A site-oriented nanosystem for active transcellular chemo-immunotherapy to prevent tumor growth and metastasis

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ABSTRACT Immunotherapy has shown promising potential in cancer therapy; however, poor delivery by nanocarriers and insufficient immune response in tumors have severely impeded its clinical application. To overcome these disadvantages, a site-specific and active transcellular drug delivery system was developed herein for chemotherapy-enhanced immunotherapy. When arriving at the tumor site, the matrix metalloproteinase 2 (MMP2)-responsive shell detached from the nanosystem, releasing positively charged cores. The cationic surface of the inner cores induced adsorption-mediated transcytosis, which facilitated transendothelial transportation and transcellular drug delivery into distal tumor cells. PD-L1 antibody and chemotherapeutic drugs were loaded in the outer layer and inner cores of the nanosystem, respectively, to be precisely delivered to target sites, thereby achieving synchronized delivery and site-oriented release of different anticancer agents. PD-L1 antibody released in the tumor microenvironment effectively blocked the binding of PD-L1 to its receptors on the T cell surface. Oxaliplatin and indoximod co-delivered in the cationic cores can induce immunogenic cell death and attenuate the immunosuppressive effect throughout the tumor tissues, recruiting a large amount of T cells and further enhancing the immunotherapy. The resulting synergistic antitumor response could not only efficiently inhibit the growth of primary tumors, but also help prevent metastasis of primary tumor to distant sites. This study offers a novel nano-enabled strategy for chemo-immunotherapy in immunosuppressive tumors.

Keywords: active transcellular drug delivery, site-oriented drug release, chemo-immunotherapy, immunosuppressive effect reversal, tumor metastasis inhibition

INTRODUCTION

Blockade of the interaction between programmed cell death-1 (PD-1) and its ligands has emerged as a promising strategy to combat malignancies [1–3]. However, so far less than one-third of patients display positive responses to this therapeutic strategy [4]. Ongoing research is focused on improving the therapeutic efficacy of PD-L1 blockade treatments [5–9]. Tumors with low immunogenicity, such as breast cancers, are characterized with

insufficient tumor-infiltrating lymphocytes (TILs) and cytotoxic T lymphocytes (CTLs) for effective tumor cell killing [10]. Emerging evidence suggests that the first line chemotherapeutic agent oxaliplatin (OX) can induce immunogenic cell death (ICD) [11], which results in the expression of calreticulin (CRT) on the dying tumor cells. CRT provides an “eat me” signal and facilitates cross-presentation in dendritic cells (DCs), subsequently promoting proliferation and activation of TILs and CTLs [12]. The increased TILs and CTLs can further enhance the sensitivity of low immunogenic tumors to PD-L1 blockade treatment. Apart from increasing the immunogenicity, it is more important to reverse the immunosuppressive micro-environment in “cold” tumors to improve the immunotherapy efficacy. Indoleamine 2,3-dioxygenase 1 (IDO) is a metabolic enzyme frequently overexpressed in breast cancer responsible for converting tryptophan (Try) to kynurenine (Kyn). Try insufficiency at tumor sites often leads to broad immunosuppression, which induces production of regulatory T cells (Tregs) [13,14], prevents effective T-cell priming [15], and decreases the functions of DCs. Indoximod (IND), an IDO inhibitor, has been reported to be able to effectively increase the Try concentration, thereby reducing immunosuppression in tumor sites. Therefore, cooperating IND-induced immunosuppressive reversal with OX-mediated ICD can improve the immunotherapy efficacy through different channels, which provides an ideal candidate for lighting the cold immune microenvironment of breast cancer.

Although both OX and IND have been proved to be effective for immunological enhancement, some clinical trials combining these enhancers with anti-PD-L1 antibody (aPDL1) did not lead to better outcome compared with that of aPDL1 monotherapy [16,17]. One of the potential reasons may be the insufficient treatment in tumor sites [18], which caused by the nonspecific distribution of these anticancer agents. Thus, precise nanocarriers are urgently needed for the combined therapy strategy. More importantly, different anticancer agents delivered by one carrier may have different therapeutic sites. Immunotherapy utilizing aPDL1 blocks the interaction of PD-L1 with PD-1 [19,20], and acts on PD-L1 expressed at the surface of tumor cells. Whereas OX and IND target DNA and IDO, respectively, both of which are located inside tumor cells. Consequently, a specific delivery system with site-oriented release is urgently needed.

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Nanosystems with core-shell structures, which can load various active substances in different layers, have become an important biotechnology for multi-drug delivery [21–24]. The anticancer agents encapsulated in the shell layer are first released when the outer shell is disintegrated, and the inner cores continue to carry cargos to the target sites and realize the second-stage release. To achieve effectively site-specific drug transportation, the shell and core layers of the nanosystems are usually designed to response to different stimuli [25,26]. Core-shell nanosystems with controlled drug release provide specific site-oriented drug delivery platforms for combination therapy. However, for most nanomedicines, the unsatisfactory performance of these nanosystems in infiltration into tumor mass impedes the drug delivery into the distal tumor cells [27], leading to poor prognosis and eventual metastasis [28]. Even though Doxil, an anticancer nanomedicine in clinical use, has been verified to have an enhanced accumulation in tumor tissues, it is seriously compromised in its ability to penetrate tumors and remains localized within the perivascular region [29]. Drug delivery systems with good infiltration into tumor tissues are urgently needed to improve the efficacy of tumor

therapy. Adjusting the particle size and other properties of the drug delivery system is one approach that could improve tumor permeation [30]. However, the penetration efficiency of such systems is still not high enough owing to the issue of simple passive diffusion through the paracellular pathway [31,32]. Therefore, the most promising solution to improve nanosystem tumor permeation is to design a drug delivery system with active transcellular transportation in addition to the passive diffusion through intercellular gap. Moreover, the active transportation may also induce endothelial cell transcytosis, which will transport the nanosystem across the vascular wall and further promote its accumulation in tumors.

Here, we have generated a site-oriented drug delivery system with active transcytosis for enhancing chemo-immunotherapy. As displayed in Fig. 1a, the proposed nanosystem (CLSN@Gel) was composed of a cross-linked gelatin (Gel) shell and *N*-lysinal-*N'*-succinyl chitosan nanogel (CLSN)-constructed cores. Particularly, CLSN was defined to be susceptible to acid-triggered protonation (Fig. 2a) due to the abundant amino groups. Anti-PD-L1 antibody and chemotherapeutic drugs (both OX and IND) were separately encapsulated in the shell layer and inner

Figure 1 Schematic depicting the site-oriented nanosystem for transcellular chemo-immunotherapy. (a) Synthesis route of OI/CLSN-P@Gel. The system was composed of an MMP2-response shell and positively charged cores. The aPD1 and chemotherapeutics were separately encapsulated in different layers of the nanosystem. (b) Active transcellular delivery of OI/CLSN-P@Gel and site-oriented controlled drug release. (I) The neutral shell degraded in MMP2-overexpressed tumor microenvironment, exposing the positively charged cores. (II) The cationic surface and smaller size facilitated the nanosystem to pass through the capillary wall by transendothelial transportation as well as (III) extravasation *via* gaps between epithelial cells. (IV) Cationization-mediated transcytosis enabled OI/CLSN to be transcellularly delivered layer by layer. (V) The aPD1 and chemotherapeutics loaded in different layers of the nanosystem were released at specific sites under various stimuli. (c) Immune activation induced by OI/CLSN. The intracellularly released OX and IND act on DNA and IDO, respectively, resulting in ICD and IDO inhibition simultaneously.

