8 μg si-NC or si-CD47 or/and si-PD-L1 (0.4 mg/kg for each) were injected to each mice through tail. Injection begins on the 14th day of inoculation and is given every 3 days. Mice were sacrificed and tissues were harvested on day 39 after inoculation.

The tissue distribution in metastasis model measurement method was consistent with subcutaneous tumor model referring to Method 2.6. The mouse blood was collected from 10 days after injection different LPP-Ep complexes. And immune cells collection method is consistent with Method 2.6. CBA assay was executed to detect the cytokines of IL-10, IL-17A, TNF, IFN-γ, IL-6, IL-4 and IL-2. The procedure was described as followed: mice blood samples were collected from the mice eyes with the capillaries. Fifty microliter of mouse blood was drawn from each mice and collected in 1.5 mL EP tube containing ethylenediaminetetraacetic acid. The supernatant was centrifuged through 300 g for 10 min. Detection steps refer to CBA instructions.

Histopathology analysis method refers to method 2.6. After treatment, whole blood collected from the mice were assayed for hematol- ogy parameters. Tissues including heart, liver, spleen, lung and kidney were collected for hematoxylin and eosin assay.

2.8. Ethics statement

All animal experiments were approved by the Institutional Animal Care and Use Committee of Fuzhou University and operated following the NSFC regulations concerning the care and use of experimental animals. Balb/C female mice (about 20 g weight, 4–6 weeks old) were obtained from Fuzhou Wushi Animal Center. The healthy human blood collection procedure was carried out in accordance with the guidelines.
verified and approved by Fujian Provincial People’s Hospital Affiliated. All donors signed an informed consent for scientific research statement.

2.9. Statistical analysis

Graphpad Prism 5.0 (Graphpad software, San Diego, CA) was used for all statistical analysis. The mean ± S.E.M. was determined for each treatment group in the individual experiments, and the standard t-test was used to determine the significances between treatment and control group(s). P-values < 0.05 were significant. Statistical analyses were performed with the SPSS statistical software package.

3. Results

3.1. Synthesis of LP, LPP and LPP-Ep

Firstly, DC-Chol, DOPE and MAL-PEG-DOPE were prepared for liposome LP and then aptamer EpCAM that specifically targets high-EP-CAM tumor cells were conjugated onto the surface of liposomes. The synthetic procedures for LP, LPP and LPP-Ep are showed in Fig. 1. Firstly, DOPE was connected to MAL-PEG-COOH by carboxyl and amino reactions. The primary amine groups of DOPE were covalently conjugated to carboxyl of MAL-PEG-COOH by EDC/NHS catalysis to generate the MAL-PEG-DOPE conjugate. The conjugation synthesis of MAL-PEG-DOPE was confirmed by Nuclear Magnetic Resonance (NMR), which was showed in Fig. S1. The characteristic peaks of DOPE showed the succeed synthesis of DOPE-PEG- MAL.

After successful synthesis of MAL-PEG-DOPE, HS-EpCam with thiol group was designed to react with MAL-PEG-DOPE containing maleimide groups at buffer solution. To confirm the conjugation of aptamer HS-EpCAM to LPP, agarose electrophoresis was run (Fig. 2a). Since aptamer EpCAM showed negative charges, LPP displayed positive charges. Aptamer EpCAM alone showed a band at the small molecular weight (Fig. 2a, line 2). When aptamer EpCam was covalently conjugated with LPP, a smeared band at the opposite loading site appeared (Fig. 2a, line 1). LPP itself did not show any band (Fig. 2a, line 3). The immediate mixture of aptamer EpCAM and LPP showed band with the same band result of LPP-Ep (Fig. 2a, line 4), indicating that the reaction was very fast. These data indicated that aptamer EpCam could strongly and rapidly connect with the surface of LPP. Besides, the connection amount between LPP and EP-CAM was also determined by fluorescence spectrometer which was about 200 μM/mL (Fig. 2b).

The synthesis steps of LPP-Ep were shown in the Method [24]. A range size and potential of liposome complexes were analyzed by DLS. All liposomes have a particle size of about 120–175 nm and a uniform particle size and positive zeta potential (Fig. 2 and Table S2). The diameters and the size distributions of LPP-Ep, LPP-P-Ep/LPP-4-Ep and LPP-P4-Ep measured by DLS showed only a slight increase after single or dual siRNA loading. The results of LPP-P4-Ep observed from TEM, SEM and AFM was also about 175 nm, which consisted to DLS data (Fig. 2c-e). The zeta potential of LPP-P4-Ep decreased to 31.7 mV showed that the biological toxicity would be reduced due to the decrease of strong positive charges [32] (Table S2).

