blood advances

American Society of Hematology
2021 L Street NW, Suite 900,
Washington, DC 20036
Phone: 202-776-0544 | Fax 202-776-0545
bloodadvances@hematology.org

Anti-BCMA-engineered Exosomes for Bortezomib Targeted Delivery in Multiple Myeloma

Tracking no: ADV-2023-012464R2

Shushu Yuan (The Second Affiliated Hospital of Soochow University, China) Qi Li (The Second Affiliated Hospital of Soochow University, China) Chuan He (The Second Affiliated Hospital of Soochow University, China) Mengli Bing (The Second Affiliated Hospital of Soochow University, China) Xinyun Zhang (Department of Hematology, the Second Affiliated Hospital of Soochow University, China) Hao Xu (The Second Affiliated Hospital of Soochow University, China) Zhiming Wang (School of Biology & Basic Medical Sciences, Soochow University, China) Meifang Zhao (The Second Affiliated Hospital of Soochow University, China) Yuchen Zhang (The Second Affiliated Hospital of Soochow University, China) Yali Chai (Suzhou Medical College of Soochow University, China) Bingzong Li (The Second Affiliated Hospital of Soochow University, China) Wenzhuo Zhuang (Suzhou Medical College of Soochow University, China)

Abstract:
Exosomes have emerged as promising vehicles for delivering therapeutic cargoes to specific cells or tissues, owing to their superior biocompatibility, reduced immunogenicity, and enhanced targeting capabilities compared to conventional drug delivery systems. In this study, we developed a delivery platform utilizing exosomes derived from monocytes, specifically designed for targeted delivery of Bortezomib (Btz) to multiple myeloma (MM) cells. Our approach involved the genetic modification of monocytes to express antibodies targeting B cell maturation antigen (anti-BCMA), as BCMA selectively expresses on myeloma cells. This modified anti-BCMA was then efficiently incorporated into the monocyte-derived exosomes. These adapted exosomes effectively encapsulated Bortezomib, leading to enhanced drug accessibility within MM cells and sustained intracellular accumulation over an extended period. Remarkably, our results demonstrated that anti-BCMA-Exo-Btz outperformed free Btz in vitro, exhibiting a more potent myeloma-suppressive effect. In orthotopic MM xenograft models, anti-BCMA-Exo-Btz exhibited a significant anti-tumor effect compared to free Btz. Furthermore, it demonstrated remarkable specificity in targeting Bortezomib to myeloma cells in vivo. Importantly, we observed no significant histological damage in mice treated with anti-BCMA-Exo-Btz and a slight effect on PBMCs. Additionally, our study highlighted the multifunctional potential of monocyte-exosomes, which induced cell apoptosis, mediated immune responses, and enhanced the osteogenic potential of mesenchymal stromal cells. In conclusion, our study suggests that exosomes modified with targeting ligands hold therapeutic promise for delivering Bortezomib to myelomas, offering substantial potential for clinical applications.

Conflict of interest: No COI declared

COI notes:

Preprint server: No;

Author contributions and disclosures: Bingzong Li and Wenzhuo Zhuang designed the research, and contributed reagents and other essential materials. Shushu Yuan and Qi Li performed research and wrote the paper, Chuan He, Mengli Bing, Hao Xu, Xinyun Zhang, Yuchen Zhang and Zhiming Wang analyzed data. Meifang Zhao and Yali Chai modified the paper.

Non-author contributions and disclosures: No;

Agreement to Share Publication-Related Data and Data Sharing Statement: emails to the corresponding author

Clinical trial registration information (if any):

Anti-BCMA-engineered Exosomes for Bortezomib Targeted Delivery in Multiple Myeloma

Shushu Yuan,[1, 2, 3, +] Qi Li,[1, +] Chuan He,[1] Mengli Bing,[1] Xinyun zhang,[1] Hao Xu,[1] Zhiming Wang,[2] Meifang Zhao,[1] Yucheng Zhang,[1] Yali Chai,[2] Bingzong Li,[1, *] Wenzhuo Zhuang,[2, *]

[1]Department of Hematology, the Second Affiliated Hospital of Soochow University, Suzhou, China.

[2]Department of Cell Biology, School of Biology & Basic Medical Sciences, Suzhou Medical College of Soochow University, Suzhou, China.

