



LRP-1/CD44-targeted regorafenib nano-delivery system leveraging anti-angiogenesis and synergistic cytotoxicity against peritoneal metastasis of colorectal cancer

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ABSTRACT

Peritoneal metastasis of colorectal cancer (PM-CRC) represents a major therapeutic challenge in advanced disease, where aberrant tumor vasculature contributes to poor prognosis. To address the pharmacological limitations of regorafenib (REG), this study developed a dual-receptor-targeted nanoplatfrom (REG@LFHA NPs) that leverages the characteristic overexpression of LRP-1 and CD44 receptors in the colorectal cancer tumor micro-environment. The nanoplatfrom was engineered through nanoprecipitation and electrostatic self-assembly, incorporating lactoferrin for LRP-1 targeting and hyaluronic acid for CD44 recognition. REG@LFHA NPs exert multifaceted antitumor effects through three coordinated mechanisms: potent suppression of tumor vasculature through VEGF-VEGFR pathway blockade, effectively disrupting blood and oxygen supply to induce tumor necrosis; direct tumor cytotoxicity via REG-mediated apoptosis and cell cycle arrest; and immune microenvironment remodeling through macrophage repolarization from pro-tumor M2 to antitumor M1 phenotypes. In PM-CRC models, REG@LFHA NPs demonstrated significantly enhanced tumor accumulation and therapeutic efficacy compared to free REG. Furthermore, the nanoplatfrom showed remarkable synergy with oxaliplatin, the first-line chemotherapeutic agent for PM-CRC, producing superior treatment outcomes through complementary mechanisms of action. This study not only establishes REG@LFHA NPs as an effective dual-targeting nanomedicine but also demonstrates their strong potential for clinical translation, particularly in combination with standard chemotherapy regimens for advanced peritoneal metastatic colorectal cancer.

1. Introduction

Colorectal cancer (CRC) represents one of the most common malignancies globally, whose adverse clinical outcomes are principally attributable to metastatic spread [1–3]. Peritoneal metastases (PM) occur in 20–25 % of patients with advanced CRC and portend an inferior prognosis, with 5-year survival rates consistently reported below 15 % [4–6]. These sobering clinical outcomes underscore the imperative for developing more effective treatment modalities.

The pathogenesis of peritoneal metastasis of colorectal cancer (PM-

CRC) involves a complex interplay between tumor cells and their microenvironment. A key pathological feature is the establishment of an abnormal vascular network that not only sustains tumor growth through nutrient supply but also facilitates metastatic dissemination [7–9]. This dysregulated angiogenesis creates a self-reinforcing cycle that promotes both local progression and peritoneal seeding.

Targeting this pathological characteristic, anti-angiogenic therapy has become an important strategy for PM-CRC. Vascular endothelial growth factor/vascular endothelial growth factor receptor (VEGF/VEGFR) pathway inhibitors represented by bevacizumab, ramucirumab,

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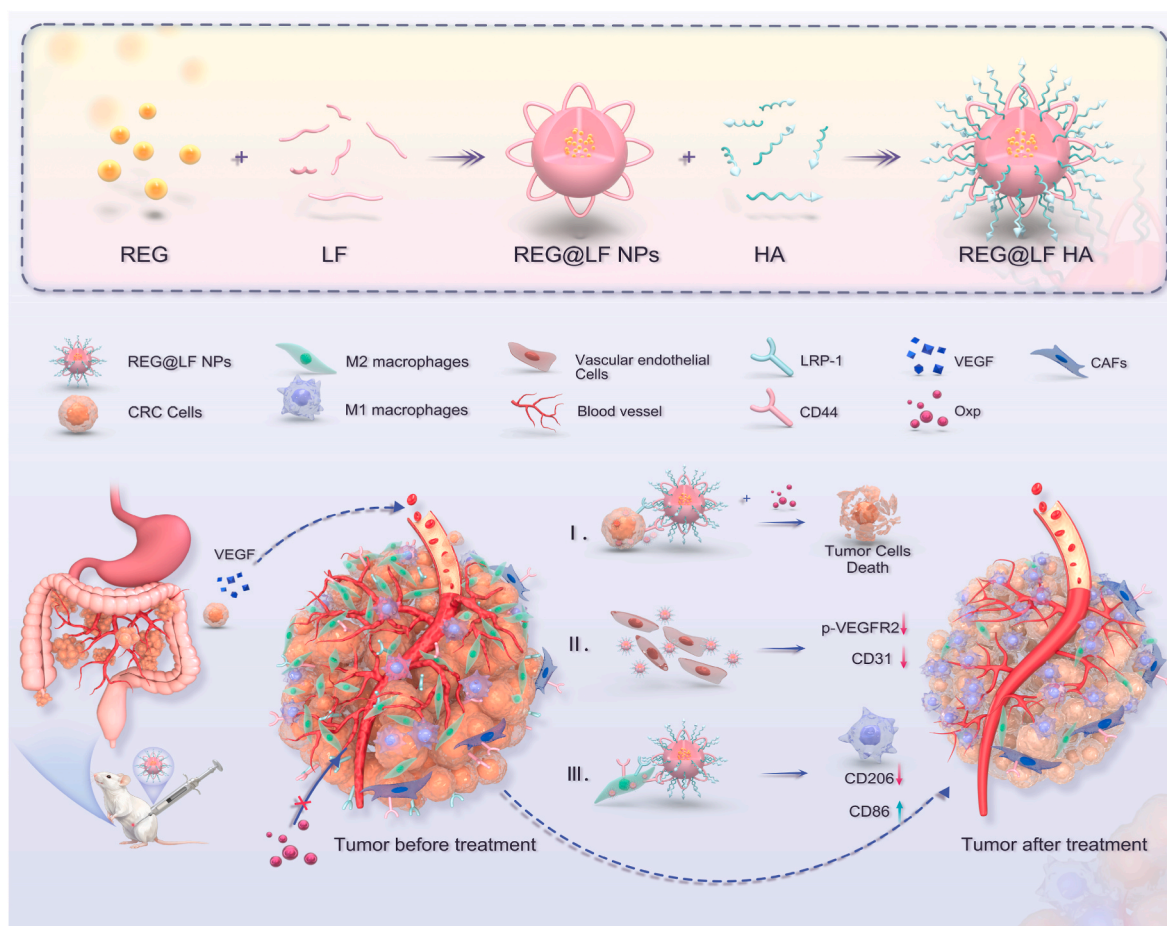
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Scheme 1. Schematic illustration of the design of REG@LFHA NPs and their synergistic therapeutic mechanism against PM-CRC: (I) Combines with Oxp to kill tumor cells; (II) Targets and prunes pathological vasculature through blockade of the VEGF-VEGFR pathway; (III) Reprograms M2 macrophages toward tumoricidal M1 phenotypes to reverse immunosuppression.

and aflibercept have demonstrated efficacy in clinical applications. Against this background, the oral multi-target inhibitor regorafenib (REG) has garnered widespread attention due to its unique mechanism of action. This drug not only effectively blocks the VEGF/VEGFR signaling pathway but also simultaneously targets multiple kinase receptors involved in tumor progression, demonstrating significant multi-target anti-tumor properties[10–12]. From the perspective of its mechanism of action, REG exerts anti-tumor effects through multiple pathways. Its potent anti-angiogenic effect significantly disrupts tumor blood and oxygen supply, while the drug can also directly induce tumor cell apoptosis and cell cycle arrest. Furthermore, recent studies have revealed that REG possesses unique immunomodulatory functions, capable of reprogramming immunosuppressive M2 macrophages into tumor-killing M1 phenotypes, thereby remodeling the tumor immune microenvironment [13,14].

However, despite REG's broad-spectrum anti-tumor activity, its therapeutic efficacy is significantly constrained by poor solubility and low permeability, which collectively impair systemic bioavailability and tumor drug accumulation. Moreover, the drug's off-target distribution induces dose-limiting toxicities such as hand-foot syndrome, consequently limiting its clinical applicability through necessitated dose reductions or treatment discontinuation. While intraperitoneal administration partially circumvents first-pass metabolism and enhances locoregional drug exposure, this approach remains suboptimal due to rapid peritoneal clearance and inefficient tumor penetration, ultimately often failing to achieve therapeutic drug concentrations at target sites[15–17].

To overcome these challenges, nanotechnology has demonstrated

remarkable potential in enhancing the delivery efficiency of various conventional chemotherapeutic and targeted agents[18–24]. The identification and utilization of key receptors represent a crucial strategy for achieving precise targeted delivery and consequently improving therapeutic outcomes. Researchers have developed multiple nanoplateforms based on the characteristic overexpression of receptors such as low-density lipoprotein receptor-related protein-1 (LRP-1), CD44, CD206, and integrin $\alpha\beta 3$ in tumor cells and various tumor microenvironment (TME) components, significantly improving intratumoral drug delivery efficiency[25–31].

For instance, LRP-1 shows marked overexpression in cancer-associated fibroblasts (CAFs) and M2-type tumor-associated macrophages (TAMs) [32,33]. Its high-affinity ligand, lactoferrin (LF), serves as an ideal carrier for constructing anti-tumor nanodelivery systems, not only mediating active targeting but also reprogramming pro-tumoral M2 macrophages into anti-tumor M1 phenotypes[34–36]. Similarly, the CD44 receptor is widely overexpressed on tumor cells, cancer stem cells, and various stromal cells. Its natural ligand, hyaluronic acid (HA), can effectively promote nanoparticle accumulation and retention in tumor tissues[15,37–39].

Based on these theoretical foundations, our study developed an innovative LF-HA hybrid nanoplateform (REG@LFHA NPs) for REG delivery. This platform was engineered through the strategic integration of LF's drug loading capacity with its inherent electrostatic interactions with HA [34,40,41]. The rationally designed nanosystem combines the complementary biological functions of both components: the LF component mediates tumor-targeted delivery through LRP-1 recognition and effectively alleviates the immunosuppressive TME via

macrophage polarization, while the HA coating enhances tumor accumulation through CD44 receptor-mediated active targeting.

To further enhance REG's therapeutic efficacy and promote its clinical translation, we systematically evaluated the combined therapeutic effects of this nano-system with the first-line clinical drug oxaliplatin (OxP). As a third-generation platinum compound, OxP induces tumor cell DNA interstrand cross-links through the formation of DNA adducts, blocking DNA replication and transcription processes, and ultimately triggering apoptosis. It serves as a core component of standard chemotherapy regimens for PM-CRC. In clinical practice, the sequential or combined use of REG and OxP has become an important treatment strategy for advanced CRC, particularly suitable for patient populations who have failed previous treatments or require intensified therapy [6, 42]. However, traditional combination regimens of the two drugs still face challenges such as pharmacokinetic mismatch and superimposed toxic side effects.

In preclinical models of PM-CRC, REG@LFHA NPs demonstrated comprehensive anti-tumor activity through multiple synergistic mechanisms, including disruption of nutrient supply and inhibition of metastatic dissemination via suppression of tumor angiogenesis, coupled with suppression of tumor cell proliferation and induction of apoptotic cell death. Additionally, the combined immunomodulatory effects of LF and REG work in concert to reverse the immunosuppressive TME. Importantly, this multifunctional nanoplatform combined with OxP exhibited significantly enhanced therapeutic efficacy, validating not only the compatibility of the nano-system with conventional chemotherapy but also demonstrating the unique value of nanotechnology-modified REG in clinical combination treatment regimens, thereby highlighting its strong translational potential for the clinical management of PM-CRC (Scheme 1).