To evaluate the dual siRNA release behavior from LPP and LPP-Ep, and LPP and LPP-Ep were incubated in pH 5.6 and 7.4, respectively, and the released FAM-siCD47 and Cy5-si-PD-L1 were analyzed at the setting time (Fig. 2g). In pH 5.6 and 7.4 conditions, the release rates of total siRNA from LPP and LPP-Ep were relatively slow and then reached a stable state after 8 h. The release percentage of total siRNA in pH 7.4 only was 10%, which was half of release percentage in pH 5.6. The standard curve lines of FAM-siCD47 and Cy5-siPD-L1 were listed in Fig. S3.

The optimal N/P ratio about siRNA and LPP-Ep is also discussed by a gel retardation assay. The N/P ratios from 1:1 to 5:1 appeared obviously bands which was agreed with the single nucleic acid bands, however, the band disappeared when the N/P ratio was up to 10:1 (Fig. S2). These results provided an optional and suitable N/P ratio between siRNA and LPP-Ep in vitro and in vivo for future biological experiments. LPP-Ep containing siRNA in 1640 medium, PBS and FBS for 24 h still maintained high concentration (Fig. S3). LPP-Ep could keep siRNA stable.

3.2. High expression of three proteins and effective cellular uptake

The expression levels of CD47, PD-L1 and EpCAM were confirmed by Flow cytometry. As show in Table 1, the expression of CD47 and PD-L1 on lung cancer cells was generally higher than that of breast cancer cells. The expression percentage of EpCAM in PC-9 cells was about 99.9%, the expression percentage of EpCAM in HELF cells was about 1.18%, and the expression percentage of EpCAM in A549 cells was about 10.6%. Based on the results, PC-9 cells were selected as experimental positive cells, and HELF cells were chosen as negative cells for experiments.

Flow cytometry was used to detect the binding ability of the aptamer EpCam to recognize EP-CAM over-expressed PC-9 cells. In Fig. 3a-b, after incubation with Cy5-EpCam aptamer, PC-9 fluorescence mean intensity was significantly higher than that of control. Meanwhile, low Cy5-fluorescence intensities were measured in the EpCam-negative HELF and A549 cells.

LPP-P4-Ep cell uptake on PC-9, A549 and Helf cells was detected by confocal laser scanning microscopy and flow cytometry. LPP and LPP-Ep loaded with FAM-siCD47 and Cy5-siPD-L1 were incubated with EpCam-positive PC-9 cells, EpCAM-low A549 and EpCAM-negative HELF cells at 37 °C for 4 h. After transfection with fluorescent labelled LPP-P4-Ep, PC-9 cells showed strong green and red fluorescence, low fluorescence on A549 cells and almost no fluorescence on HELF cells. (Fig. 3). Therefore, in the following experiments, PC-9 cells were selected as target cells, because they were highly expressed in CD47 and PD-L1 and could be effectively recognized by aptamer EpCAM.
3.3. LPP-P4-Ep cytotoxicity assays, cells gene silencing and co-incubation toxicity

It is commonly believed the relation to an electrostatic interaction between the positive charge of cationic liposomes and the negatively charged glycoalyx of endothelial cells. In vitro cytotoxicity of blank cationic liposomes and liposomes encapsulated CD47 and PD-L1 siRNA was determined using the MTT assay [33]. After incubation with LP, LPP and LPP-Ep complexes for 24 h, the survival rate of 4 T1 cancer cells and normal cells HELF maintained nearly 100%, but there were slightly toxic to PC-9 cells. (Fig. 4a). However, after being incubation with LPP-Ep contained CD47 and PD-L1 dual siRNA, the cells viability maintained nearly 95% for PC-9 cells (Fig. 4b). These results indicated that LPP-P4-Ep complexes were less cytotoxic to PC-9 cells, 4 T1 cells and HELF cells.