[3]Department of Oncology, Affiliated Tumor Hospital of Nantong University, Nantong, China

[+]: These authors contributed equally to this work

[*]: Corresponding Authors

Bingzong Li, Tel: 86−512−67784069, E-mail: lbzwz0907@hotmail.com, Department of Haematology, The Second Affiliated Hospital of Soochow University, San Xiang Road 1055, Suzhou, 215006, China.

Wenzhuo Zhuang, Tel: 86−512−65880103, E-mail: zhuangwenzhuo@suda.edu.cn, Department of Cell Biology, School of Biology & Basic Medical Sciences, Suzhou Medical College of Soochow University, Ren Ai Road 199, Suzhou, 215123, China

Availability of data and materials

The data supporting the findings of this study can be found in the article or available from the corresponding author upon reasonable request.

Conflict of Interest：The authors declare that they have no competing interests.

Key point

1. Anti-BCMA-Exo-Btz precisely targets myeloma cells, effectively inhibiting multiple myeloma and mitigating bone destruction.

Word count for text: 3951

Word count for abstract: 228

Figure count: 7

Reference count: 31

Abstract

Exosomes have emerged as promising vehicles for delivering therapeutic cargoes to specific cells or tissues, owing to their superior biocompatibility, reduced immunogenicity, and enhanced targeting capabilities compared to conventional drug delivery systems. In this study, we developed a delivery platform utilizing exosomes derived from monocytes, specifically designed for targeted delivery of Bortezomib (Btz) to multiple myeloma (MM) cells. Our approach involved the genetic modification of monocytes to express antibodies targeting B cell maturation antigen (anti-BCMA), as BCMA selectively expresses on myeloma cells. This modified anti-BCMA was then efficiently incorporated into the monocyte-derived exosomes. These adapted exosomes effectively encapsulated Bortezomib, leading to enhanced drug accessibility within MM cells and sustained intracellular accumulation over an extended period. Remarkably, our results demonstrated that anti-BCMA-Exo-Btz outperformed free Btz in vitro, exhibiting a more potent myeloma-suppressive effect. In orthotopic MM xenograft models, anti-BCMA-Exo-Btz exhibited a significant anti-tumor effect compared to free Btz. Furthermore, it demonstrated remarkable specificity in targeting Bortezomib to myeloma cells in vivo. Importantly, we observed no significant histological damage in mice treated with anti-BCMA-Exo-Btz and a slight effect on PBMCs. Additionally, our study highlighted the multifunctional potential of monocyte-exosomes, which induced cell apoptosis, mediated immune responses, and enhanced the osteogenic potential of mesenchymal stromal cells. In conclusion, our study suggests that exosomes modified with targeting ligands hold therapeutic promise for delivering Bortezomib to myelomas, offering substantial potential for clinical applications.

Introduction

Multiple Myeloma (MM) is a hematological malignancy marked by the abnormal proliferation of plasma cells, resulting in myeloma formation in the bone marrow, leading to osteolytic bone disease, anemia, acute kidney injury, and various clinical manifestations[1].

Bortezomib (Btz), a first-generation proteasome inhibitor, has been approved for the treatment of MM patients and significantly prolongs their survival time [2]. Btz-based combination therapy has gradually become the first-line regimen for MM patients [2,3]. However, the treatment efficacy of Btz is hindered by issues such as lack of specificity, poor permeability, limited bioavailability, and drug resistance with long-term use [4]. While increasing the Btz dose slightly improves therapeutic effects, it also leads to significantly increased toxicity[5]. Therefore, a novel approach is required to address these limitations and further enhance the efficacy of Btz in myeloma treatment.

The utilization of exosomes as drug-delivery systems has gained significant attraction in the field of delivering chemotherapeutic drugs[6,7]. Unlike free drug molecules, exosomes encapsulation improves drug stability, reducing degradation and inactivation in the circulation, thereby enhancing overall efficacy [8]. Due to their capacity to reshape the microenvironment and attract other cell populations to inhibit tumor progression, exosomes derived from immune cells are ideal candidates as delivery vehicles for chemotherapy drugs [9]. Exosomes derived from immune cells, particularly monocyte-derived exosomes, show reduced immunogenicity and are seldom cleared by the mononuclear phagocyte system, making them ideal vehicles for drug delivery [10].