2. Methods

2.1. Materials and animals

Regorafenib (REG), coumarin-6 (C6), and oxaliplatin (OxP) were purchased from Aldrich (Shanghai, China). DiR (1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide) and fetal bovine serum (FBS) were obtained from Yeasen (Shanghai, China). Bovine lactoferrin (LF) was sourced from Shanghai Yuanye Bio-Technology (Shanghai, China). Hyaluronic acid (HA, 100 kDa) was provided by Bloomage Biotech (Jinan, China). Macrophage colony-stimulating factor (M-CSF) and interleukin-4 (IL-4) were acquired from UA BIOSCIENCE (Nanjing, China). Cell Counting Kit-8 (CCK-8) was procured from LABLEAD (Beijing, China). The Calcein/PI Cell Viability/Cytotoxicity Kit, Annexin V-FITC/PI Apoptosis Detection Kit, RIPA Lysis Buffer, BCA Protein Assay Kit, and enhanced chemiluminescence (ECL) substrate were supplied by Beyotime Biotechnology (Shanghai, China). D-Luciferin potassium salt was purchased from MeilunBio (Dalian, China), and prestained protein molecular weight markers were obtained from Servicebio (Wuhan, China). Ceturegel® LDEV-Free Matrix Gel was sourced from Yeasen (Shanghai, China). The protease inhibitor cocktail was provided by MedChemExpress (Monmouth Junction, NJ, USA). DMEM high-glucose medium, penicillin-streptomycin solution (100 ×), and phosphate-buffered saline (PBS) were obtained from Gibco (Grand Island, NY, USA). Endothelial Cell Medium (ECM) was purchased from ScienCell (Carlsbad, CA, USA). APC-conjugated anti-mouse F4/80 (rat source), FITC-conjugated anti-mouse CD206 (rat source), and PE-conjugated anti-mouse CD86 (rat source) were obtained from BioLegend (San Diego, CA, USA). Rabbit anti-mouse LRP-1 polyclonal antibody and mouse anti-mouse CD44 monoclonal antibody were purchased from Abiowell (Changsha, China). Rabbit anti-mouse CD31 polyclonal antibody and rabbit anti-mouse CD206 polyclonal antibody were acquired from Servicebio (Wuhan, China).

Female BALB/c mice (6–8 weeks old) were purchased from Chengdu Dashuo Biotechnology Co., Ltd. (Chengdu, China) and housed under

specific pathogen-free (SPF) conditions with ad libitum access to sterile irradiated rodent diet and autoclaved water. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the State Key Laboratory of Biotherapy, Sichuan University (Approval No.: 20241127008) and conducted by the ARRIVE guidelines.

2.2. Preparation of REG@LF NPs and REG@LFHA NPs

REG@LF NPs were prepared using a solvent displacement method. Briefly, 10 mg of LF was dissolved in 10 mL of ultrapure water under continuous magnetic stirring (1000 rpm). To this solution, 120 µL of REG in DMSO (10 mg/mL) was added dropwise. The mixture was stirred for an additional 10 min to ensure complete nanoparticle formation. Free REG was then removed by ultracentrifugation (MWCO: ≤100 kDa) to obtain purified REG@LF NPs. For the preparation of REG@LFHA NPs with varying HA/LF mass ratios, different volumes (100 µL, 250 µL, 500 µL, 1 mL, or 2 mL) of HA (100 kDa) aqueous solution (10 mg/mL) were added dropwise to 10 mL of the prepared REG@LF NPs dispersion. The mixtures were stirred at room temperature for 1 h to allow HA coating, followed by ultrafiltration centrifugation (MWCO: 100 kDa) to remove unbound REG and HA, yielding the final REG@LFHA NPs.

2.3. Characterization of REG@LFHA NPs

The physicochemical properties of REG@LF NPs and REG@LFHA NPs with different HA modification ratios (10 %, 25 %, 50 %, 100 % and 200 % w/w relative to LF) were systematically characterized. Hydrodynamic diameter, polydispersity index (PDI), and zeta potential were measured by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS instrument at 25 °C, with all measurements performed in triplicate and reported as mean values ± standard deviation. Morphological examination was conducted by transmission electron microscopy (TEM, Tecnai G2 F20 S-TWIN) after negative staining with 2 % phosphotungstic acid. Crystalline properties were analyzed by X-ray diffraction (XRD, Rigaku Ultima IV) using Cu Kα radiation ($\lambda = 1.54060 \text{ \AA}$) at 40 kV and 40 mA, with scanning from 5° to 85° (2θ) at a rate of 5°/min. XRD patterns were obtained for raw REG material, pure LF, pure HA, a physical mixture of REG, LF, and HA, and lyophilized REG@LFHA NPs to evaluate crystallinity changes during nanoparticle formation.

2.4. Determination of Drug Loading (DL) and Encapsulation Efficiency (EE)

The REG content in REG@LFHA NPs was quantified using UV–Vis spectrophotometry. Briefly, an aliquot of purified REG@LFHA NPs dispersion was mixed with anhydrous methanol to disassemble the nanoparticle structure and dissolve the encapsulated REG. After centrifugation at 12,000 rpm for 10 min, the absorbance of the supernatant was measured at 263 nm (λ_{max} of REG) using anhydrous methanol as a blank. The REG concentration was determined from a pre-established standard curve of REG in anhydrous methanol (concentration range: 0–10 µg/mL, $R^2 > 0.999$). All measurements were performed in triplicate, and results were expressed as mean ± standard deviation.

2.5. Cell culture

The mouse colon carcinoma cell line CT26-luc (stably expressing luciferase) was cultured in DMEM supplemented with 10 % (v/v) fetal bovine serum (FBS) and 1 % (v/v) penicillin-streptomycin solution (containing 100 U/mL penicillin and 100 µg/mL streptomycin). Human umbilical vein endothelial cells (HUVECs) were maintained in endothelial cell-specific medium containing 10 % (v/v) FBS and 1 % (v/v) penicillin-streptomycin solution.

Bone marrow-derived macrophages (BMDMs) were generated by aseptically isolating bone marrow cells from the femurs and tibias of 6–8 weeks old male C57BL/6 mice under specific pathogen-free (SPF)

conditions. After red blood cell lysis, cells were resuspended in DMEM medium with 10 % (v/v) FBS and 1 % (v/v) penicillin streptomycin, then seeded in 12-well plates at a density of 1×10^6 cells/well. Cells were differentiated into unpolarized macrophages (M0) by culturing for 72 h in the presence of 20 ng/mL macrophage colony-stimulating factor (M-CSF) at 37 °C in a humidified 5 % CO₂ incubator. For M2 macrophage polarization, the medium was replaced with fresh medium containing 20 ng/mL interleukin-4 (IL-4) for an additional 24 h.

2.6. Cellular uptake and tumor spheroid penetration

For monolayer cellular uptake studies, CT26-luc, HUVEC, and BMDM cells were seeded in 12-well plates and cultured until reaching 70–80 % confluence. The cells were divided into the following treatment groups and incubated in serum-free medium for 2 h: C6-labeled LF NPs or C6-labeled LFHA NPs. To investigate receptor-mediated competitive inhibition, two additional groups of CT26-luc cells were pre-treated with an excess of free LF (500 µg/mL) or HA (500 µg/mL) for 0.5 h before the addition of C6@LFHA NPs. After incubation, all cells were washed three times with cold PBS to remove unbound C6. Cellular uptake efficiency across the different cell lines and treatment groups was quantitatively analyzed using flow cytometry (NovoCyte, Agilent, China). Intracellular distribution and competitive inhibition effects in CT26-luc cells were further counterstained with Hoechst 33342 for 15 min visualized by confocal laser scanning microscopy (CLSM; A1R, Nikon, Japan).

For tumor spheroid penetration studies, CT26-luc multicellular tumor spheroids (MCTS) were prepared using a 3D cell culture coating kit (Beyotime). Briefly, CT26-luc cells were seeded at 1×10^4 cells/well in U-bottom 96-well plates pre-coated with the coating solution and cultured for 5 days at 37 °C with 5 % CO₂ to form compact and uniformly-sized MCTS. The formed MCTS were then treated with C6@LF NPs or C6@LFHA NPs for 24 h. To investigate receptor-mediated competitive inhibition, some MCTS were pre-treated with excess free LF (500 µg/mL) or HA (500 µg/mL) for 0.5 h before adding C6@LFHA NPs. After treatment, MCTS were gently washed three times with PBS. Z-stack imaging was performed using a confocal microscope (IXplore SpinSR, Olympus) to analyze nanoparticle penetration at different depths of the spheroids.

2.7. CCK-8 assay

CT26-luc cells or HUVECs were seeded in 96-well plates at a density of 5000 cells/well (100 µL complete medium) and cultured overnight for adherence. After removing the medium, fresh medium (100 µL/well) containing different concentrations of REG@LFHA NPs or Oxp was added, followed by incubation for 48 h. Subsequently, 100 µL of fresh medium containing 10 % (v/v) CCK-8 reagent was added to each well, and the plates were incubated at 37 °C for 1 h. The absorbance (OD450) was measured using a microplate reader. Cell viability was calculated relative to untreated controls (set as 100 %). Experiments were independently repeated at least three times.

2.8. Apoptosis/necrosis assessment

Fluorescence Microscopy (Calcein AM/PI Staining): CT26-luc cells or HUVECs were seeded in confocal dishes at 2×10^5 cells/dish (2 mL medium) and cultured overnight. After treatment with REG for 24 h, cells were washed twice with PBS and stained with 2 µM Calcein AM (green fluorescence, live cells) and 4.5 µM propidium iodide (PI, red fluorescence, dead cells) in PBS for 15–30 min at room temperature in the dark. After washing, images were acquired using an upright fluorescence microscope (Axio Imager M2, ZEISS).

Flow Cytometry (Annexin V-FITC/PI Staining): Cells were seeded in 12-well plates (1×10^5 cells/well, 1 mL medium) and treated with drugs for 24 h. Suspended and adherent cells were collected, washed with cold PBS, and stained using an Annexin V-FITC/PI Apoptosis Detection Kit

(Liji Bio) for 15 min in the dark. Apoptosis was analyzed immediately by flow cytometry (NovoCyte, ACEA). Experiments were repeated at least three times.

2.9. Cell cycle analysis

CT26-luc cells or HUVECs (1×10^5 cells/well) were treated with REG@LFHA NPs for 24 h, harvested, and fixed in cold 70 % ethanol at 4 °C overnight. After washing, cells were resuspended in 500 µL PBS containing 50 µg/mL PI and 200 µg/mL RNase A, incubated for 15 min in the dark, and analyzed by flow cytometry (NovoCyte, ACEA).

2.10. In vitro anti-angiogenic assay

In the tube formation assay, HUVECs and SVEC4-10 cells were plated onto Matrigel® (1 % w/v, Corning)-coated 24-well plates at the following densities: 9×10^4 cells/well for HUVECs and 15×10^4 cells/well for SVEC4-10 and cultured in complete endothelial cell medium containing different concentrations of REG (1, 2.5 and 5 µM). After incubation at 37 °C with 5 % CO₂ for 8 h, tube-like structures were observed and photographed using an inverted fluorescence microscope, followed by quantitative analysis with ImageJ Angiogenesis Analyzer. For VEGFR2 signaling analysis, performed according to the method of Wilhelm et al. [12]. With minor modifications, HUVECs were pretreated with REG@LFHA NPs for 2 h, followed by VEGF stimulation (30 ng/mL, 20 min). Subsequently, VEGFR2 and pVEGFR2 expression levels in cell lysates were assessed by Western blot analysis.

2.11. BMDM polarization analysis

M2 polarized BMDMs, prepared as described in Section 2.5, were treated for 24 h with LPS (100 ng/mL), LF (100 µg/mL), free REG (7.5 µM), or equivalent concentrations of REG@LFHA NPs. After treatment, cells were collected and washed twice with cold PBS, then stained with fluorescently-labeled antibodies against F4/80, CD206, and CD86 for 30 min at room temperature in the dark. Following a further two washes, the cells were resuspended in buffer and analyzed by flow cytometry (NovoCyte, ACEA) to determine the percentages of M1 (F4/80⁺CD86⁺) and M2 (F4/80⁺CD206⁺) macrophage populations. Culture supernatants were collected for measurement of IL-6 and TNF-α levels using ELISA kits. All experiments were independently repeated at least three times.