Figure 2 Characterization of OI/CLSN-P@Gel. (a) Illustration of the drug release profile based on the response to stimulus. (b) TEM images of OI/CLSN-P@Gel; images were taken after treatment with (c) MMP2 and then (d) pH was adjusted to 5.0. Changes in (e) size and (f) zeta potential of OI/CLSN-P@Gel with sequential treatments. Drug release profiles of (g) OX, (h) IND, and (i) aPDL1 in response to MMP2 and acidic conditions.

cores (OI/CLSN-P@Gel). Once arriving at the tumor site, the outer shell detached from the positively charged cores. Cationic charges facilitated active transportation of the nanosystem across the vascular wall *via* endothelial cell transcytosis, as well as the enhanced permeation and retention (EPR) effect (Fig. 1b). Anti-PD-L1 antibody encapsulated in the Gel shell would be released in the matrix metalloproteinase 2 (MMP2)-abundant tumor microenvironment for PD-1/PD-L1 blockade, which afterward activated the immune response [33]. Meanwhile, the positively charged inner cores could actively transport OX and IND *via* adsorption-mediated transcytosis. The active layer by layer transportation facilitated the delivery of drug-loaded cores (OI/CLSN) into deep tumors. When the pH value rapidly declined in lysosomes, the positive charges of the nanosystem increased owing to the protonation of amino groups in CLSN, leading to extensive volume expansion and then intracellular drug release. Site-oriented co-delivery of IND and OX into tumor cells was expected to effectively enhance the immune response and further increase the antitumor effect of immunotherapy (Fig. 1c). To verify our hypothesis, the *in vivo* anticancer effect of the combined strategy was evaluated in orthotopic 4T1 tumor-bearing mice and distant metastasis model. This site-oriented drug delivery system with cascade release of synergistic anticancer agents has presented a promising strategy for improving clinical cancer immunotherapy.

EXPERIMENTAL SECTION

Materials

Anti-PDL1 antibody (aPDL1) was purchased from BioXcell (Lebanon, USA). Boc-Lysine (Boc-Lys), succinic anhydride (Suc), chitosan (CS) and Gel were purchased from Aladdin (Shanghai, China). *N*-hydroxysuccinimide (NHS), *N*-hydroxysulfosuccinimide (Sulfo-NHS) and 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide hydro (EDC) were purchased from Sigma-Aldrich (Shanghai, China). Sodium persulfate (APS), *N,N'*-methylenebisacrylamide (MBA), *N*-isopropyl acrylamide (NIPAM), and dimethyl sulfoxide (DMSO) were purchased from Energy Chemical (Shanghai, China).

Fibroblasts (3T3) and murine breast carcinoma (4T1) cell lines were obtained from American Type Culture Collection, and cultured in a humidified incubator with 5% CO₂ at 37°C.

BALB/c mice were purchased from Daren Laboratory Animal Co. Ltd. (Qingdao, China). All animal experiments were performed according to the Animal Management Rules of the Ministry of Health of the People's Republic of China (document No. 55, 2001).

Synthesis and characterization of the nanosystem

As displayed in Fig. S1, 2.39 g of CS was dissolved in *N,N*-dimethylformamide and stirred overnight. Boc-Lys, NHS and

EDC (mole ratio = 1:1.5:1.5) were dissolved in methanol, and then added into the above CS solution, followed by stirring for three days. The obtained *N*-lysinal chitosan (CS-Lys) was dialyzed against distilled water and freeze dried. CS-Lys and Suc were dissolved in *N,N*-dimethylformamide. The pH of the solution was adjusted to 8 using NaOH, followed by stirring for another 24 h. The obtained solution was then precipitated in acetone, and the obtained precipitate was redissolved in *N,N*-dimethylformamide, and hydrochloric acid was added dropwise. After stirring for 30 min, the obtained *N*-lysinal-*N'*-succinyl chitosan (CS-Lys-Suc) was dialyzed and freeze dried. Both CS-Lys and CS-Lys-Suc were characterized by ^1H nuclear magnetic resonance (NMR) and Fourier transformed infrared (FTIR). To prepare CS-Lys-Suc based Nanogels (CLSN), NIPAM and CS-Lys-Suc (mass ratio 2:1) were reacted in deionized water with APS as an initiator and MBA as a crosslinker. To fabricate the OX/IND-loaded CLSN (OI/CLSN), OX and IND were added to the CS-Lys-Suc solution.

Gel shell-coated OI/CLSN (OI/CLSN@Gel) was synthesized as follows: OI/CLSN was added into the Gel aqueous solution, and 10 mL of acetone was added dropwise. After standing for 1 min, the floc formed was collected and redissolved in deionized water, and the pH was adjusted to 2.7. Then 16 mL of acetone was added at a speed of 1 mL min^{-1} , and the solution was stirred for 4 h at room temperature. Glutaraldehyde (20% acetone solution) was then added dropwise, followed by stirring at 40°C for 8 h. Acetone solvent was removed by a rotary evaporator. The product was characterized by transmission electronic microscope (TEM, HITACHI-7650) and dynamic light scattering (DLS, Nano ZS90). The aPDL1-loaded OI/CLSN@Gel (OI/CLSN-P@Gel) was obtained by adding aPDL1 into the OI/CLSN@Gel solution and stirring for 1 h. EDC and sulfo-NHS were then added to the mixture solution with the mole ratio of 1:1. After stirring for 4 h, the extra aPDL1 was removed through centrifugation and dialysis. The amounts of the loaded therapeutics were measured by ultraviolet (UV) and fluorescence analysis. Stability of OI/CLSN-P@Gel was evaluated by monitoring the size changes in pH 7.4, pH 5.5 and in media with $0.5\text{ }\mu\text{g mL}^{-1}$ MMP2.

Stimuli-responsive ability of the nanosystem

The double sensitivity of CLSN@Gel was evaluated by measuring the diameter of the nanosystem as follows: MMP2 was added to the medium ($0.5\text{ }\mu\text{g mL}^{-1}$), and the pH of the medium was adjusted to 5.0–7.4. The medium was stabilized for 6 h. The morphology change of the CLSN@Gel was investigated by TEM. The zeta potential of the CLSN@Gel solution was measured during adjusting pH value from 7.4 to 4.0. To characterize the drug release profile of the nanosystem, OI/CLSN-P@Gel was dialyzed in different media. The concentrations of OX and IND were determined by detecting the UV absorption of the media. For the test of aPDL1 releasing, aPDL1 was incubated with Alexa Fluor 488-labeled goat anti-rat IgG, and then OI/CLSN-P@Gel was dissolved in different media. At certain time points, aPDL1 released in the solution was separated through centrifugation and detected by fluorospectrophotometry.