The inhibition ability of LPP-P4-Ep in functional CD47 and PD-L1 siRNA was confirmed by dual protein expressions in PC-9 cells (Fig. 4c-h). Both LPP and LPP-Ep contained target siRNA can effectively interfere with the proteins expression of CD47 and PD-L1, and the LPP-Ep group has obvious interference effects than LPP group (Fig. 4c-d). Compared to negative control, CD47 protein expression decreased at 48 h and reduced significantly till 72 h after transfection with LPP-4-Ep (si-CD47 at a dose of 25 nM). In addition, there was no significant change in the expression level of CD47 protein treated with LPP-NC-Ep or si-CD47 alone. The inhibitory result of PD-L1 protein is the same as that of CD47 (Fig. 4g-h). These results implied that LPP-P4-Ep can effectively inhibit CD47 and PD-L1 proteins expression through RNAi regulation. Besides, the expression of EpCAM on PC-9 cells was detected by flow cytometry which was inhibited by aptamer EpCAM and decreased gradually with the increase concentration of EpCAM (Fig. 4i). After confirming the interference effect of LPP-P4-Ep, monocytes were added to the PC-9 cells at a ratio of 10:1. After 36 h all cells were collected and stained with PI. Data were collected regarding CFSE-positive, PI-positive, and double-positive events (Fig. 4j), the last events interpreted as immune effect between monocyte effectors and tumor cell targets and shown in the column diagram (Fig. 4k). PC-9 cells transfected by either CD47 or PD-L1 groups showed strongly toxicity compared to the control group. It is exciting that double RNA interference cells showed lower survival rate compared with single RNA interference. These results indicated that CD47 coordination with PD-L1 may have more effective immunotherapy effect.

3.4. Systemic delivery of CD47 and PD-L1 siRNA in LPP-NPs inhibited solid tumor model

After confirming that CD47 and PD-L1 silencing could induce immune activation in vitro, we next evaluated the effect of systemic delivery of CD47/PD-L1 siRNA in Balb/c mice. First, in vitro target function and inhibition CD47 and PD-L1 proteins efficient of LPP-P4-Ep on 4 T1 were also determined in the same methods as PC-9 cells (Fig. S6). The results indicated that CD47 and PD-L1 proteins could effectively inhibited by LPP-P4-Ep.

In left leg of Balb/c mice were injected with 5×10⁵ 4 T1 cells. The mice received subcutaneous injections of LPP-P4-Ep every two days for a total of six injections. As was reported by Chono et al. [34], LPP-NPs loaded siRNA was minimally immune-stimulatory over a broad
range of dose (0.15–1.2 mg/kg). As shown in Fig. 5a, there was no significant difference in body weight between the six groups, indicating the biocompatibility and safety of LPP-Ep complex in vivo. In terms of tumor growth, the delivery of CD47 and PD-L1 siRNA in an LPP-P4-Ep formulation can effectively inhibit solid tumor growth. After treatment, the volume of tumors decreased by 87% (P<0.001) compared with the untreated group. And the tumor volume treated by dual siRNA was reduced by 49% and 52% respectively compared with the use of siRNA CD47 or siRNA PD-L1 alone (Fig. 5b and d). Anti-CD47/PD-L1 antibody immunofluorescence assay was used to detect CD47/PD-L1 protein knockout level in tumor tissues. Fluorescence images, mRNA data and western blot results (Fig. 5c and Fig. S7) showed that CD47 and PD-L1
were effectively silenced after LPP-P4-Ep treatment. Besides, spleen weight decreased slightly in each group, but there were no significant difference (Fig. 5e-f).

The distributions of LPP-Ep-Cy5 on heart, liver, spleen, lung, kidney and tumor were determined by confocal microscope at 2, 4 and 12 h. As shown in Fig. 6a, LPP-Ep showed relatively lower distribution in heart and kidney during 12 h. And LPP-Ep showed relatively high distribution in liver and lung in 2 h, and then reduced rapidly after 4 h. The accumulation of LPP-Ep in the spleen and tumor was significantly lower at 4 h but enriched at 12 h. These results demonstrated that LPP-Ep containing siRNA could be effectively delivered to tumor.