2.12. Establishment of the CT26-luc PM-CRC model

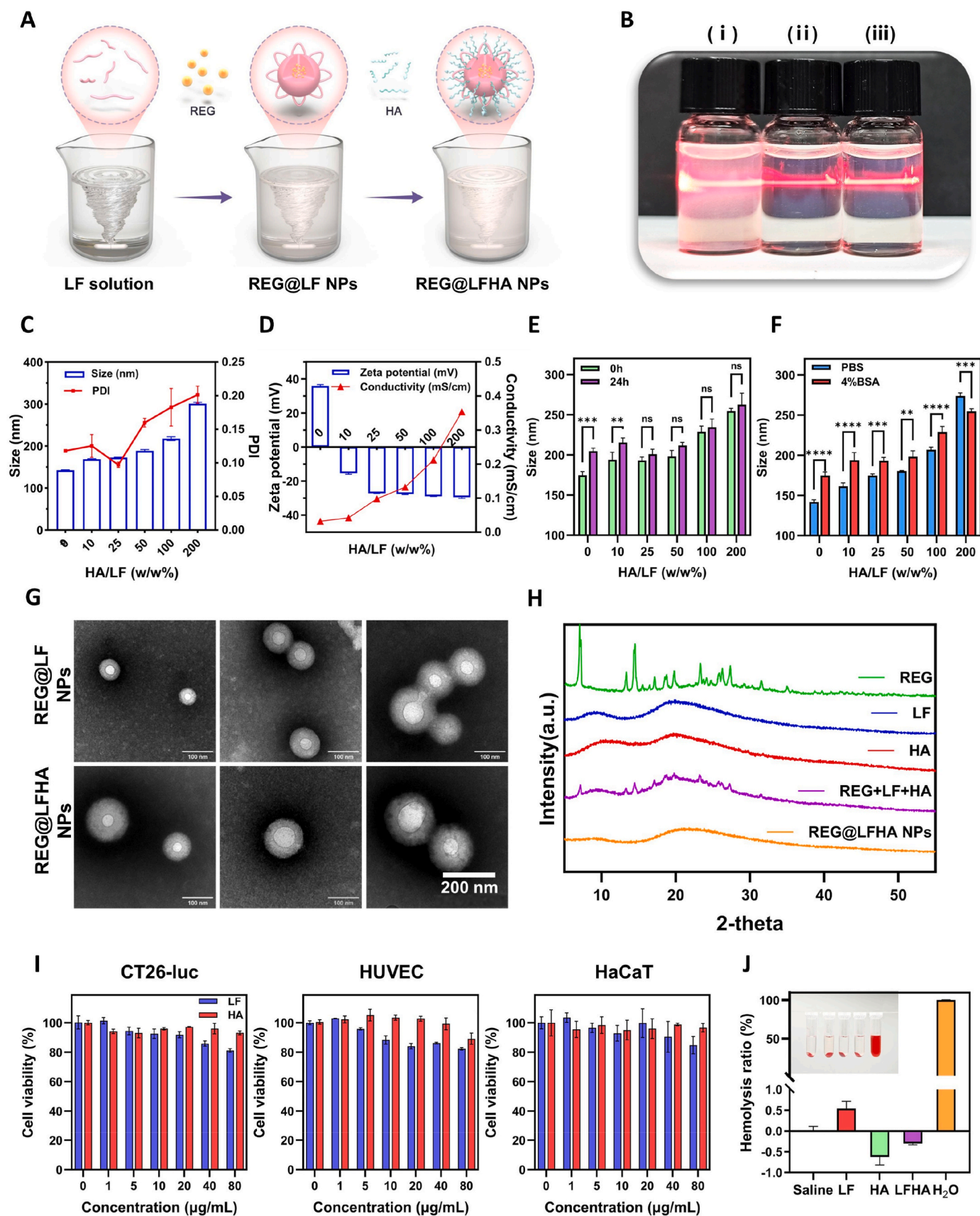
The PM-CRC model was established by intraperitoneal injection (i. p.) of CT26-luc cells (5×10^5 cells/mouse) into 8-week-old female Balb/c mice. Successful establishment of the PM-CRC model was confirmed by in vivo bioluminescence imaging (BLI) prior to subsequent experiments.

2.13. In vivo biodistribution

Tumor-bearing mice were randomly divided into groups receiving either free DiR, DiR-labeled LF NPs, or LFHA NPs at an equivalent dose of 0.5 mg/kg via i.p. In vivo distribution was monitored at predetermined time points (2, 4, 8, 12, and 24 h) using the AniView 100 imaging system. At 24 h post-injection, mice were sacrificed, and major organs (heart, liver, spleen, lungs, kidneys), along with peritoneal tumors, were excised for ex vivo imaging. Radiant efficiency of regions of interest (ROI) was quantified using the accompanying software.

2.14. Immunofluorescence Co-staining for LRP-1 and CD44

Paraffin-embedded sections (5 µm) of tumor tissues and major organs (heart, liver, spleen, lungs, kidneys, intestine) were subjected to antigen retrieval in citrate buffer. After blocking with 5 % BSA, sections



(caption on next page)

Fig. 1. Physicochemical characterization and biocompatibility evaluation of REG@LF NPs and REG@LFHA NPs. (A) Schematic illustration of the preparation process for REG@LFHA NPs. (B) Photographs demonstrating the colloidal stability of (i) free REG, (ii) REG@LF NPs, and (iii) REG@LFHA NPs under laser irradiation. (C) Hydrodynamic diameter and polydispersity index (PDI) of nanoparticles prepared with different HA/LF mass ratios. (D) Zeta potential and conductivity of nanoparticles formulated with varying HA/LF mass ratios. (E) Comparison of the hydrodynamic diameter of nanoparticles (different HA/LF mass ratios) in PBS containing 4 % BSA at 0 h and 24 h. (F) Hydrodynamic diameter of nanoparticles after 1 h incubation in PBS with or without 4 % BSA. (G) Representative TEM images of REG@LF NPs (upper panel) and REG@LFHA NPs (lower panel). Scale bar = 200 nm. (H) XRD patterns of the raw materials and the formulated nanoparticles, indicating their crystalline structures. (I) In vitro cytotoxicity assessment of LF and HA using the CCK-8 assay, including CT26-luc cells, HUVECs, and HaCaTs. (J) Hemolysis assay of LF and HA. The photograph and quantitative analysis demonstrate their hemocompatibility. All statistical data are represented as mean $\pm$ SD ($n = 3$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

were co-incubated with primary antibodies against LRP-1 (rabbit, 1:250) and CD44 (rat, 1:200) overnight at 4 °C. Following washes, binding was detected using a mixture of Alexa Fluor 594 anti-rabbit and Alexa Fluor 488 anti-rat secondary antibodies (1:500). Nuclei were visualized with DAPI, and imaging was performed on an Olympus VS200 system.

2.15. In vivo anti-tumor effects

The PM-CRC model was established by i.p. of CT26-luc cells into BALB/c mice as described in Section 2.12. Following confirmation of successful model establishment by bioluminescence imaging, mice were randomly allocated into six treatment groups on day 6 post-inoculation. ($n = 4$): PBS, free REG (10 mg/kg), REG@LF NPs (10 mg/kg REG equivalent), REG@LFHA NPs (10 mg/kg REG equivalent), OxP (4 mg/kg), and combination treatment of OxP and REG@LFHA NPs (4 mg/kg OxP and 10 mg/kg REG equivalent). All treatments were administered via i.p. every other day for a total of four doses. Tumor progression was monitored by bioluminescence imaging (AniView 100) at baseline and study endpoint. Mice received an i.p. of D-luciferin potassium salt (150 mg/kg) 10–15 min before imaging. Total flux (photons/s) within tumor regions was quantified using the imaging software. Body weights were recorded every other day to assess treatment toxicity. At humane endpoints, mice were euthanized for tumor collection and weighing. Major organs and tumor samples were fixed in 4 % PFA for subsequent histopathological and immunofluorescence analyses.

2.16. Immunofluorescence staining

Tumor tissues were fixed in 4 % PFA for 24 h, paraffin-embedded, and sectioned into 5 μ m thickness for immunofluorescence staining. For the simultaneous detection of microvessels and macrophages, sections were incubated with a mixture of rabbit anti-CD31 (1:200, AbioWell) and rabbit anti-CD206 (1:300, Servicebio) primary antibodies at 4 °C overnight. After washing, the sections were incubated with appropriate Alexa Fluor-conjugated secondary antibodies (1:500) at room temperature for 1 h. To assess hypoxia levels within the tumor microenvironment, the expression of HIF-1 α was detected on separate sections. These sections were subjected to antigen retrieval followed by incubation with a rabbit anti-HIF-1 α primary antibody (1:250, Abcam) at 4 °C overnight, and then with an Alexa Fluor-conjugated secondary antibody (1:500) at room temperature for 1 h.

For all staining procedures, nuclei were counterstained with DAPI (1 μ g/mL). Slides were scanned using an Olympus VS200 system, and microvessel density (MVD), CD206⁺ cell infiltration, and HIF-1 α positive areas were quantified with ImageJ software.

2.17. Flow cytometric analysis of M1 macrophages in tumor tissues

To analyze the infiltration of M1 macrophages in tumor tissues, flow cytometry was performed. Briefly, tumor tissues from tumor-bearing mice were collected and digested with an enzyme cocktail containing collagenase, hyaluronidase, and DNase at 37 °C for 30–45 min to prepare single-cell suspensions. After red blood cell lysis, cells were washed twice with cold PBS. The cells were then resuspended in staining buffer and incubated with the following fluorescently-labeled antibodies for

cell surface staining: anti-CD45, anti-F4/80, and anti-CD86, for 30 min at room temperature in the dark. Following incubation, the cells were washed twice to remove unbound antibodies and finally resuspended in flow cytometry buffer. Data acquisition was carried out using a flow cytometer. The leukocyte population was first gated as CD45⁺ events, from which macrophages were identified as F4/80⁺ cells. M1 macrophages were defined as the F4/80⁺CD86⁺ population, and their percentages were quantified. All experiments were independently repeated at least three times.

2.18. Histopathology and biosafety evaluation

Tumor and major organs (heart, liver, spleen, lungs, kidneys) were fixed in 4 % PFA for 24 h. After the organs were embedded in paraffin, they were sliced into thin sections and stained with hematoxylin and eosin (H&E). The sections were then evaluated using a 3DHISTECH Panoramic system for pathological analysis.

Blood samples collected via retroorbital plexus were centrifuged (3,000g, 15 min, 4 °C) to obtain plasma. Liver function (ALT, AST) and renal function (UREA) parameters were measured using an automated biochemistry analyzer.

2.19. Statistical analysis

All data represent mean $\pm$ SD from ≥ 3 independent experiments. Statistical analyses were performed using GraphPad Prism 7.0. Multiple comparisons were analyzed by one-way ANOVA with Tukey's equal variance post-hoc tests. Significance levels, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3. Results and discussion

3.1. Preparation, characterization, and biocompatibility evaluation of REG@LF NPs and REG@LFHA NPs

REG@LF NPs were successfully prepared via a nanoprecipitation method. Subsequent electrostatic adsorption of HA onto the cationic REG@LF NPs yielded REG@LFHA NPs (Fig. 1A). Under laser illumination, free REG in aqueous solution showed strong light scattering and visible flocculent precipitates, whereas both REG@LF NPs and REG@LFHA NPs formed homogeneous colloidal dispersions with distinct Tyndall effects, confirming stable nanoformulations (Fig. 1B).

Dynamic light scattering (DLS) revealed that REG@LF NPs had a hydrodynamic diameter of 142.50 ± 0.87 nm and a zeta potential of 35.86 ± 0.85 mV (Fig. S1C). HA surface modification induced a charge reversal (to -29.46 ± 0.72 mV at 200 % HA/LF mass ratio) and increased particle size (to 301.07 ± 3.17 nm at 200 % HA/LF mass ratio), verifying successful HA coating (Fig. 1C and D) (Table S1).