MMP2-triggered disintegration of the nanosystem

To explore the disintegration of CLSN@Gel in tumor cells, fluorescein isothiocyanate isomer (FITC)-labeled CLSN ($^{\text{FITC}}\text{CLSN}$) and rhodamine-labeled Gel ($^{\text{RhB}}\text{Gel}$) were used to

construct the nanosystem ($^{\text{FITC}}\text{CLSN}@^{\text{RhB}}\text{Gel}$). 4T1 cells were seeded in a cell dish at a density of 1×10^5 cells per dish and cultured for 12 h. The cells were then incubated with $^{\text{FITC}}\text{CLSN}@^{\text{RhB}}\text{Gel}$ ($+0.5\text{ }\mu\text{g mL}^{-1}$ MMP2) or $^{\text{FITC}}\text{CLSN}@^{\text{RhB}}\text{Gel}$ for 4 h, washed three times with phosphate buffer saline (PBS), and detected using laser confocal fluorescence microscopy (LSCM, Zeiss LSM800).

The *in vivo* disintegration of $^{\text{FITC}}\text{CLSN}@^{\text{RhB}}\text{Gel}$ was also measured. MMP2 was pre-injected in tumor tissues. Then, $^{\text{FITC}}\text{CLSN}@^{\text{RhB}}\text{Gel}$ was intravenously (I.V.) injected into 4T1 tumor-bearing mice (with or without MMP2 pre-injection). After 2 h, the mice were sacrificed, and tumor tissues were harvested, sliced and examined by LSCM (ex: 488 and 560 nm).

Intercellular delivery and cytotoxicity of the nanosystem

4T1 cells (I) were treated with $^{\text{RhB}}\text{CLSN}@^{\text{Gel}}$, washed with PBS, and co-incubated with fresh cells (II) for 20 h in a new culture dish. Then the cells (II) were washed and again co-incubated with fresh cells (III). The procedure was repeated on cells (III) to obtain cells (IV). The cells were washed with PBS and observed under LSCM. To distinguish the different batches of cells, cells (I) and cells (III) were transfected with green fluorescent protein (GFP).

The cytotoxicity of CLSN and CLSN@Gel was investigated using 4T1 and 3T3 cells. The cells were seeded in 96-well plates (2×10^3 cells per well) and cultured for 12 h. Then the cells were treated with a series of concentrations of nanocarriers for 72 h. After the addition of 20 μL MTT solution to each well, the cells were incubated for another 4 h. Afterwards, the medium was replaced by 150 μL DMSO, and the absorption at 570 nm was measured using a microplate reader (SpectraMax M3).

Transwell permeability assay

Human umbilical vein endothelial cells (HUVECs) were seeded on a Transwell insert (2×10^4 cells per well) until confluent. The monolayer HUVECs were exposed to $^{\text{RhB}}\text{CLSN}@^{\text{Gel}}$ with or without MMP2 ($0.5\text{ }\mu\text{g mL}^{-1}$). The media in the lower compartment of the transwell were collected at each time point (0, 1, 2, 4, and 8 h) and measured using fluorospectrophotometry.

Three-dimensional (3D) tumor spheroids of human pancreatic carcinoma (BxPC-3) cells were developed as follows: 80 μL of sterile agarose-based Dulbecco's modified eagle medium (2%, w:v) was added in each well of 96-well plates. Then, the cells were seeded in the pre-coated 96-well plate (2×10^3 cells per well) and cultured for seven days. The spheroids were seeded in the lower compartment of the transwell and observed by LSCM.

In vivo biodistribution of the nanosystem

The 4T1 tumor-bearing mouse model was developed by subcutaneously injecting $\sim 1 \times 10^6$ 4T1 cells to each mouse. CLSN was labeled with a near-infrared (NIR) dye Cy7 to obtain $^{\text{Cy}}\text{CLSN}$ through the interaction between carboxyl and amino. $^{\text{Cy}}\text{CLSN}$ and $^{\text{Cy}}\text{CLSN}@^{\text{Gel}}$ were I.V. injected into the 4T1 tumor-bearing mice and fluorescence images were captured using an *in vivo* imaging system. The fluorescence intensity ratio of tumor to normal tissues was analyzed using the Scion Image software. The mice were sacrificed at 24 h post-injection and the tumors and main organs (heart, liver, spleen, lung, and kidney) were harvested and imaged. The sections of tumors and the main organs were further imaged by LSCM.

OI/CLSN-P@Gel (5 mg kg⁻¹ OX, 14.5 mg kg⁻¹ IND, 10.82 mg kg⁻¹ aPDL1) was intravenously injected into tumor-bearing mice ($n = 3$). Plasma samples were collected at 5 min, 2, 8, 24 and 48 h. The mixture was treated through high-power sonication to break the structure of the nanoparticles, resulting in the drug release. Then, the free OX and IND were extracted and studied by UV. The loaded aPDL1 was labeled with Alexa Fluor 488-labeled goat anti-rat IgG and detected by fluorospectrophotometry. The pharmacokinetic (PK) parameters, half life ($t_{1/2}$) and area under curve (AUC), were calculated through WinNonlin.

Suppression of tumor growth *in vivo*

The orthotopic implantation tumor model was developed by injecting 1×10^6 4T1 cells into the right breast pad of each mouse. Then the mice were randomly divided into six groups ($n = 10$). Five days after inoculation, different reagents (saline, CLSN@Gel, aPDL1, OX + IND, aPDL1 + OX + IND and OI/CLSN-P@Gel) were injected *via* the tail vein of mice. The administration was repeated every three days. The tumor volume, body weight and survival of animals in each group were recorded every other day. Fifteen days after administration, the mice were sacrificed, blood samples were collected for biochemical parameters test, and the tumors and main organs (heart, liver, spleen, lung, and spleen) were harvested, sliced and analyzed using haematoxylin and eosin (H&E) staining. For terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, the tumor tissues from different groups were cut into 4 μ m in thickness, pretreated, and stained using a TUNEL apoptosis detection kit by following the manufacturer's instructions. The fluorescence images were analyzed using LSCM. The median survival time was calculated by SPSS.

Immune response study

The recruitment of CD4+ T cells and CD8+ T cells was measured using flow cytometry. Briefly, the tumor tissues were harvested, digested and passed through a filter. The obtained cells were incubated with anti-CD8-PE (PE: Phycoerythrin) and anti-CD4-FITC antibodies at 4°C for 30 min, washed three times with PBS, and measured using flow cytometry. The memory T cells (CD44 + CD62L-) and Tregs (CD4 + FoxP3+) were also evaluated by flow cytometry. Immunohistochemical analysis was also conducted to investigate the expression of CRT and CD103 in tumors. Briefly, the tumor tissues were cut into 4 μ m in thickness, subjected to deparaffinization, hydration and antigen retrieval, and then incubated with anti-CRT or anti-CD103 antibodies, followed by incubation with 3,3'-diaminobenzidine.