The IFN-γ and IL-6 cytokines in tumors and blood were determined by ELISA. We found that the dual siRNA treatment LPP-P4-Ep group mice contained significantly higher levels of both IFN-γ and IL-6 than those of the PBS control mice or single siRNA treatment mice (Fig. 6e-f). As shown in Fig. S8. a-b, the percentage of NK cells slightly increased in LPP-P4-Ep group compared to other group. These results indicated that immunization with dual checkpoints inhibition induced immune
Fig. 5. In vivo antitumor effects of LPP-P4-Ep for solid tumor. (a) Mice weight changes. Each data point was represented as mean ± SEM, n = 6. (b) Tumor size changes of 4 T1 model mice after treated with PBS, LPP-Ep, LPP-NC-Ep, LPP-4-Ep, LPP-P-Ep and LPP-P4-Ep. *P < 0.05, **P < 0.01, ***P < 0.001 compared to the control group by ANOVA test. (c) Immunofluorescence images of CD47 and PD-L1 on tumor tissue. The nucleus was stained with Hoechst 33258. (d) Collecting tumor tissue after administration. (e-f) The spleens were isolated and weighted, and spleen weight index were calculated as organ weight (milligram, mg) per gram (g) of mouse body weight. All error bars expressed as mean ± SEM (n = 3).
response that cellular immunity and cytokines secretion may further promote NK cell immune response and antibody production.

The H&E staining of heart, liver, spleen, lung, kidney and tumor was conducted to analyze the toxicity of LPP-Ep complexes in vivo. Significant metastases can be seen in each group lung tissue except LPP-P4-Ep group. Normal tissues, such as heart, liver, spleen, lung, kidney and tumor were shown in Fig. 7. Histological features of control group mouse showed normal structures. Moreover, main viscera tissues including heart, liver, spleen and kidney show no remarkable histopathological abnormalities or lesions in LPP-Ep treated group. However, compared to the other four therapeutic groups, the PBS and LPP-Ep group appeared much more hypercellular and meanwhile exhibited higher level of unclear polymorphism in H&E strained lung and tumor sections (Fig. 7b). Moreover, tumor tissue from the LPP-P4-Ep group showed the lowest cancer cell density.

3.5. Systemic delivery of LPP-NPs containing si-CD47 or si-PD-L1 to suppress tumor metastasis model

In order to evaluate whether the targeted delivery of si-CD47 and si-PD-L1 to cancer cells can be therapeutically applied to combat the homing of circulating cells in the lung, we used a well-established 4 T1 metastasis model, which spontaneously generated lung metastasis [35]. LPP-Ep containing CD47 or/and PD-L1 was injected into Balb/c animals every 3 days, 6 times (dose = 0.4 mg/kg) from 2 days after inoculation. As shown in Fig. 8a, there was no significant difference in body weight among six groups, indicating that tail vein injection of these liposome complexes was also safe and biocompatible with subcutaneous injection. And lung micro-metastasis was significantly lower in the LPP-P4-Ep group than in the untreated group or the LPP-P-Ep/LPP-4-Ep group. (Fig. 8b-c). The results demonstrated that LPP-P4-Ep could significantly decrease tumor metastasis, ~85% of that of the untreated control. The distributions of LPP-P4-Ep in tissues were detected in fluorescence signal of Cy5-EpCAM at 2, 4 and 12 h by fluorescence spectrophotometer (Fig. 8d) and confocal microscopy (Fig. 8e). The accumulation of LPP-P4-Ep in heart, liver, spleen, lung and kidney reached the maximum values at 4 h, and decreased significantly at 12 h, which may due to the metabolism in blood of mice. The results revealed that LPP-P4-Ep (red) were still accumulated in the lungs after 12 h.

The histological analysis of the lung tissues showed fewer and smaller lung micro-metastases observed in LPP-P4-Ep-treated animals, compared to the LPP-P-Ep-treated/ LPP-4-Ep-treated animals (Fig. 9a). H&E results were consistent with the metastasis number results. Checkpoints stimulation can change local cytokine microenvironment that will in turn influence the differentiation of T helpers, whose appropriate reaction is essential to the development and function of B cells. Therefore, we assessed the percentage of T cells, NK cells, NKT cells and the cytokine profile in mice blood. The cytokines in blood were determined
by CBA assay. The LPP-P4-Ep group mice maintained higher level in T cells (Fig. 9b) compared to the control, but among the six group the percentage of NK cells and NKT cells maintained the same levels (Fig. 9c-d). The LPP-P4-Ep group mice also contained significantly higher levels of immune cytokines including IL-10, IFN-γ, IL-4 and IL-6 than those of the PBS control mice (Fig. 9f-l), but no significant different in TNF, IL-17A and IL-2 cytokines. These results indicated that cellular immunity and cytokine secretion induced by blockade CD47 and PD-L1 would further promote T cell differentiation and mixed cytokines immune responses. The detection method of different cytokines was showed in Fig. 9e. The standard curve lines of IL-10, IFN-γ, TNF, IL-17A, IL-4, IL-1 and IL-6 were listed in Fig. S9.