Based on a comprehensive evaluation of colloidal stability, active targeting capability, and passive targeting potential, the 50 % HA/LF mass ratio was ultimately selected as the optimized REG@LFHA NPs formulation for subsequent investigations. This ratio achieved an optimal balance among these critical parameters.

Firstly, in stability assessments mimicking the ascites environment (using a 4 % BSA solution), the 50 % HA/LF NPs maintained excellent colloidal stability, with no statistically significant change in particle size

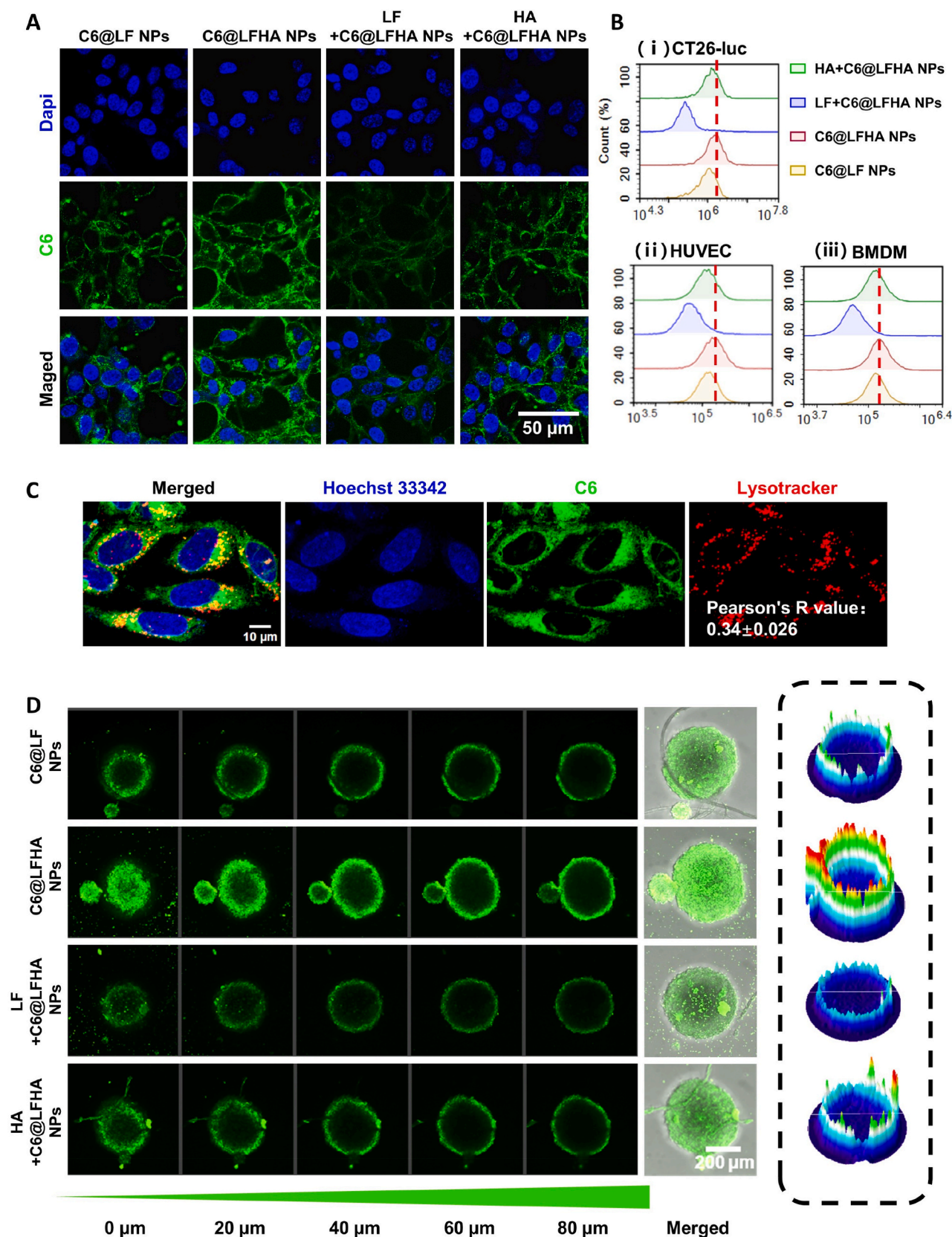
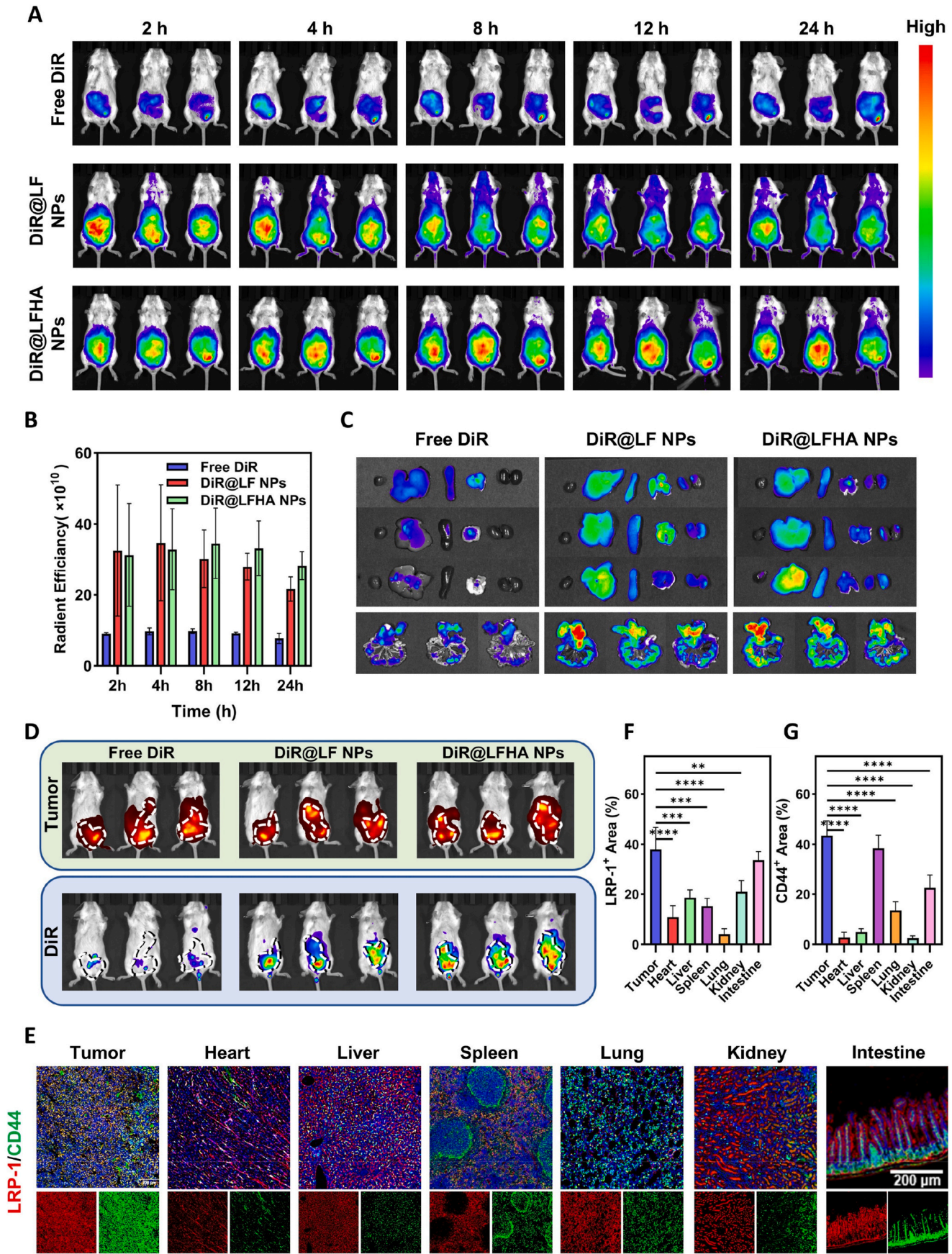


Fig. 2. Cellular uptake and tumor spheroid penetration. (A) Confocal microscopy images of CT26-luc cells after 2 h of incubation with C6@LF NPs, C6@LFHA NPs, and competitive inhibition groups pre-treated with free LF or HA. Scale bar: 50 μ m. (B) Flow cytometry histograms of (i) CT26-luc cells, (ii) human umbilical vein endothelial cells (HUVECs), and (iii) M2-polarized bone marrow-derived macrophages (M2-BMDMs) treated with C6@LF NPs, C6@LFHA NPs, and competitive inhibition groups. (C) Lysosomal co-localization of C6@LFHA NPs in HUVECs after 8 h incubation. Quantitative analysis showed a Pearson's correlation coefficient of 0.34 ± 0.026 . (D) Penetration of C6@LFHA NPs into CT26-luc tumor spheroids, as shown by Z-stack slices (Scale bar: 20 μ m), maximum intensity projection, and 3D reconstruction (generated using ImageJ). Scale bar: 200 μ m. All data are presented as mean $\pm$ SD (n = 3).



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Fig. 3. Biodistribution with Receptor Expression Analysis. (A) In vivo fluorescence imaging of tumor-bearing mice at different time points (2–24 h) after intra-peritoneal (i.p.) injection of free DiR, DiR@LF NPs, and DiR@LFHA NPs. (B) Quantitative analysis of the average fluorescence intensity in the abdominal region at various time points (equivalent DiR dose: 0.5 mg/kg). (C) Ex vivo fluorescence imaging of major organs (heart, liver, spleen, lungs, kidneys) and tumors excised at 24 h post-injection. The gastrointestinal tract is included. (D) (Upper panel) Bioluminescence imaging of tumor-bearing mice, indicating tumor location. (Lower panel) In vivo fluorescence imaging at 8 h post-injection of free DiR, DiR@LF NPs, and DiR@LFHA NPs. Tumor areas are delineated with dashed circles, showing significant co-localization of DiR fluorescence (lower panel) with bioluminescent tumor signals (upper panel) in the DiR@LF NPs and DiR@LFHA NPs groups. (E) Immunofluorescence staining of LRP-1 (red) and CD44 (green) in tumor tissues and major organs (heart, liver, spleen, lungs, kidneys, intestine). Scale bar: 200 μ m. (F) Quantitative statistical analysis of the LRP-1-positive area in various tissues from (E). (G) Quantitative statistical analysis of the CD44-positive area in various tissues from (E). All statistical data are represented as mean $\pm$ SD ($n = 3$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

after 24 h of incubation (Fig. 1E). The strong negative zeta potential (-27.59 ± 0.35 mV) provided electrostatic stabilization, while the moderate HA surface density conferred effective steric hindrance, collectively ensuring the long-term stability of the nanoparticles under complex physiological conditions. Notably, unlike NPs with lower HA ratios (0 %, 10 %), which showed significant aggregation in BSA, the 50 % ratio NPs effectively resisted protein-induced aggregation. Concurrently, compared to NPs with a very high HA ratio (such as 200 %) which completely suppressed interactions with BSA, the moderate initial size increase observed for the 50 % ratio NPs in BSA (Fig. 1F) suggested that the LF targeting moieties (LRP-1 ligands) on their surface were not entirely shielded by the HA chains and remained partially accessible and potentially functional.

Secondly, regarding active targeting capability, flow cytometry analysis demonstrated that C6@LFHA NPs with the 50 % HA/LF mass ratio exhibited the highest cellular uptake efficiency in CT26-luc cells (Fig. S1A). This result can be attributed to the sufficient HA surface density at this ratio for effective CD44-mediated targeting, coupled with the likely partial exposure and functional availability of LF's LRP-1 targeting sites, enabling synergistic targeting towards both CD44 and LRP-1 receptors.

Furthermore, the hydrodynamic diameter of 188.57 ± 2.84 nm (Fig. S1D) fell within the ideal size range (100–200 nm) for optimal tumor accumulation through the enhanced permeability and retention (EPR) effect, thereby maximizing the passive targeting potential of the nano-carrier system [44]. In conclusion, the REG@LFHA NPs with a 50 % HA/LF mass ratio integrate superior colloidal stability, efficient CD44/LRP-1 mediated active targeting, and promising passive targeting potential, thus being selected as the ideal nanopatform for further in vitro and in vivo studies.