Suppression of tumor metastasis *in vivo*

Lung metastasis model was established to evaluate the anti-metastasis effect of the reagents. First, an osteosarcoma *in situ* tumor was constructed as primary tumor. The mice were randomly divided into six groups ($n = 10$) and administrated with saline, CLSN@Gel, aPDL1, OX + IND, aPDL1 + OX + IND or OI/CLSN-P@Gel. Five days after treatment, 4T1 cells (1×10^5) were I.V. injected into each mouse. On day 25 of the experiment, the mice were sacrificed, the lungs of different groups were harvested and the metastasis nodules were counted. The lung sections were stained with H&E for further pathological analysis. Immunohistochemical analysis of Ki67 was conducted as described above. The secretions of interferon (IFN)- γ and IL-10

in serum were measured using an enzyme linked immunosorbent assay (ELISA).

Statistical analysis

All data are expressed as mean $\pm$ standard deviation (SD). Statistical significance was analyzed using Student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Synthesis and characterization of OI/CLSN-P@Gel

The nanosystem consisted of an MMP2 responsive Gel shell and positively charged cores (CLSN). The CLSN was composed of CS-Lys-Suc-based nanogel, which displayed acid-triggered volume expansion due to the amino group protonation at low pH values. The structure of CS-Lys-Suc was confirmed by ¹H NMR and FTIR (Figs S2 and S3). CLSN was synthesized by polymerizing NIPAM with CS-Lys-Suc. Cationization can mediate active transportation; however, positively charged nanosystems are more likely to be cleared from the circulation. The neutral Gel-coated outside of the nanosystem prevented its clearance during circulation. The morphology of CLSN@Gel is shown in Fig. 2b, with a particle size of ~140 nm. The enlarged view of the TEM clearly shows the CLSN cores coated inside the Gel layer. Once arriving at the MMP2-abundant tumor site, the core-shell structure collapsed and the positively charged cores that are smaller in size were released (Fig. 2c), which facilitated penetration *via* active transcytosis as well as paracellular diffusion. Moreover, the abundant amino groups of CLSN induced prompt protonation in acidic lysosomes, and the zeta potential of CLSN also showed a change from 4.36 mV at pH 7.4 to 16.13 mV at pH 5.0 (Fig. 2f). Due to the electrostatic repulsion between CS-Lys-Suc chains, the CLSN cores obviously swelled to nearly 1 μ m when the pH decreased to 5.0 (Fig. 2d), along with rapid release of the loaded drugs in tumor cells. The cationic charged surface and the extensive volume expansion of CLSN triggered the lysosomal bursting and brought CLSN into the cytosol, where most CLSN shrank back with pH returned to 7.4 (Fig. 2e, Fig. S4).

To site-specifically control the release of the anticancer agents, OX/IND and aPDL1 were loaded in CLSN and the outer Gel shell, respectively (OI/CLSN-P@Gel). The successful encapsulation of aPDL1, which was labeled with the fluorescent dye, was verified by the emission signal at 526 nm of the nanosystem (Fig. S5). The drug loading efficacy of OX, IND and aPDL1 were 7.55%, 21.88% and 16.32%, respectively, and the corresponding encapsulation efficiencies were 93.17%, 73.5% and 87.16%. The contents of major elements were evaluated, and the ratios of C, H, O, N, S, Pt were about 33.58%, 5.85%, 9.63%, 3.03%, 1.7%, 4.8%. Moreover, the stability of OI/CLSN-P@Gel was investigated (Fig. S6). In the media of pH 7.4, the particle size of OI/CLSN-P@Gel displayed minimal changes during 10 days' storage, indicating that OI/CLSN-P@Gel was stable enough for further investigations. However, OI/CLSN-P@Gel cannot maintain its stability in acidic solution or MMP2-abundant environment.

Owing to the dual protection by the core-shell structure, the presence of MMP2 and an acidic pH value were both necessary for the release of OX and IND. In MMP2-abundant media, more than 80% of both drugs were released when the pH was lower than 5.0 (Fig. 2g, h). The release of aPDL1, which was encapsulated

sulated in the space outside CLSN, was only related to the concentration of MMP2 (Fig. 2i). The outer shell was dissociated by MMP2, leading to a quick release of aPD1L1.

Intracellular behavior of CLSN@Gel

Fig. S7a displayed the LSCM images of 4T1 cells, which were incubated with CLSN@^{RhB}Gel. After 4 h incubation, obvious red fluorescence was observed in the cytoplasm, indicating that CLSN@^{RhB}Gel had entered the tumor cells. To evaluate the endocytosis mechanism, clathrin was inhibited before CLSN@^{RhB}Gel incubation. The results demonstrated that the internalization of CLSN@^{RhB}Gel was significantly inhibited, which implied that the uptake of the nanosystem by tumor cells was mainly *via* clathrin-mediated endocytosis. Furthermore, to study the intracellular fate, the localization of CLSN@^{RhB}Gel was observed by LSCM. As shown in Fig. S7b, the fluorescence signal of CLSN@^{RhB}Gel was colocalized with lysosomes, demonstrating that the nanosystem entered lysosomes after endocytosis.

To realize site-oriented drug delivery, the nanosystem should be sensitive enough to allow for degradation of the outer layer in MMP2-abundant environment. The intracellular behavior of the nanosystem in 4T1 cell line was investigated through a fluorescence double labeling method. FITC-labeled CLSN (^{FITC}CLSN) and RhB-labeled Gel (^{RhB}Gel) were used to construct ^{FITC}CLSN@^{RhB}Gel. After incubation with 4T1 cells in the presence of MMP2, the green fluorescence signal of ^{FITC}CLSN was detected to diffuse away from the overlapped yellow signal of ^{FITC}CLSN@^{RhB}Gel (Fig. 3a), indicating the dissociation of ^{FITC}CLSN@^{RhB}Gel. In contrast, in the MMP2-insufficient environment, the green and red fluorescence signals were almost completely overlapped, indicative of the intact structure of ^{FITC}CLSN@^{RhB}Gel. The semi-quantitative result further confirmed this conclusion (Fig. 3b).

Flow cytometry was used to evaluate the cellular uptake with

or without MMP2. As shown in Fig. S8, the fluorescence intensity of ^{FITC}CLSN@Gel in 4T1 cells in the presence of MMP2 was much stronger than that without MMP2 activity. These results indicated that the nanosystem maintained its structure when MMP2 was not present, and the larger particle size thereby reduced the uptake of the nanosystem by normal cells. CLSN also exhibited some toxicity to both tumor and normal cells (Fig. S9), which might be attributed to the swelling of CLSN in the acidic environment of lysosomes, thereby causing damage to lysosomes. However, the Gel layer coated outside the nanosystem could protect the cells from side effects caused by CLSN, thus decreasing its toxicity.

To evaluate the transcellular delivery of CLSN to the adjacent cells, 4T1 cells (I) pre-incubated with ^{RhB}CLSN were collected and incubated with fresh cells (II). The process was repeated in cells (III) and (IV) (Fig. S10). In order to distinguish between different batches of cells, cells (I) and (III) were transfected with GFP. As shown in Fig. 3c, the red fluorescence signal in 4T1 cells (I) implied that ^{RhB}CLSN widely distributed in the cytoplasm. After incubation with fresh cells, significant fluorescence signal was detected in the infected cells (II), and additionally, the red signal was still observed in the infected cells (III) and (IV). These results demonstrated that some ^{RhB}CLSN internalized in the first treated cells (I) was exocytosed into the culture medium and subsequently taken by cells (II), (III) and (IV), indicative of the excellent transcellular delivery ability of CLSN. We speculated that the effective transcellular delivery of CLSN was due to the prompt protonation of amino groups in lysosomes. The electrostatic repulsion in CLSN led to the swelling of the drug-loaded cores, resulting in a rapid drug release. Lysosomal escape of the amino-abundant CLSN brought it back to the cytosol and contracted it back to its original size. After liberation from dead cells or exocytosis from live cells, CLSN was taken by adjacent tumor cells, leading to a layer-by-layer mechanism of transcellular drug delivery.