3.6. Systemic delivery of si-CD47 or/and si-PD-L1 in LPP-Ep caused no anemia in blood

Systemic administration can cause a certain degree of side effects, and one of the primary goals of pharmaceutical science is to reduce undesirable side effects by improving the pharmacokinetic profile and biodistribution of the therapeutic agent. To evaluate whether systemic administration of si-CD47 or/and si-PD-L1 produced any side effects to circulating hematopoietic cells, a hematology examination was performed on the blood samples collected after animals receiving injections of LPP-P4-Ep for 6 injections. The WBC, HCT, PLT, RBC and HGB cell counts of LPP-P4-Ep treated mice were not significantly different from the control groups (Fig. 10). These results indicated that LPP-P4-Ep was the safety and effective gene delivery for the treatment of cancer.

4. Discussion

High invasiveness and metastasis of each kind of tumor requires the cells to be able to survive and evade immune system once they are detached from the immunosuppressive primary microenvironment and until they establish a metastatic site. PD-L1 is a transmembrane protein involved in the immune system suppression [36]. CD47 is a receptor that is ubiquitously expressed on cell surfaces; it conveys a “self” signal to the macrophages and T cells to limit clearance from the system [16,37]. CD47 and PD-L1 serve as critical innate and adaptive checkpoints, respectively, which are critical to the immune system. The expression of CD47 and PD-L1 was elevated in various cancer cells including lung cancer cells, breast cancer cells, melanoma cells [38] and esophageal cancer [39], but except normal cells (Table 1). Si-CD47 and si-PD-L1 co-loaded LPP-P4-Ep complexes were modified by aptamer EpCAM (Figs. 1-2 and Fig.S1). The enhanced cancer targeting (Fig. 3), increased down-regulation of immune-related proteins...
expression, and induced cancer cells apoptosis with immune cells obviously demonstrated the effectiveness of LPP-P4-Ep in high CD47 and PD-L1 expression immune evasion cells (Fig. 4). In Fig. 4, the CD47 and PD-L1 proteins inhibition by siRNA are small, but there are statistical differences among each group. After incubating CD47-inhibited and PD-L1-inhibited cells with monocyte cells, IFN-γ and IL-6 secreted by monocyte cells were highest (Fig. S4), which are critical to CD47/SIPR-α [40] and PD-L1/PD-1 [41] axis immune response.

In vitro data demonstrated that inhibition of CD47 and PD-L1 proteins induced immune cell activation and cytokine secretion, and decreased tumor growth and tumor metastasis. This reduction was observed in 4T1 subcutaneous models (Figs. 5-7 and Figs. S6-7) and
4 T1 metastasis models (Fig. 8), when the immune-associated proteins were blocked by injection of LPP-PE-Ep. After injected with LPP-P4-Ep, in subcutaneous models IFN-γ and IL-6 on mice blood and tumors were significant higher. And in metastasis model (Fig. 9) the secretion of IL-10, IFN-γ, IL-6 and IL-4 cytokines on blood were higher, too. These cytokines IL-10, IFN-γ, IL-6 and IL-4 are critical to immune response, specially IFN-γ is essential for anti-tumor response, stimulates the development of cytotoxic T lymphocytes, improves antigen presentation and many more [42].

Interestingly, there are few reports on the simultaneous targeting of CD47 and PD-L1, and most of the physiological activity on CD47 or PD-L1 is studied by using monoclonal antibodies, which was just mechanically injected into the body. The LPP-Eps accumulated in the proximity of the cancer cells (Figs. 6a and 8d-e), prolonged half-life and controlled the tropism of therapeutics [43]. In our research, even though repeated injections of the LPP-Ep loading si-CD47 or/and si-PD-L1 effectively inhibited tumor growth while maintaining blood parameters even after 6 administrations (Fig. 10).