Transmission electron microscopy imaging demonstrated spherical REG@LFHA NPs with a dry-state diameter of 138 ± 0.97 nm (smaller than the DLS measured hydrodynamic size due to solvation effects) (Fig. 1G). REG concentrations were quantified based on absorbance at 263 nm using the established standard curve in anhydrous methanol (Fig. S1E), from which the drug loading (DL) and encapsulation efficiency (EE) for REG@LFHA NPs were calculated as 9.33 ± 0.46 % and 77.8 ± 3.52 %. Respectively, X-ray diffraction (XRD) analysis confirmed the amorphous state of REG in REG@LFHA NPs. While distinct crystalline peaks were observed in both free REG and physical mixture groups, these characteristic peaks disappeared in the REG@LFHA NPs (Fig. 1H).

The excellent biocompatibility of the carrier materials system was systematically validated through comprehensive in vitro evaluations. CCK-8 assays demonstrated minimal cytotoxicity of LF and HA across multiple cell lines, including CT26-luc cells, HUVEC cells, and HaCaT cells (Fig. 1I), suggesting broad-spectrum cellular compatibility. Furthermore, hemolysis tests revealed outstanding blood compatibility of the nanoformulations. The hemolytic rates for both the carrier components (LF and HA) and the drug-loaded REG@LFHA NPs were very low (Fig. 1J and Fig. S1B). These collective findings provide compelling evidence for the exceptional biosafety profile of the LF and HA, meeting critical requirements for potential clinical translation.

3.2. Cellular uptake and tumor spheroid penetration

A comprehensive evaluation of LFHA NPs' cellular internalization was performed through multiple experimental approaches. Confocal microscopy revealed significantly enhanced fluorescence accumulation of C6@LFHA NPs in CT26-luc cells compared to C6@LF NPs (Fig. 2A, Fig. S2D), demonstrating superior cellular uptake efficiency. This uptake could be markedly inhibited by pretreatment with free HA or LF, confirming an internalization mechanism co-mediated by CD44 and LRP-1 receptors. Flow cytometric quantification (Fig. 2B, Fig. S2A) further validated the superior uptake of C6@LFHA NPs in key cellular components of the PM-CRC TME—including CT26-luc cells, HUVECs, and M2-polarized BMDMs—where they consistently exhibited the highest cellular uptake efficiency across the different cell types.

Lysosomal co-localization analysis in HUVECs (Fig. 2C, Fig. S2B and C) demonstrated significant lysosomal escape capability for both C6@LF NPs and C6@LFHA NPs after 8 h of incubation, as evidenced by low Pearson's correlation coefficients of 0.34 ± 0.045 and 0.34 ± 0.026 , respectively. The underlying mechanism for this observation likely resides in the unique composition of the nanoparticles: while C6@LF NPs primarily rely on their positively charged core to mediate escape via the “proton sponge effect” and direct membrane interaction in the acidic lysosomal environment, C6@LFHA NPs employ a more sophisticated cascade mechanism—the acidic pH of the lysosome triggers the dissociation of the HA shell, exposing the internal positively charged LF core, which subsequently exerts its membrane-disruptive activity [45,46]. This efficient lysosomal escape property ensures the effective cytoplasmic delivery of the therapeutic cargo, a critical prerequisite for subsequent drug activity.

Tumor spheroid penetration assays utilizing Z-stack scanning and 3D reconstruction (Fig. 2D, Fig. S2E), coupled with quantitative penetration depth analysis (Fig. S2F), confirmed the superior spatial distribution of C6@LFHA NPs compared to C6@LF NPs and competitive inhibition groups, characterized by deeper penetration depth and stronger fluorescence signals.

Collectively, these results validate the exceptional targeting and penetration capabilities of LFHA NPs, which originate from the synergistic combination of LRP-1/CD44 dual-receptor-mediated active targeting, pH-responsive endo/lysosomal escape properties, and the inherent advantages of the nano-delivery system.

3.3. Biodistribution with receptor expression analysis

To evaluate the in vivo targeting capability and biodistribution of LFHA NPs, we monitored the fluorescence distribution in a murine model of peritoneal metastasis after intraperitoneal injection of different DiR formulations. As shown in Fig. 3A and B, both DiR@LFHA NPs and DiR@LF NPs exhibited stronger fluorescence signals and longer retention time in the peritoneal tumor region compared to free DiR. Ex vivo organ imaging at 24 h (Fig. 3C) further demonstrated that DiR@LFHA NPs maintained the strongest fluorescence signal in tumor tissue while showing low distribution in major organs, indicating favorable tumor targeting and potential safety.

Real-time imaging at 8 h post-injection (Fig. 3D) provided more compelling evidence: the fluorescence signals of DiR@LF NPs and DiR@LFHA NPs showed significant spatial co-localization with

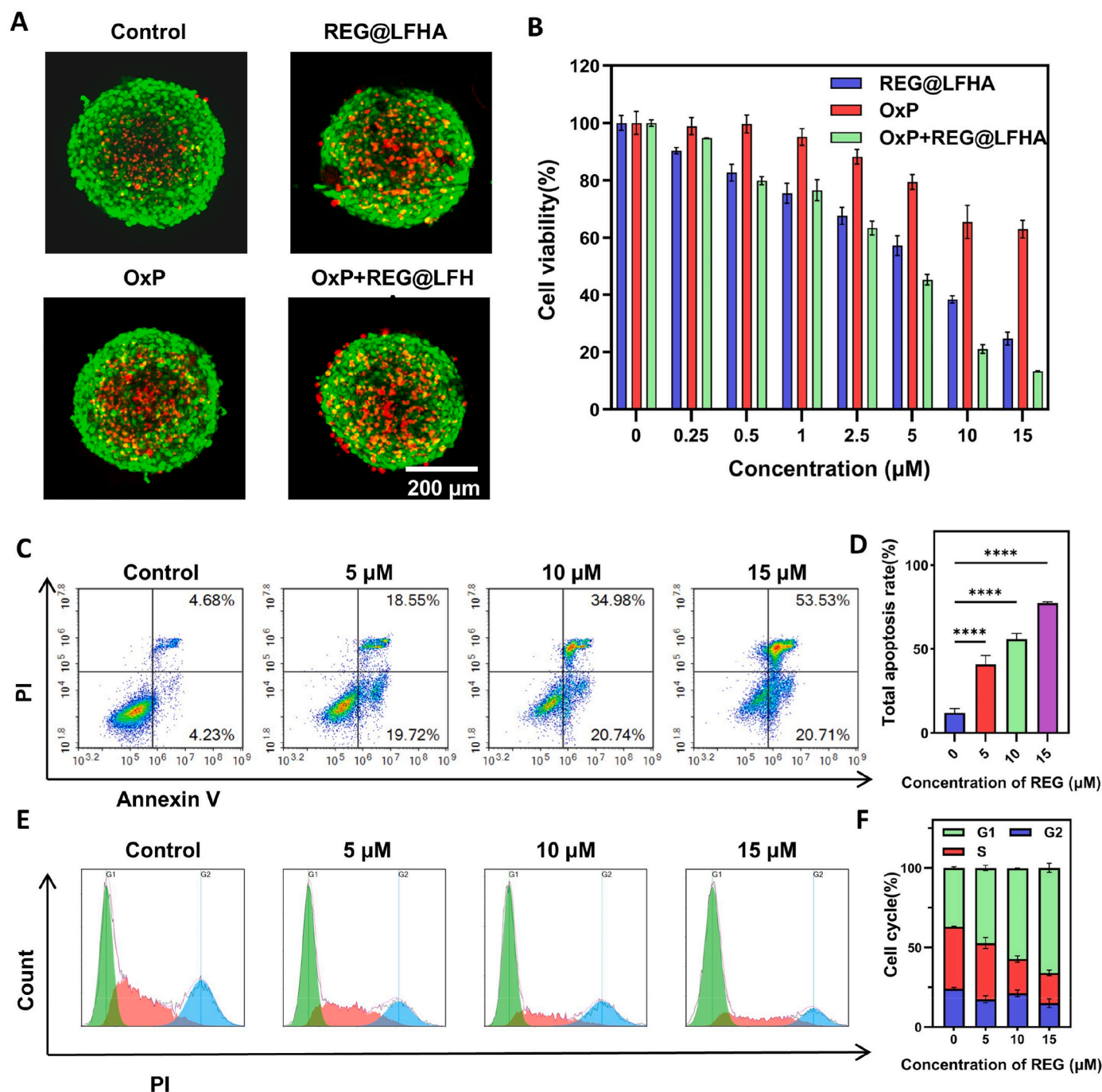
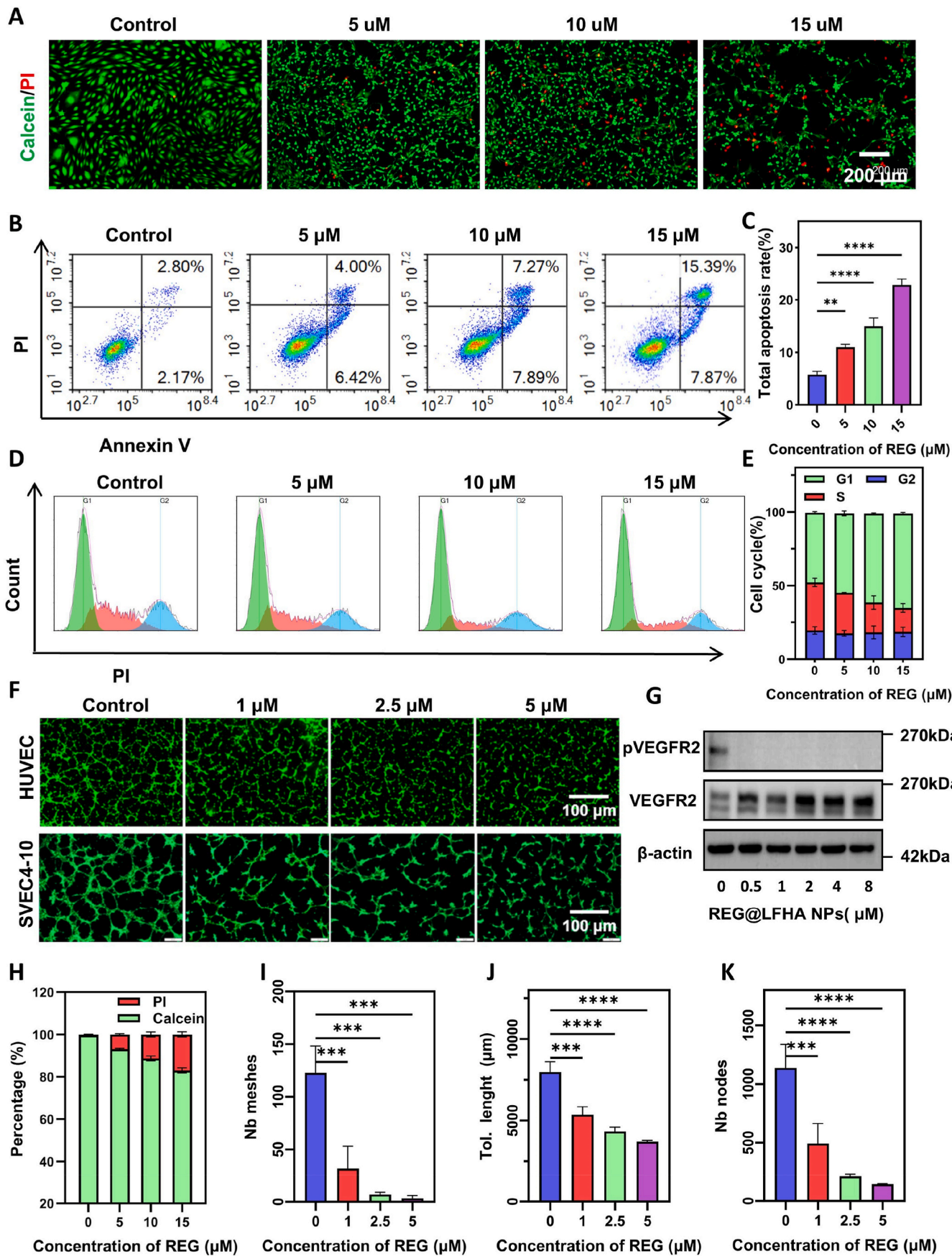


Fig. 4. In vitro anti-tumor activity. (A) Live/Dead staining (Calcein-AM, green for live cells; Propidium Iodide (PI), red for dead cells) of CT26-luc tumor spheroids after treatment with different formulations. Scale bar: 200 μm . (B) Cell viability of CT26-luc cells treated with OxP, REG@LFHA NPs, or their combination for 48 h, by the CCK-8 assay. REG@LF means REG@LFHA NPs, REG@LFHA means REG@LFHA NPs. (C) Apoptosis induction by gradient concentrations of REG@LFHA NPs, measured via Annexin V-FITC/PI flow cytometry. (D) Statistical analysis of the apoptosis rates quantified from (C). (E) Cell cycle distribution was analyzed by flow cytometry with PI staining. (F) Statistical analysis of the percentage of cells in each cell cycle phase (G1, S, and G2/M). All statistical data are represented as mean $\pm$ SD ($n = 3$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

bioluminescent tumor signals (indicating precise tumor location), while such specific distribution was absent in the free DiR group. This phenomenon directly demonstrates the active targeting capability of nanoparticles toward tumor tissue.