Figure 3 Responsiveness to stimuli and transcellular transportation of CLSN@Gel. (a) Dissociation of ^{FITC}CLSN@^{RhB}Gel in 4T1 cells after incubation with the media with or without additional MMP2 (0.5 μg mL⁻¹). The FITC signal is shown in green and the RhB signal is shown in red. (b) Fluorescence intensity profiles of ^{RhB}Gel and ^{FITC}CLSN at the central axis of 4T1 cells with or without MMP2 incubation (red: ^{RhB}Gel; green: ^{FITC}CLSN). (c) Intercellular transfer of ^{RhB}CLSN as observed by LSCM. 4T1 cells (I) were incubated with ^{RhB}CLSN for 6 h, then washed with PBS and co-cultured with cells (II) for 20 h in fresh medium. The process was repeated to obtain cells (III) and (IV) (red: ^{RhB}CLSN; green: GFP transfected 4T1 cells).

Penetration and *in vivo* dissociation of CLSN@Gel

Inspired by the results of transcellular delivery of CLSN to adjacent tumor cells, we proposed that it may promote the active transportation across the capillary wall into tumor tissues through endothelial cell transcytosis, in addition to the passive paracellular pathway. The transwell assay across a dense HUVEC barrier was conducted to evaluate the transcytosis of RhB@CLSN@Gel (Fig. 4a). As shown in Fig. 4b, in the presence of MMP2, a significant fluorescence signal was detected in the lower compartment of the transwell. Moreover, the fluorescence intensity increased over time. In contrast, in the MMP2-insufficient environment, the fluorescence signal was much lower than that in the MMP2-abundant group. We speculated that RhB@CLSN@Gel decorticated the outer layer and exposed the inner cores with high concentrations of MMP2. The smaller cores with positive charges were more easily internalized by the endothelial cells and further leading to transendothelial transportation. Moreover, to further investigate the behavior of the nanosystems after passing through the endothelial cell barrier, 3D multicellular tumor spheroids were incubated in the lower compartment. As shown in Fig. S11, in the presence of MMP2, the released RhB@CLSN cores effectively penetrated throughout the center of tumor spheroid, which was attributed to the smaller size and positive charges. In sharp contrast, limited tumor penetration was observed in the spheroid treated with RhB@CLSN@Gel . The tumor spheroid only showed weak red fluorescence distribution at the periphery area. These results demonstrated that the degradation of the outer shell promoted the nanosystem to actively transport across endothelial cell barriers and further penetrate into deep tumors.

The intratumoral behavior of the nanosystem was also examined. As displayed in Fig. S12, the green signal of FITC@CLSN was separated from the red signal of RhB@Gel at 6 h post-injection, indicating that the structure of FITC@CLSN@RhB@Gel collapsed into smaller particles in the presence of MMP2 in the tumor microenvironment. The inner cores with smaller particle sizes and positive charges penetrated deeply into the tissues and were distributed throughout the tumor. To verify the MMP2

responsiveness of FITC@CLSN@RhB@Gel , tumor tissues with increased local MMP2 concentration were obtained by pre-injection of MMP2. As shown in Fig. 4d, the green fluorescence of FITC@CLSN and red fluorescence of RhB@Gel were separated at 2 h, which was attributed to the quickly degradation of the Gel shell with high-level MMP2. Meanwhile, without the extra addition of MMP2, most fluorescence signals of FITC@CLSN and RhB@Gel were overlapped and localized in the perivascular region with little penetration at 2 h, and only a tiny proportion of FITC@CLSN was observed to be separated from RhB@Gel (Fig. 4c). These observations demonstrated that FITC@CLSN@RhB@Gel could devolve into smaller particles in tumor tissues. The speed of disintegration was closely related to the concentration of MMP2. Released FITC@CLSN infiltrated tumor tissues distal from the vessel centers through both active transcellular transport and passive paracellular diffusion. In normal tissues, however, it kept the integrate structure and restricted to the perivascular regions. Therefore, the design of this drug delivery system ensured efficient transport and specific release of different cargos to appropriate sites in the tumor environment.

In vivo biodistribution and tumor accumulation of CLSN@Gel

The biodistribution of CLSN@Gel was investigated in 4T1 tumor-bearing mice, using CLSN as control. NIR dye Cy7-labeled CLSN (Cy7@CLSN) was used to construct Cy7@CLSN@Gel for *in vivo* fluorescence imaging at the desired time points. Following intravenous injection, both Cy7@CLSN@Gel and Cy7@CLSN rapidly spread throughout the whole body (Fig. 5a). An obvious fluorescence signal from each group was detected in the tumor area after 4 h. According to semi-quantitative analysis, the fluorescence intensity in tumors peaked at 8 h post-injection and the signal remained detectable until 24 h post-injection (Fig. 5d). The mice were euthanized 24 h post-injection, and the main organs were collected for *ex vivo* fluorescence imaging. As shown in Fig. 5b, a higher signal was observed in tumor tissues from the Cy7@CLSN@Gel group, owing to the low clearance of neutral nanoparticles in tumor tissues. To ascertain the biodistribution of Cy7@CLSN@Gel , mice were sacrificed and the fluor-

Figure 4 The dissociation and penetration of CLSN@Gel. (a) Experimental scheme of the penetration assay. Dense HUVECs were seeded on the Transwell insert and exposed to RhB@CLSN@Gel for different time points. (b) Time-dependent fluorescence intensity changes of the nanosystem translocated across the HUVEC monolayer with or without MMP2. The dissociation and penetration of FITC@CLSN@RhB@Gel in tumor tissues (c) without or (d) with local injection of MMP2 at 2 h (green: FITC@CLSN ; red: RhB@Gel ; blue: Hoechst 33258-stained nucleus).

Figure 5 *In vivo* biodistribution of ^{64}Cu -CLSN@Gel in 4T1 tumor-bearing mice. (a) *In vivo* NIR whole body fluorescence images following I.V. administration of ^{64}Cu -CLSN@Gel and ^{64}Cu -CLSN. (b) *Ex vivo* fluorescence images of extracted tissues (heart, liver, spleen, lung, kidney and tumor) from 4T1 tumor-bearing mice 24 h post-injection. (c) Fluorescence images of tissue sections from ^{64}Cu -CLSN@Gel-treated mice. (d) Tumor to normal tissue ratios of ^{64}Cu -CLSN@Gel and ^{64}Cu -CLSN in 4T1 tumor-bearing mice at different time points. Quantitative analysis of major tissues at different time points post-injection of (e) CLSN@Gel and (f) CLSN.

escence intensity in homogenates of the major organs was estimated at each time point. Fig. 5e shows the fluorescence intensity in the liver and kidney, which gradually decreased after 8 h. The fluorescence intensity in kidney of the ^{64}Cu -CLSN group declined more quickly than that of the ^{64}Cu -CLSN@Gel-treated group (Fig. 5e, f). These results are consistent with those observed from fluorescence images. We speculated that ^{64}Cu -CLSN with positive charges is more likely to be excreted into the urine. However, neutral ^{64}Cu -CLSN@Gel was trapped in the perivascular regions, leading to a slower clearance. Sections from tumors and major organs were imaged. Strong fluorescence signal was distributed throughout tumor tissues (Fig. 5c), further demonstrating the efficient tumor accumulation and significant deep penetration of ^{64}Cu -CLSN@Gel. The AUC and $t_{1/2}$ of the loaded therapeutics were accessed after injection of OI/CLSN-P@Gel. As shown in Table S1, the circulatory of $t_{1/2}$ of OX, IND and aPDL1 were 11.5, 13.7 and 16.3 h respectively, displaying a long circulation time in blood.