Our study has revealed the critical coordination of tumor-cell relation CD47 and PD-L1 for tumor evasion. Based on this finding, we have developed EpCAM-targeted LPP-Ep complexes that simultaneously decrease both CD47 and PD-L1 proteins on tumor cells, while reducing their off-target binding to healthy cells. The strategy results in significantly showed the enhanced anti-tumor effects in both CD47 and PD-L1 inhibition compared to either CD47 or PD-L1 inhibition alone. Synergistic effects of CD47 and PD-L1 were only reported in recent years using antibody therapies [17,18]. Balb/c mice repeatedly injected with LPP-P4-Ep containing si-CD47 and si-PD-L1 did not cause anemia effect. This might be expected because aptamer EpCAM-modified LPP-Ep can selectively target to tumor cells and PEGylated complexes can reduce uptake of nanoparticles by leukocytes [44]. The major concern regarding the antibody therapy was solved by utilization the LPP-Ep nanoparticle formulation to avoid side effects. CD47 and PD-L1 serve as critical innate and adaptive checkpoints, respectively, both of them are critical to the immune system. Therefore, we believe this dual-targeting strategy will provide insight into tumor immunotherapy for better tumor control. Meanwhile, LPP-Ep formulation could be a versatile platform that co-deliver gene/drugs/chemo into individual cancer cells to target multiple signaling pathways.

Acknowledgments

This research was supported by the Ministry of Science and Technology of the People’s Republic of China (grant number 2015CB931804); the National Natural Science Foundation of China (NSFC, grant numbers 81703555, U1505225 and 81773063), and the China Postdoctoral Science Foundation (grant number 2017M620268).

Fig. 9. The immunohistochemistry and CBA assay. (a) Immunohistochemistry of heart, liver, spleen, lung and kidney collected from the endpoint from the endpoint of the experiment, amplification × 40. (b-d) The percent of T cells, NK cells and NKT cells in leukocyte after nanoparticle complexes treatment ten days. *P < 0.05, **P < 0.01, ***P < 0.001 compared to the control group by ANOVA test. (e-l) The levels of cytokines including IFN-γ, IL-17A, TNF, IL-2, IL-4, IL-6 and IL-10 in sera of immunized mice were detected by quantitative CBA. *P < 0.05, **P < 0.01, ***P < 0.001 compared to the control group by ANOVA test. All error bars expressed as mean ± SEM (n = 3).

Fig. 10. LPP-P4-Ep caused no anemia. Whole blood analysis about hematology collected from Balb/c mice treated with LPP-P4-Ep loading anti-CD47 or anti-PD-L1 siRNA (n = 5). HCT, hematocrit; HGB, hemoglobin; PLT, platelet; RBC, red blood cell; WBC, white blood cell. *P < 0.05 compared to the control group by ANOVA test. All error bars expressed as mean ± SEM (n = 3).
Author contributions
S.L. and Y.L. conceived and designed the experiments. S.L., R.X., Y.Y., X.X., Y.C., B.L. and S.L. performed the experiments. S.L. and X.X. acquired and analyzed the experimental data. S.L., X.X. and L.J. wrote the manuscript. All authors reviewed the manuscript.

Competing Interests
The authors declare no competing interests.

Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.03.018.