To elucidate the molecular mechanism of active targeting, we systematically evaluated the expression profiles of LRP-1 and CD44 receptors in tumor tissues and various normal organs. Immunofluorescence staining (Fig. 3E) and quantitative analysis (Fig. 3F and G) clearly showed that the expression levels of both LRP-1 and CD44

in tumor tissue were significantly higher than those in most normal organs. Specifically, the expression of LRP-1 in tumor tissue was 3.49-fold, 2.49-fold, 9.33-fold, 1.81-fold, 2.03-fold, and 1.13-fold higher than that in the heart, spleen, lungs, kidneys, liver, and intestine, respectively. The expression of CD44 in tumor tissue was 15.70-fold, 8.86-fold, 3.21-fold, 17.61-fold, 1.13-fold, and 1.93-fold higher than that in the heart, liver, lungs, kidneys, spleen, and intestine, respectively. Although basal LRP-1 expression was observed in the liver and intestine, and moderate CD44 expression was detected in the spleen and



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Fig. 5. In vitro anti-angiogenic activity and mechanisms. (A) Live/Dead staining (Calcein-AM, green for live cells; Propidium Iodide, red for dead cells) of HUVECs after 24 h of treatment with different formulations. (B) Apoptosis of HUVECs induced by REG@LFHA NPs, as analyzed by flow cytometry with Annexin V-FITC/PI double staining. (C) Quantitative analysis of the apoptosis rates from (B). (D) Cell cycle distribution of HUVECs after treatment, analyzed by PI staining and flow cytometry. (E) Statistical analysis of the proportion of cells in G1, S, and G2/M phases from (D). (F) Representative fluorescent images of (Upper) HUVEC and (Lower) SVEC4-10 endothelial cell tube formation on Matrigel after treatment with various concentrations of REG@LFHA NPs (1, 2.5, 5 μ M). Cells were stained with Calcein-AM. (G) Western blot analysis of VEGFR2 and phospho-VEGFR2 (pVEGFR2) protein levels in HUVECs. Cells were pre-treated with increasing doses of REG@LFHA NPs (0.5–8 μ M) for 2 h, followed by stimulation with 30 ng/mL VEGF for 20 min. (H) Quantitative analysis of the percentage of live and dead cells from (A). (I–K) Quantitative analysis of the tube formation assay for (I) number of nodes, (J) total tube length, and (K) number of meshes in HUVECs, corresponding to the images in (F). Abbreviations: Nb. nodes, number of nodes; Tol. length, total tube length; Nb. meshes, number of meshes. All statistical data are represented as mean $\pm$ SD ($n = 3$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

intestine, the expression levels of both receptors in tumor tissue reached the maximum. Flow cytometry analysis (Fig. S3A and B) further validated the dual high expression of LRP-1 and CD44 in PM-CRC tumor tissues at the single-cell level, showing that $73.69\% \pm 0.78\%$ of cells were LRP-1-positive and $79.59\% \pm 2.24\%$ were CD44-positive.

The discovery of this receptor expression profile perfectly corroborates the specific accumulation of nanoparticles at the tumor site. The results conclusively demonstrate that the highly efficient in vivo targeting of DiR@LFHA NPs is attributable to their ability to simultaneously utilize the overexpressed LRP-1 (targeted by LF) and CD44 (targeted by HA) in tumor tissue for dual active recognition.

In summary, this study establishes a complete chain of evidence through in vivo distribution experiments and receptor expression analysis: based on the characteristic dual overexpression of LRP-1/CD44 in PM-CRC tumor tissue, our designed LFHA NPs can achieve efficient and specific drug delivery to tumor sites via receptor-ligand mediated active targeting mechanism. This “dual-targeting” strategy lays a solid foundation for the precise treatment of peritoneal metastatic cancer.

3.4. In vitro anti-tumor activity

To evaluate the combined therapeutic potential of REG@LFHA NPs with the first-line chemotherapeutic agent OxP at the cellular level, we first employed 3D CT26-luc tumor spheroids to visually assess treatment efficacy. Live/dead staining revealed that while individual treatments with REG@LFHA NPs or OxP induced only moderate cell death, the combination group exhibited a significant increase in dead cells (red fluorescence), indicating superior tumor-killing efficacy (Fig. 4A). This observation was confirmed quantitatively, showing a significantly higher percentage of dead cells in the combination group compared to all monotherapy groups (Fig. S4A). In 2D cultures, the CCK-8 assay further validated the enhanced cytotoxicity of the combination regimen; across a range of concentrations, it resulted in significantly greater growth inhibition than any single agent (Fig. 4B). Synergy analysis determined a Combination Index (CI) of 0.82 at the IC₅₀ level (<1) (Fig. S4B), confirming a definite synergistic interaction between REG@LFHA NPs and OxP, which was also supported by the corresponding dose-effect curves (Fig. S4C).

To elucidate the mechanism underlying this synergy, we investigated the respective modes of action of each agent. REG@LFHA NPs demonstrated a potent, concentration-dependent ability to induce late-stage apoptosis. Flow cytometric analysis with Annexin V-FITC/PI staining showed that a high concentration (15 μ M) drastically increased the total apoptosis rate from $11.97 \pm 2.59\%$ in the control to $77.54 \pm 0.53\%$ (Fig. 4C and D). Concurrently, cell cycle analysis indicated that REG@LFHA NPs primarily induced a profound G1 phase arrest. Treatment with 15 μ M REG@LFHA NPs increased the G1 population to 1.79-fold that of the control (Fig. 4E and F), suggesting an effective blockade at the G1/S checkpoint, thereby halting cell cycle progression. Comparative analysis of cell cycle perturbations induced by the different treatment regimens provided key insights into the synergistic mechanism (Fig. S4D and E). REG@LFHA NPs alone significantly increased the G1 phase fraction to $63.19 \pm 3.81\%$ (control: $42.36 \pm 1.41\%$). Notably, OxP monotherapy also caused a noticeable accumulation of cells in the G1 phase ($55.00 \pm 2.68\%$), which contrasts with the classic S/G2 arrest

typically associated with platinum-based agents. This phenomenon can be mechanistically explained by the activation of the DNA damage response (DDR) pathway: although OxP-induced DNA crosslinks primarily cause S-phase arrest, a subset of cells with severe, irreparable damage may activate p53-dependent and p21-mediated checkpoints, leading to sustained G1 arrest to prevent the replication of damaged DNA. Consequently, as both agents converged on reinforcing G1 arrest, the combination therapy achieved the most pronounced G1 phase blockade ($73.63 \pm 3.56\%$).

In summary, the potent synergy between REG@LFHA NPs and OxP stems from their complementary mechanisms of action: REG@LFHA NPs actively drive G1 arrest and apoptosis, whereas OxP indirectly triggers G1 checkpoint activation via DNA damage; their combined action significantly enhances the anti-tumor efficacy.

3.5. In vitro anti-angiogenic activity and mechanisms

Beyond direct tumor cell cytotoxicity, we evaluated the anti-angiogenic potential of REG@LFHA NPs using human umbilical vein endothelial cells (HUVECs), a well-established model for studying vascular biology. REG@LFHA NPs demonstrated significant multifaceted anti-angiogenic activity in vitro. Initial evaluation using HUVECs revealed potent inhibition of endothelial cell viability by the nanoformulation. CCK-8 assays showed a clear concentration-dependent inhibitory effect (Fig. S5A), a trend further validated by live/dead staining, where increasing concentrations of REG@LFHA NPs led to progressively higher dead cell ratios (red fluorescence) while control cells maintained excellent viability (green fluorescence) (Fig. 5A and H).

Investigating the cell death mechanisms revealed that REG@LFHA NPs exerted anti-proliferative effects through dual pathways. Flow cytometric analysis demonstrated effective induction of both early and late apoptosis in HUVECs (Fig. 5B and C). Furthermore, cell cycle analysis revealed that REG@LFHA NPs arrested cells in G1 phase, significantly reducing the proportion of cells entering S and G2/M phases (Fig. 5D and E), providing insights into the deep-seated mechanisms of proliferation inhibition from the perspective of cell cycle regulation.

Angiogenic function studies demonstrated that REG@LFHA NPs effectively disrupted endothelial tube-forming capacity. Using both HUVECs and mouse SVEC4-10 endothelial cells, we found the nanoformulation concentration-dependently inhibited tubular structure formation, manifested by comprehensive reductions in key parameters including node number, total tube length, and mesh number (Fig. 5F and 5I–K; Fig. S5D–J). Particularly noteworthy, within the 5–10 μ M concentration range after 8 h of treatment, CCK-8 detection showed no significant impact on cell viability (Fig. S5C), clearly confirming that the angiogenesis inhibition stemmed from specific pharmacological actions rather than cytotoxicity.

At the molecular level, Western blot analysis confirmed that REG@LFHA NPs directly targeted key angiogenic pathways. Experiments showed that merely a 2-h pretreatment with 0.5 μ M significantly inhibited VEGF-induced VEGFR2 phosphorylation (Fig. 5G, Fig. S5B). As a core regulator of angiogenesis, inhibition of the VEGFR2 signaling pathway provides a molecular-level explanation for its anti-angiogenic activity.