Evaluation of anticancer efficacy

The therapeutic effect of the nanosystem on breast cancer was evaluated on 4T1 tumor-bearing mice. As illustrated in Fig. 6a, saline, CLSN@Gel, aPDL1, OX + IND, aPDL1 + OX + IND and OI/CLSN-P@Gel were respectively injected into mice every three days. The tumor volumes, body weights, and survival rates of different groups were recorded over the 15 days of treatment. As shown in Fig. 6b–d, aPDL1 had a weak inhibitory effect on growth of xenograft tumors as the result of the immunosuppressed microenvironment. OX + IND moderately suppressed tumor growth, and aPDL1 + OX + IND induced improved antitumor performance with stronger inhibition of tumor growth. Remarkably, the combination of chemotherapy and immunotherapy delivered by OI/CLSN-P@Gel had the most pronounced antitumor effect when compared with other treat-

ment groups. As shown in Fig. 6f, the body weight of OI/CLSN-P@Gel-treated mice gradually increased during the treatment, maintaining a good quality of life. The median survival time of each group (saline, CLSN@Gel, aPDL1, OX + IND, aPDL1 + OX + IND and OI/CLSN-P@Gel) was shown in Table S2. Compared with other treatment groups, OI/CLSN-P@Gel demonstrated a notable benefit in extending the lifespan of the tumor-bearing mice, with 60% of mice in the OI/CLSN-P@Gel group surviving while mice in the other therapeutic groups were mostly dead at the end of the study (Fig. 6g).

To further evaluate the antitumor efficacy of the nanosystem, sections of tumor tissues were stained with TUNEL and H&E to analyze the cell death. The OI/CLSN-P@Gel-treated group showed the most severe necrosis and apoptosis in the tumor cells. As shown in Fig. 6e, TUNEL staining of tumor tissues from the OI/CLSN-P@Gel group revealed a large number of green dots and remarkable cell condensation that was also observed by H&E staining. As shown in Fig. 6e and Fig. S13, inflammatory cell infiltration and vacuolar degeneration of liver were observed in the OX + IND + aPDL1 group, implying that there was sight damage in liver. Moreover, congestion of lung and renal edema was observed, which indicated the side effect caused by free drugs combination. In the OI/CLSN-P@Gel-treated mice, the systemic toxicity was reduced. The low toxicity to normal organs might be attributed to the specific drug release. As shown in Fig. S14, tumor tissues displayed obviously higher expression of MMP2 compared with normal organs, which facilitated precise drug release in tumor tissues.

Anticancer immune response study

To evaluate the immune response induced by the nanosystem treatment, CD8+ T cells and CD4+ T cells were analyzed by flow cytometry. Tumor-infiltrating cytotoxic CD8+ T cells are known to kill tumor cells directly, while helper CD4+ T cells could

Figure 6 Antitumor effects of OI/CLSN-P@Gel ($n = 10$). (a) Diagram of the 4T1 orthotopic implantation model and administration method for tumor therapy. (b) Tumor images and (c) tumor weight of 4T1 tumor-bearing mice after the treatment of saline, CLSN@Gel, aPDL1, OX + IND, aPDL1 + OX + IND and OI/CLSN-P@Gel at day 15. (d) Tumor growth in mice during the 15 days treatment. (e) TUNEL analysis of tumor tissue and H&E staining of tumor, liver and lung. (f) Weight changes of tumor-bearing mice during the experiment. (g) The survival rates of mice bearing 4T1 tumors treated with different administrations.

regulate immunosuppression. As displayed in Fig. 7a, we counted the CD8⁺ T cells and CD4⁺ T cells in tumor tissues after various administrations. Interestingly, the OX + IND group displayed obvious expression of CD8⁺ T cells, owing to the generation of CTLs in response to the synergy between OX and IND. The amount of CD8⁺ T cells was further increased with the blockade of PD-1/PD-L1 by aPDL1. Notably, OI/CLSN-P@Gel group demonstrated the highest CD8⁺ T cell concentration (Fig. 7b), which fully consolidated the efficient synergy achieved by the novel drug delivery system and the combination therapy delivered. Moreover, due to intratumoral stimulation of the immune response, the percentage of CD4⁺ T cells in the OI/CLSN-P@Gel-treated mice was 5.89%, which was much higher than that in the control group (Fig. 7c). These data suggest that OI/CLSN-P@Gel could successfully recruit tumor-infiltrating cytotoxic CD8⁺ T cells and helper CD4⁺ T cells to establish an effective immune response at the tumor site (Fig. 7d). Additionally, the memory T cells (Tems, CD44

+CD62L-) and the regulatory T cells (Tregs, CD4 + FoxP3+) were evaluated (Fig. S15). OI/CLSN-P@Gel treatments enriched the Tems population by 4.87-fold higher than that of control. Meanwhile, although OX-induced ICD activates CTLs and enhances immunotherapy, the secreted IFN- γ would upregulate IDO expression, leading to the immunosuppressive effect in tumors (Fig. S16). As displayed in Fig. S15, Tregs of OX-treated group increased by 2.37 times compared with saline group. However, combined with IND, the OX-induced immunosuppression was reversed, and OI/CLSN-P@Gel-treated group displayed an obvious decline of Tregs.

In addition to influencing the adaptive immune response, innate parameters, which could reflect the induction of ICD, were also assessed (Fig. 7e). A significant increase in CRT expression in tumor tissues from the OI/CLSN-P@Gel group was observed. Integrin receptor CD103, which can be used by DC to facilitate CD8⁺ T cell proliferation and activation, was detected as a biomarker for DC activation. As shown in Fig. 7e,

Figure 7 The immune response evaluation of OI/CLSN-P@Gel. (a) Flow cytometry analysis of CD8+ T cells and CD4+ T cells in tumor tissues after administration of saline, CLSN@Gel, aPDL1, OX + IND, aPDL1 + OX + IND and OI/CLSN-P@Gel. Quantitative flow cytometry analysis of (b) CD8+ T cells and (c) CD4+ T cells in tumor tissues. (d) Illustration of the synergistic effect of OX, IND and aPDL1 in inducing the tumor immune response. (e) Immunohistochemical staining of CD103 and CRT in tumor tissues after different treatments.