References
[1] Mellman I, Coukos G, Dranoff G. Cancer immunotherapy comes of age. Nature 2011;480:480–9. https://doi.org/10.1038/nature10673.
[2] Zhu EF, et al. Synergistic innate and adaptive immune response to combination immunotherapy with anti-tumor antigen antibodies and extended serum half-life IL-2. Cancer Cell 2015;27:485–91. https://doi.org/10.1016/j.ccell.2015.03.004.
[3] Park S, et al. The therapeutic effect of anti-HER2/neu antibody depends on both innate and adaptive immunity. Cancer Cell 2010;18:160–70. https://doi.org/10.1016/j.ccell.2010.03.004.
[4] Yang X, et al. Cetuximab-mediated tumor regression depends on innate and adaptive immune responses. Mol Ther 2013;21:91–100.
[5] Dong H, et al. Velcro engineering of high affinity CD47-based ligands containing novel ocular delivery systems for ta-crolimus (FK506): in vitro characterization and improved corneal permeation. Int J Nanomed 2011;6:1921–33. https://doi.org/10.3892/ijn.2011.24847.
[6] Li L, et al. Nucleolin-targeting liposomes guided by aptamer SA1411 for the delivery of siRNA for the treatment of malignant melanomas. Biomater 2014;35:3840–50. https://doi.org/10.1016/j.biomaterials.2014.01.019.
[7] Gao J, et al. The promotion of siRNA delivery to breast cancer overexpressing epidermal growth factor receptor through anti-EGFR antibody conjugation by immunoliposomes. Biomater 2011;32:3459–70. https://doi.org/10.1016/j.biomaterials.2011.01.034.
[8] Su S, et al. CRISPR-Cas9-mediated disruption of PD-1 on human T cells for adoptive cellular therapies of EBV positive gastric cancer. Oncol Immunother 2017;6:12495538. https://doi.org/10.1016/j.jci.2017.03.004.
[9] Belz GT, et al. The CD11b/CD18 (++) dendritic cell is responsible for inducing peripheral self-tolerance to tissue-associated antigens. J Exp Med 2002;196:999–104.
[10] Cheng Y, et al. Metastatic cancer cells compensate for low energy supplies in hostile microenvironments with bioenergetic adaptation and metabolic reprogramming. J Int Oncol 2018;5:2590–604. https://doi.org/10.3892/ji.2018.4582.
[11] Lin S, et al. S. nitroscopatociporin interrupts adhesion of cancer cells to vascular endothelium by suppressing cell adhesion molecules via inhibition of the NF-κB, Cdk4, and JAK/STAT signal pathways in endothelial cells. Eur J Pharmacol 2017;791:62–71. https://doi.org/10.1016/j.ejphar.2016.08.018.
[12] Fischer D, Li Y, Ahlemeyer B, Krieglstein J. In vitro cytotoxicity testing of polyacrylates: influence of polymer structure on cell viability and hemolysis. Biomater 2003;24:1121–31.
[13] Emmert MY, et al. Human stem cell-based three-dimensional microtissues for advanced cardiac cell therapies. Biomaterials 2013;34:6339–54. https://doi.org/10.1016/j.biomaterials.2013.04.034.
[14] Chono S, Li SD, Conwell CC, Huang L. An efficient and low immunostimulatory nanoparticle formulation for systemic siRNA delivery to the tumor. J Control Release 2008;131:64–9. https://doi.org/10.1016/j.jconrel.2008.07.006.
[15] Tang Q, et al. A novel co-drug of aspirin and ursolic acid interrupts adhesion, invasion and migration of cancer cells to vascular endothelium via regulating EMT and HIF-1alpha in triple negative breast cancer. Oncotarget 2016;7:13114–29. https://doi.org/10.18632/oncotarget.12232.
[16] Zhao Y, et al. Antigen-presentation cell-intrinsic PD-1 neutralizes PD-L1 in cis to attenuate PD-1+ Signaling in T cells. Cell Rep 2018;24. https://doi.org/10.1016/j.celrep.2018.06.054. 379–390 e376.
[17] Weiskopf K, Cancer immunotherapy targeting the CD47/SIRPalpha axis. Eur J Cancer 2017;76:100–9. https://doi.org/10.1016/j.ejca.2017.02.013.
[18] Cerezo M, et al. Translational control of tumor immune escape via the eIF4F–STAT1-PD-1 axis in melanoma. Nat Med 2018. https://doi.org/10.1038/s41591-018-0217-z.
[19] Zhao X, et al. Characterization of cluster of differentiation 47 expression and its potential as a therapeutic target in esophageal squamous cell cancer. Oncol Lett 2018;15:2017–20. https://doi.org/10.3892/ol.2017.7447.
[20] Fidyk W, et al. Evaluation of proinflammatory and immunosuppressive cytokines in blood and bone marrow of healthy hematopoietic stem cell donors. Cytokine 2018;102:181–6. https://doi.org/10.1016/j.cyto.2017.09.001.
[21] Bardwell DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer 2012;12:252–64. https://doi.org/10.1038/nrc3329.
[22] Yang YG, Wang H, Asavaroengchai W, Dey BR. Role of interferon-gamma in GVHD of advanced cardiac cell therapies. Biomaterials 2013;34:6339–54. https://doi.org/10.1016/j.biomaterials.2013.04.034.
[23] Allen TM, Brandeis E, Hansen CB, Kao GY, Zalipsky S. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumorotropic accumulation of proteins and the antitu- mor agent smans. Cancer Res 1986;46:6387–92.
[24] Gref K, et al. Biodegradable long-circulating polymeric nanospheres. Science 1994;263:1600–3.