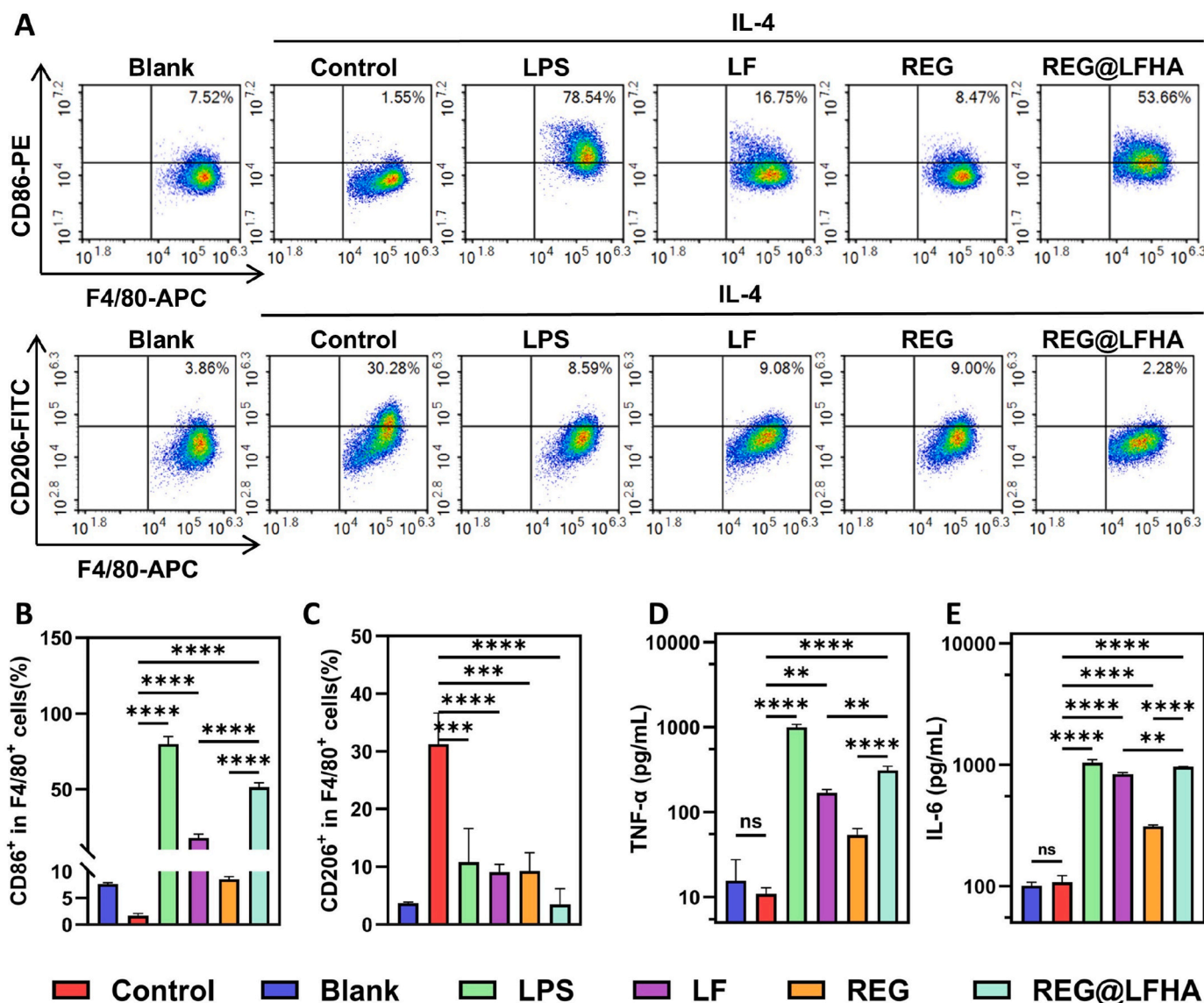


Fig. 6. Immunomodulatory effects on bone marrow-derived macrophages. (A) Flow cytometric analysis of M1 macrophage (CD86⁺) and M2 macrophage (CD206⁺) subsets gated on F4/80⁺ BMDMs. (B) Quantitative analysis of M1 macrophage proportions. (C) Quantitative analysis of M2 macrophage proportions. (D) TNF-α concentration in cell supernatants (ELISA). (E) IL-6 concentration in cell supernatants (ELISA). REG@LF means REG@LFHA NPs, REG@LFHA means REG@LFHA NPs. All statistical data are represented as mean ± SD (n = 3; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

In summary, REG@LFHA NPs exert anti-angiogenic effects through multiple mechanisms, including induction of apoptosis, G1-phase arrest, inhibition of vascular formation, and blockade of key signaling pathways.

3.6. Immunomodulatory effects on bone marrow-derived macrophages

REG@LFHA NPs demonstrated significant immunomodulatory capacity by effectively promoting the polarization of bone marrow-derived macrophages (BMDMs) toward the anti-tumor M1 phenotype. Flow cytometry analysis (Fig. 6A–C) revealed that compared to free REG, REG@LFHA NPs-treated cells showed a substantially increased CD86⁺ (M1 marker) population and a markedly decreased CD206⁺ (M2 marker) population. Notably, the carrier material LF alone exhibited an inherent ability to promote M1 polarization, indicating its intrinsic immunostimulatory properties, which form an important basis for the immunomodulatory effects of REG@LFHA NPs.

This immunomodulatory effect was further validated at the cytokine level. Cytokine profiling (Fig. 6D and E) demonstrated that REG@LFHA

NPs treatment significantly enhanced the secretion of pro-inflammatory cytokines TNF-α and IL-6 in cell supernatants, reaching 28.47-fold and 8.94-fold of the control levels, respectively. Importantly, LF alone elevated TNF-α and IL-6 to 15.44-fold and 7.77-fold, while free REG resulted in 4.92-fold and 2.89-fold increases, respectively. This graded enhancement clearly indicates that LF plays a predominant role in driving M1 polarization, while REG@LFHA NPs build upon this foundation through a synergistic interaction between drug and carrier to achieve the strongest immunostimulatory effect.

These findings not only highlight the value of LF itself as an immunomodulator but also demonstrate that REG@LFHA NPs can effectively remodel the tumor immune microenvironment through a synergistic mechanism, providing new insights for nanocarrier-based immunotherapy strategies.

3.7. In vivo anti-tumor efficacy and systemic safety evaluation

Using a CT26-luc-derived PM-CRC mouse model, we systematically evaluated the antitumor efficacy of different therapeutic regimens

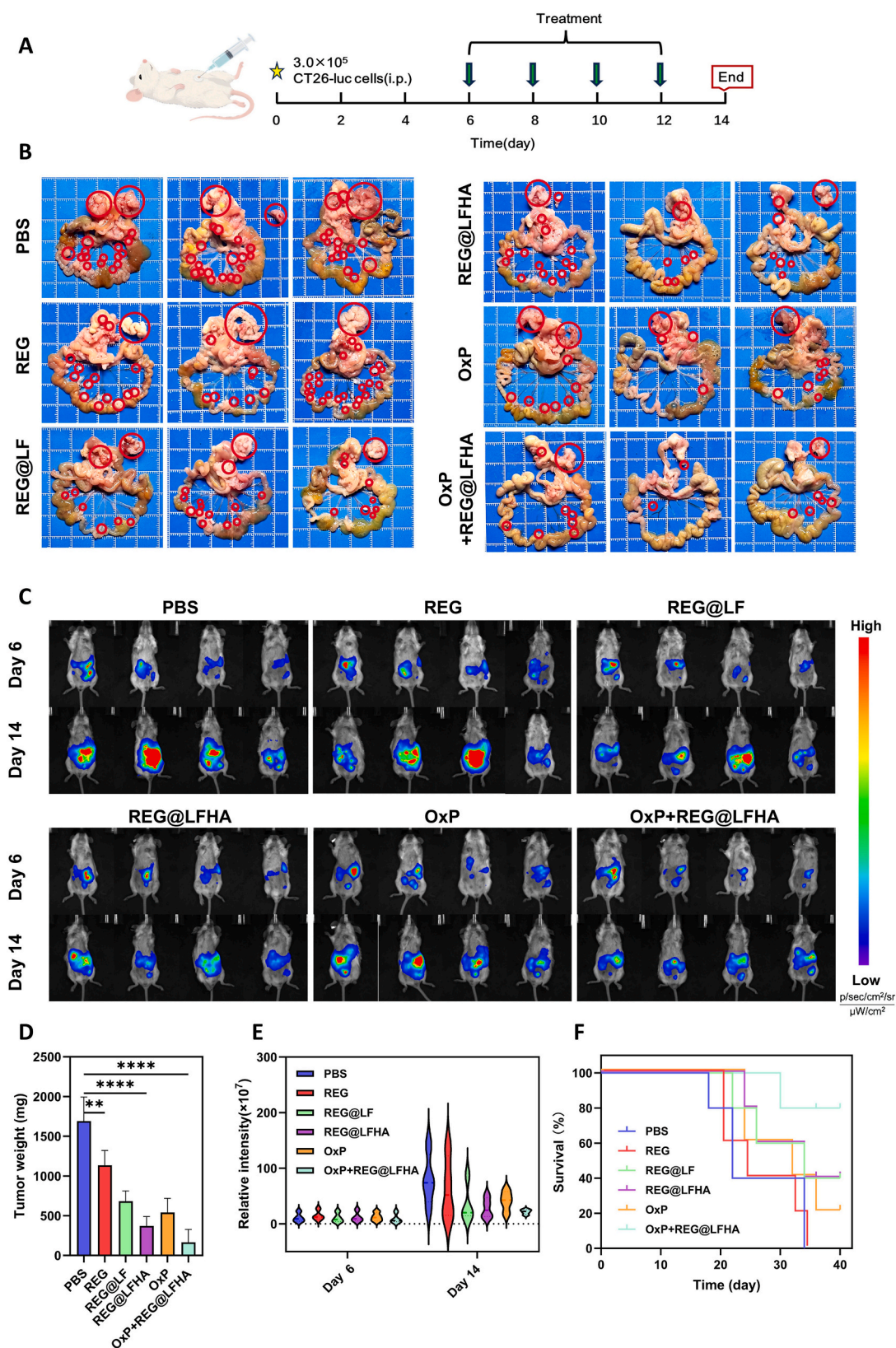
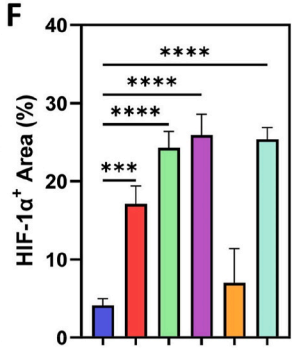
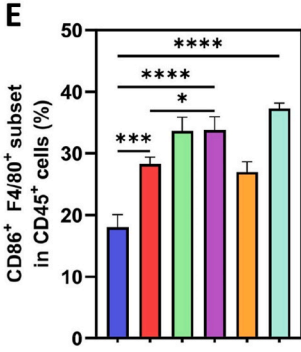
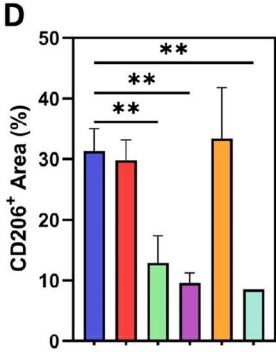
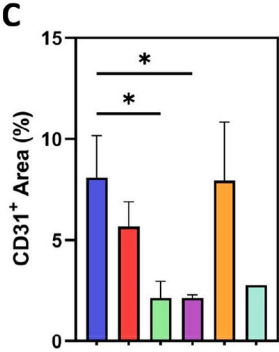
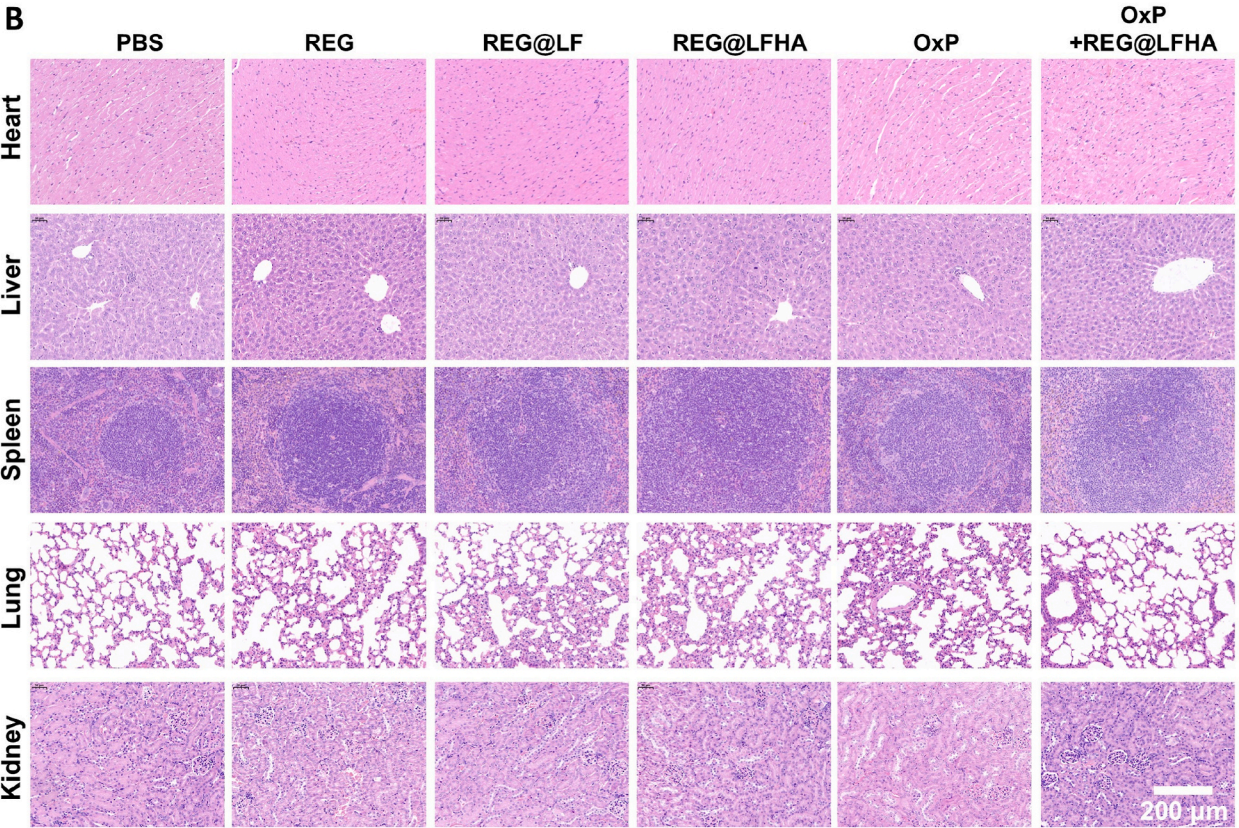
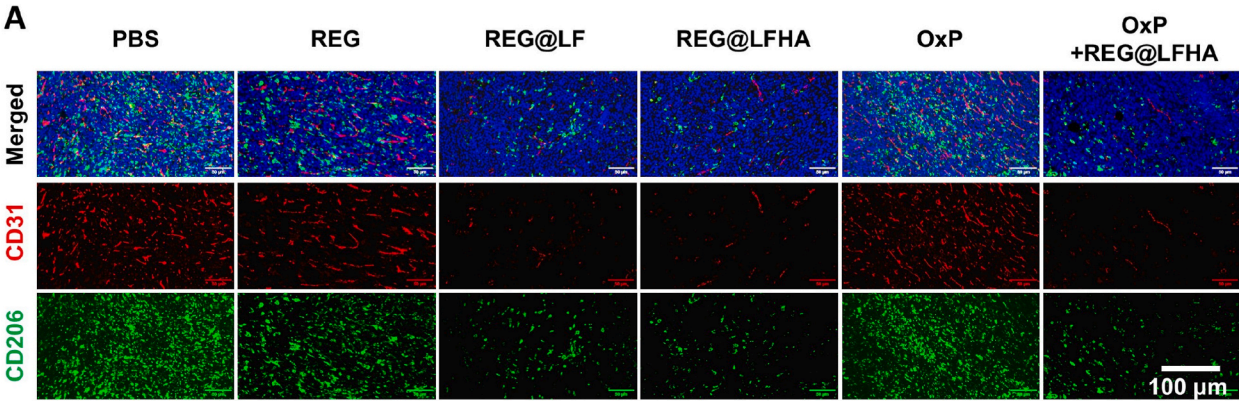