OI/CLSN-P@Gel notably improved CD103 expression when compared with aPDL1, OX + IND or aPDL1 + OX + IND treatment. Furthermore, increased IFN- γ and granzyme B in tumor tissues were observed for OI/CLSN-P@Gel-treated group, implying the enhanced proliferation of TILs (Fig. S17). We also measured the expression of phosphorylated S6 kinase (P-S6), a key indicator of reversal of IDO-induced immunosuppression. As shown in Fig. S18, OI/CLSN-P@Gel treatment had an exaggerated effect on P-S6 generation, indicating efficient delivery of a high level of IND by the nanosystem. These findings well demonstrated that OX-induced ICD and IND-mediated reversal of immunosuppression can effectively recruit CTLs and TILs, reversing the cold immune environment at the tumor site. Moreover, the abundant amount of intratumoral T cells expressing immune checkpoint receptors that are present with treatment boosted the effects of the PD-L1 blockade immunotherapy (Fig. 7d).

Lung metastasis inhibition

Metastasis is the main cause of death for cancer patients. To study the potential of OI/CLSN-P@Gel to inhibit tumor metastasis, a lung metastatic tumor model was established. 4T1 cells were orthotopically injected into the breast pads of mice as primary tumors, and the cells were intravenously injected into the mice 5 days after treatment to drive formation of metastatic tumors (Fig. 8a). The lungs of each treatment group were excised to analyze tumor metastasis. As shown in Fig. 8b, e, aPDL1 + OX + IND had a preventative effect on the development of lung metastasis with only a few tumor nodules observed in the lungs. Moreover, the combined therapy delivered through the synthesized drug delivery nanosystem had significantly decreased

metastasis, with no obvious metastases observed in the lungs. These results were confirmed by H&E staining (Fig. 8c). Large areas of tumor metastases were observed in lung sections from all treatment groups except for the OI/CLSN-P@Gel group, indicating the effective inhibition of development of lung metastases. Similar results were observed in Ki67 staining of lung sections, which is closely correlated with the proliferation of tumors (Fig. 8d).

The inhibitory mechanism of the various treatments on lung metastasis was further studied. The proinflammatory cytokine IFN- γ is regarded as an important antitumor cytokine; however, its secretion is suppressed in immunosuppressed breast tumors. IFN- γ is reported to be generated as a result of immune checkpoint blockade [34]. We therefore collected blood samples from mice 5 days after receiving different treatments and measured IFN- γ levels. We observed that the OI/CLSN-P@Gel treatment group had the highest serum concentration of IFN- γ , which was maintained for 5 days post-administration (Fig. 8f). Moreover, IL-10, an immunosuppressive marker [35] decreased remarkably following OI/CLSN-P@Gel treatment (Fig. 8g). These results demonstrated the strong immune anticancer activity of OI/CLSN-P@Gel and capacity of the nanosystem to prevent development of tumor metastases.

CONCLUSIONS

In summary, we developed a site-specific and active transcellular drug delivery nanosystem, OI/CLSN-P@Gel, as a combined chemo-immunotherapy approach to inhibit breast tumor growth and metastasis. The nanosystem we developed could precisely deliver different anticancer agents to their specific target sites. To achieve more efficient drug delivery, the nano-

Figure 8 Antitumor effects of OI/CLSN-P@Gel in a lung metastasis model ($n = 10$). (a) Schematic depicting establishment of the metastasis model and therapy procedure. (b) Images of lung metastases in 4T1 tumor-bearing mice after treatment of saline, CLSN@Gel, aPDL1, OX + IND, aPDL1 + OX + IND and OI/CLSN-P@Gel. (c) H&E and (d) Ki67 staining of the lungs from different treatment groups. (e) Number of lung metastatic nodules in different groups at the end of the experiment. ELISA analyses of (f) IFN- γ and (g) IL-10 in blood samples.

system was designed to change in size during delivery and to be charge reversible, endowing OI/CLSN-P@Gel with both passive paracellular diffusion capability and active transcellular transport capability. The nanosystem delivers OX deeply into tumor cells to stimulate ICD as well as IND to inhibit the immunosuppressive effects of the IDO pathway. Cooperating OX and IND, the cold immune breast cancer environment was reversed, recruiting a large amount of T cells. Meanwhile, aPDL1 released in the tumor microenvironment could effectively block the interaction between tumor cell-expressed PD-L1 and its receptors on the surface of T cells. The excellent delivery performance and cooperative effects of this therapy made OI/CLSN-P@Gel a promising therapeutic strategy in breast tumor therapy. More importantly, the treatment was effective in prevention of tumor metastasis to the lung, which could also be applied to other cancers.

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- Doroshov DB, Bhalla S, Beasley MB, *et al.* PD-L1 as a biomarker of response to immune-checkpoint inhibitors. *Nat Rev Clin Oncol*, 2021, 18: 345–362
- Wan C, Keany MP, Dong H, *et al.* Enhanced efficacy of simultaneous PD-1 and PD-L1 immune checkpoint blockade in high grade serous ovarian cancer. *Cancer Res*, 2020, 81: 158–173
- Yu JX, Hodge JP, Oliva C, *et al.* Trends in clinical development for PD-1/PD-L1 inhibitors. *Nat Rev Drug Discov*, 2020, 19: 163–164
- Park SE, Lee SH, Ahn JS, *et al.* Increased response rates to salvage chemotherapy administered after PD-1/PD-L1 inhibitors in patients with non-small cell lung cancer. *J Thoracic Oncol*, 2018, 13: 106–111
- Su Z, Xiao Z, Wang Y, *et al.* Codelivery of anti-PD-1 antibody and paclitaxel with matrix metalloproteinase and pH dual-sensitive micelles for enhanced tumor chemoimmunotherapy. *Small*, 2020, 16: 1906832

- Hu C, He X, Chen Y, *et al.* Metformin mediated PD-L1 downregulation in combination with photodynamic-immunotherapy for treatment of breast cancer. *Adv Funct Mater*, 2021, 31: 2007149
- Ferrara R, Naigeon M, Auclin E, *et al.* Circulating T-cell immunosenescence in patients with advanced non-small cell lung cancer treated with single-agent PD-1/PD-L1 inhibitors or platinum-based chemotherapy. *Clin Cancer Res*, 2021, 27: 492–503
- Wang C, Shi X, Song H, *et al.* Polymer-lipid hybrid nanovesicle-enabled combination of immunogenic chemotherapy and RNAi-mediated PD-L1 knockdown elicits antitumor immunity against melanoma. *Biomaterials*, 2021, 268: 120579
- Chen Y, Ma H, Wang W, *et al.* A size-tunable nanoplatform: Enhanced MMP2-activated chemo-photodynamic immunotherapy based on biodegradable mesoporous silica nanoparticles. *Biomater Sci*, 2021, 9: 917–929
- Timaner M, Kotsofruk R, Raviv Z, *et al.* Microparticles from tumors exposed to radiation promote immune evasion in part by PD-L1. *Oncogene*, 2020, 39: 187–203
- Zheng J, Sun J, Chen J, *et al.* Oxygen and oxaliplatin-loaded nanoparticles combined with photo-sonodynamic inducing enhanced immunogenic cell death in syngeneic mouse models of ovarian cancer. *J Control Release*, 2021, 332: 448–459
- Gao J, Wang WQ, Pei Q, *et al.* Engineering nanomedicines through boosting immunogenic cell death for improved cancer immunotherapy. *Acta Pharmacol Sin*, 2020, 41: 986–994
- Kesarwani P, Prabhu A, Kant S, *et al.* Tryptophan metabolism contributes to radiation-induced immune checkpoint reactivation in glioblastoma. *Clin Cancer Res*, 2018, 24: 3632–3643
- Volarevic V, Zdravkovic N, Harrell CR, *et al.* Galectin-3 regulates indoleamine-2,3-dioxygenase-dependent cross-talk between colon-infiltrating dendritic cells and T regulatory cells and may represent a valuable biomarker for monitoring the progression of ulcerative colitis. *Cells*, 2019, 8: 709
- Wang J, Li Z, Wang Z, *et al.* Nanomaterials for combinational radio-immuno oncotherapy. *Adv Funct Mater*, 2020, 30: 1910676
- Long GV, Dummer R, Hamid O, *et al.* Epacadostat plus pem-