Fig. 7. In vivo anti-tumor efficacy. (A) Schematic illustration of the animal model establishment and therapeutic regimen. (B) Representative photographs of excised tumors from each group at the experimental endpoint (Day 14). Tumor masses are outlined in red. Tumors from REG-containing treatment groups exhibit a characteristic pale color, suggesting extensive necrosis. (C) In vivo bioluminescence imaging before treatment (Day 6) and at the endpoint (Day 14), showing changes in tumor burden. (D) Quantitative analysis of tumor weight at the endpoint (Day 14). (E) Quantitative analysis of bioluminescence signal intensity at Day 14. (F) Survival curves of different treatment groups (n = 5). Data in (D) and (E) are presented as mean $\pm$ SD (n = 4). REG@LF means REG@LFHA NPs, REG@LFHA means REG@LFHA NPs. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; statistical significance was determined by one-way ANOVA. Survival analysis (F) was performed using the log-rank test.



■ PBS ■ REG ■ REG@LF ■ REG@LFHA ■ OxP ■ OxP+REG@LFHA

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Fig. 8. Analysis of tumor microenvironment and systemic safety evaluation. (A) Representative immunofluorescence staining of tumor tissues from different treatment groups, showing CD31⁺ blood vessels (red), CD206⁺ M2 macrophages (green), and DAPI (blue) for nuclei. Scale bar = 100 μ m. (B) Representative H&E-stained histological sections of major organs (heart, liver, spleen, lungs, and kidneys). Scale bar = 200 μ m. (C) Quantitative analysis of microvessel density (MVD) based on CD31-positive areas. (D) Quantitative analysis of CD206-positive areas. (E) Statistical summary of the percentage of F4/80⁺CD86⁺ cells within CD45⁺ cells in tumor tissues, as determined by flow cytometry. (F) Quantitative analysis of the HIF-1 α -positive area percentage in tumor tissues from different groups. REG@LF means REG@LFHA NPs, REG@LFHA means REG@LFHA NPs. All statistical data are represented as mean $\pm$ SD ($n = 3$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

(Fig. 7A). Following four times treatment, all groups demonstrated significant tumor growth inhibition. The REG@LFHA NPs group exhibited particularly remarkable antitumor activity, reducing tumor weight and bioluminescence intensity by 78.0 % and 64.5 %, respectively, compared to controls (Fig. 7B–E). Macroscopic examination revealed marked pallor in tumor tissues across all REG-treated groups, indicating compromised vasculature and subsequent necrosis (Fig. 7B). Survival analysis confirmed the sustained therapeutic benefit of REG@LFHA NPs (Fig. 7F). While free REG showed moderate antitumor efficacy (32.8 % reduction in tumor weight), its effectiveness was constrained by rapid peritoneal clearance and poor tumor penetration. In contrast, REG@LFHA NPs, leveraging optimized nanoscale properties, achieved prolonged intraperitoneal retention and enhanced tumor accumulation through LRP-1/CD44 dual-targeting functionality.

One of the primary study objective was to assess the combinatorial potential of REG@LFHA NPs with OxP. The combination therapy produced substantial synergistic antitumor effects (Fig. 7D and E), achieving a 90.3 % tumor inhibition rate that significantly surpassed all monotherapies. This synergy stems from complementary mechanisms: OxP induces DNA damage in proliferating tumor cells, while REG@LFHA NPs exert multi-targeted effects through angiogenesis inhibition and immune microenvironment modulation.

Immunofluorescence analysis provided comprehensive insights into the therapeutic mechanisms of our nanotherapy. Using dual-label immunofluorescence targeting vascular endothelial cells (CD31) and M2 macrophages (CD206), we systematically evaluated tumor vascularization and immunosuppressive cell infiltration. The analysis revealed that untreated tumors exhibited characteristic pathological features of advanced malignancy, including aberrant, disorganized vasculature indicative of pathological angiogenesis, along with dense infiltration of M2-polarized macrophages that establish an immunosuppressive microenvironment.

Treatment with REG@LFHA NPs effectively reversed these pathological alterations through dual mechanisms. First, the nanoformulation potently suppressed tumor angiogenesis, reducing CD31-positive areas by 73.6 %—significantly exceeding the 29.8 % reduction achieved by free REG (Fig. 8A–C). This profound anti-angiogenic effect created ischemic conditions within tumors, triggering compensatory HIF-1 α upregulation to 6.25-fold of control levels (Fig. 8F–S6I). The strong negative correlation between HIF-1 α expression and CD31-positive areas confirms treatment-induced hypoxia resulting from successful vascular disruption [43,44].

Concurrently, REG@LFHA NPs demonstrated remarkable immunomodulatory capabilities, transforming the immunosuppressive microenvironment into an antitumor milieu. The treatment significantly reduced M2 macrophage infiltration by 69.4 % as determined by CD206 immunofluorescence analysis, while increasing M1 macrophage polarization by 87.3 % as quantified through flow cytometric analysis, with these complementary methodologies providing robust validation of macrophage repolarization (Fig. 8D, E, S6G, H). This coordinated reprogramming of macrophage polarization represents a crucial mechanism contributing to the overall antitumor efficacy.

The integrated design of REG@LFHA NPs proved to be essential for achieving these comprehensive therapeutic outcomes. It is worth noting that while the lactoferrin carrier alone demonstrated certain immunomodulatory potential in vitro, it failed to show significant tumor suppression efficacy in vivo (Fig. S6A and B). This finding indicates that immunomodulation alone is insufficient within the complex tumor

microenvironment, and that the combination of immunomodulation with other therapeutic strategies—including anti-angiogenesis and direct tumor cell killing—is necessary for effective tumor control. This understanding forms the fundamental rationale behind our development of the multifunctional REG@LFHA NPs system. Safety assessment showed preserved histology in major organs (Fig. 8B) and normal serum biochemical parameters (Fig. S6D–F), confirming REG@LFHA NPs' biocompatibility.

In conclusion, this study establishes that REG@LFHA NPs enhance tumor drug accumulation, suppress angiogenesis, and modulate the immune microenvironment, while demonstrating remarkable synergy with OxP. This nanocarrier-based strategy successfully integrates targeted and standard therapies, overcoming pharmacokinetic limitations of free REG while enhancing therapeutic outcomes, providing a promising translational approach for peritoneal metastatic colorectal cancer.

4. Conclusion

The pathological vasculature in PM-CRC not only provides essential nutritional support for tumor survival but also facilitates metastatic dissemination. To overcome the pharmacological limitations of conventional REG therapy, we developed REG@LFHA NPs employing a dual LRP-1/CD44 targeting strategy, which significantly enhanced tumor-specific drug accumulation while minimizing systemic exposure. This innovative nanosystem exerts comprehensive antitumor effects through three synergistic mechanisms: suppression of tumor angiogenesis to disrupt blood and oxygen supply, thereby inhibiting tumor growth and reducing metastatic potential; enhanced tumor cell killing through synergistic effects with OxP; and promotion of macrophage repolarization from immunosuppressive M2 to antitumor M1 phenotypes via the combined action of REG and LF. In vivo results demonstrated that the combination therapy of REG@LFHA NPs with OxP significantly suppressed tumor progression, as evidenced by remarkable reductions in both tumor weight and bioluminescence signals. This combinatorial approach effectively controlled primary tumor growth and metastatic dissemination while maintaining a favorable safety profile. Our study provides valuable preclinical evidence to support further development of REG@LFHA NPs as a potential therapeutic strategy for PM-CRC.

CRedit authorship contribution statement

Yutong Qian: Writing – original draft, Software, Project administration, Methodology, Conceptualization. **Meng Wang:** Supervision, Methodology, Formal analysis, Data curation. **Yicong Li:** Validation, Software, Methodology. **Mei Zhu:** Validation, Software, Methodology. **Meng Pan:** Validation, Software, Methodology. **Xicheng Li:** Validation, Software, Methodology. **Wen Chen:** Methodology. **Yujia Wei:** Methodology. **Ran Li:** Methodology. **Danrong Hu:** Conceptualization. **Zhiyong Qian:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Ethics approval and consent to participate

All animal experiments were performed following the protocols approved by the Ethics Committee of the Animal Experimental Center of State Key Laboratory of Biotherapy of Sichuan University (Checking number: 20241127008), and were carried out in compliance with all

relevant ethical regulations.

Declaration of competing interest

Zhiyong Qian is an editorial board member for *Bioactive Materials* and was not involved in the editorial review or the decision to publish this article. All authors declare that there are no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioactmat.2025.12.015>.

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