- brolizumab versus placebo plus pembrolizumab in patients with unresectable or metastatic melanoma (ECHO-301/KEYNOTE-252): A phase 3, randomised, double-blind study. *Lancet Oncol*, 2019, 20: 1083–1097
- 17 Breakstone R. Colon cancer and immunotherapy—Can we go beyond microsatellite instability? *Transl Gastroenterol Hepatol*, 2021, 6: 12
 - 18 Muller AJ, Manfredi MG, Zakharia Y, *et al.* Inhibiting IDO pathways to treat cancer: Lessons from the ECHO-301 trial and beyond. *Semin Immunopathol*, 2019, 41: 41–48
 - 19 Akinleye A, Rasool Z. Immune checkpoint inhibitors of PD-L1 as cancer therapeutics. *J Hematol Oncol*, 2019, 12: 92
 - 20 Hellmann MD, Paz-Ares L, Bernabe Caro R, *et al.* Nivolumab plus ipilimumab in advanced non-small-cell lung cancer. *N Engl J Med*, 2019, 381: 2020–2031
 - 21 Luo L, Li L, Cong C, *et al.* Catalase-like nanosystem for interlocking trimodal cancer therapy with hypoxia relief. *Sci China Mater*, 2021, 64: 1021–1034
 - 22 Xue X, Li J, Fan Y, *et al.* Gene silencing-mediated immune checkpoint blockade for tumor therapy boosted by dendrimer-entrapped gold nanoparticles. *Sci China Mater*, 2021, 64: 2045–2055
 - 23 Cheng G, Yin C, Tu H, *et al.* Controlled co-delivery of growth factors through layer-by-layer assembly of core-shell nanofibers for improving bone regeneration. *ACS Nano*, 2019, 13: 6372–6382
 - 24 Liu P, Liu X, Cheng Y, *et al.* Core-shell nanosystems for self-activated drug-gene combinations against triple-negative breast cancer. *ACS Appl Mater Interfaces*, 2020, 12: 53654–53664
 - 25 Lin F, Chen J, Lee M, *et al.* Multi-responsive ibuprofen-imprinted core-shell nanocarriers for specific drug recognition and controlled release. *ACS Appl Nano Mater*, 2020, 3: 1147–1152
 - 26 Albinali KE, Zagho MM, Deng Y, *et al.* A perspective on magnetic core-shell carriers for responsive and targeted drug delivery systems. *IJN*, 2019, Volume 14: 1707–1723
 - 27 Yang H, Tong Z, Sun S, *et al.* Enhancement of tumour penetration by nanomedicines through strategies based on transport processes and barriers. *J Control Release*, 2020, 328: 28–44
 - 28 Zinger A, Koren L, Adir O, *et al.* Collagenase nanoparticles enhance the penetration of drugs into pancreatic tumors. *ACS Nano*, 2019, 13: 11008–11021
 - 29 Yuan F, Leunig M, Huang SK, *et al.* Microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumor xenograft. *Cancer Res*, 1994, 54: 3352–3356
 - 30 Huo D, Jiang X, Hu Y. Recent advances in nanostrategies capable of overcoming biological barriers for tumor management. *Adv Mater*, 2019, 32: 1904337
 - 31 Zhou Q, Dong C, Fan W, *et al.* Tumor extravasation and infiltration as barriers of nanomedicine for high efficacy: The current status and transcytosis strategy. *Biomaterials*, 2020, 240: 119902
 - 32 Blanco E, Shen H, Ferrari M. Principles of nanoparticle design for overcoming biological barriers to drug delivery. *Nat Biotechnol*, 2015, 33: 941–951
 - 33 Yang X, Hu C, Tong F, *et al.* Tumor microenvironment-responsive dual drug dimer-loaded PEGylated bilirubin nanoparticles for improved drug delivery and enhanced immune-chemotherapy of breast cancer. *Adv Funct Mater*, 2019, 29: 1901896
 - 34 Sanmamed MF, Chen L. Inducible expression of B7-H1 (PD-L1) and its selective role in tumor site immune modulation. *Cancer J*, 2014, 20: 256–261
 - 35 Ruan S, Xie R, Qin L, *et al.* Aggregable nanoparticles-enabled chemotherapy and autophagy inhibition combined with anti-PD-L1 antibody for improved glioma treatment. *Nano Lett*, 2019, 19: 8318–8332

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Conflict of interest The authors declare that they have no conflict of interest.

Supplementary information Supporting data are available in the online version of the paper.

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主动穿细胞转运的定向纳米载体用于化学-免疫治疗抑制肿瘤生长及转移

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摘要 目前免疫治疗已经显示出了极大的肿瘤治疗潜力, 然而纳米载体较低的转运能力以及肿瘤免疫响应性低等问题严重阻碍了免疫治疗的临床应用. 为了解决这些问题, 本课题研制了一种具有主动穿细胞转运能力的定向纳米载体用于化疗增强的免疫治疗. 当该纳米载药系统到达肿瘤部位, 金属基质蛋白酶2响应的纳米外壳崩解, 释放出带有正电荷的纳米内核. 正电荷促使纳米内核产生吸附介导的胞吞, 进而促进纳米载药系统的跨血管内皮细胞运输和跨细胞药物递送, 并最终将药物递送到远端肿瘤细胞中. PD-L1抗体和化疗药物分别被载于纳米载体的外壳和内核中进行精准递送, 从而达到对具有不同治疗靶点的药物同步递送、定向释药的目的. 肿瘤微环境中释放的PD-L1抗体作用于T细胞表面的特异性受体, 阻断了T细胞与肿瘤细胞的结合. 正电荷纳米内核同步运载奥沙利铂和吡哆美辛进入深层肿瘤细胞, 在整个肿瘤组织中引发免疫原性死亡、逆转免疫抑制作用, 招募大量的T细胞并最终增强免疫治疗疗效. 本研究中所构建的纳米载药体系不仅能够抑制原位瘤生长, 还能够阻止肿瘤的转移. 这为化疗增强的免疫治疗提供了一种新的纳米递送方案, 提高了免疫治疗在免疫响应率低的肿瘤中的疗效.