Targeting modified exosomes exhibit remarkable cell specificity, facilitating interactions with specific cells through surface proteins or other biomarkers, enabling precise drug delivery and minimizing potential adverse effects on normal cells [6,11]. B-cell maturation antigen (BCMA) serves

as a prime target antigen for myeloma immunotherapy due to its highly selective expression on malignant plasma cells and absence in other tissues, rendering it an ideal candidate for immunotherapeutic intervention in multiple myeloma compared to CD38 and SLAMF7 [12]. Significant breakthroughs have been made in utilizing BCMA as a target for immunotherapy. BCMA-directed CAR-T cell therapy has demonstrated outstanding efficacy in the treatment of multiple myeloma [13].

In this study, monocyte-derived exosomes demonstrated a high capacity to modulate the tumor microenvironment, as evidenced by their ability to regulate macrophage polarization, enhance the cytotoxicity of NK cells, and promote osteogenic differentiation of MSCs. Based on the advantages mentioned above, monocyte-derived exosomes were modified with anti-BCMA and used as the drug carrier to deliver Btz, named anti-BCMA-Exo-Btz. These exosomes were then used for targeted imaging and treatment of myeloma cells and orthotopic xenograft. Results showed that anti-BCMA-Exo-Btz could accumulate in bone marrow, facilitating accurate myeloma recognition, improving the curative effect, and repairing bone lesions. Overall, our results indicated that the proposed drug delivery system represents a viable approach against myeloma, overcoming the limitations of conventional chemotherapy.

Methods

Preparation of Anti-BCMA modified monocytes

For the genetic modification of anti-BCMA to monocytes, anti-BCMA lentivirus with GFP labeling were added to the cells. Then polybrene with a final concentration of 8 μg/ml was added. After 48 h of culture, GFP positive cells were isolated by flow cytometry, further collected and cultured to obtain anti-BCMA expressing cells.

The synthesis of anti-BCMA-Exo-Btz

Anti-BCMA-EXO (200 μg) obtained by differential centrifugation was gently mixed with Btz (100 μg) and incubated in a constant temperature shaking bed at 37 °C for 48 h. PBS solution was added to the mixture and centrifuged (130,000 ×g, 2 hours) to remove the free Btz. The pellet was resuspended in PBS and subsequently centrifuged at 130,000 ×g for another 2 hours. The pellets were resuspended in PBS and stored at -80°C for further use.

Loading capacity and in vitro release

The content of Btz in exosomes was determined by HPLC. Anti-BCMA-EXO-Btz was mix with 1 ml acetonitrile and thoroughly mixed, after ultrasound, centrifuging at 16500 g for 20 minutes. The supernatant was detected by HPLC. The supernatant was filtered with a 0.22 μm syringe filter and 20 μL aliquots were transferred into HPLC autosampler vials. The loading efficiency represents the ratio of loaded Btz dose to exosome dose, while encapsulation efficiency denotes the ratio of loaded Btz dose to the initially used dose of Btz for loading. To measure Btz release, anti-BCMA-EXO-Btz was loaded in a dialysis bag in pH=7.35 and pH=7.45 cell buffers. Samples were taken at different time points and analyzed using HPLC, expressed as the percentage of Btz released divided by total Btz.

Intracellular Btz accumulation

To quantify the amount of Btz accumulation in MM cells, anti-BCMA-EXO-Btz was added to and incubated with MM cells for 12 hours or 24 hours. Then the cells were washed with PBS and lysed with Triton x-100 and ultrasound was performed on ice. The lysed cell fluid was centrifuged at 16500 g for 20 minutes, and the supernatant (20 μL) was determined by HPLC.

In vivo studies

Animals were housed and maintained in accordance with the institutional guidelines for the use of laboratory animals and after acquiring permission from the ethics committee of Soochow University for animal experimentation. Four-week-old female NOD-Prkdcscid Il2rgtm1/Bcgen (NSG) mice were purchased from a biocytogen company (Beijing, China), and were acclimated one week prior to tumor cell inoculation. A total of 1×10^6 luciferase labeled LP-1 cells were injected via the lateral tail vein. Tumors were allowed to grow for a week, and then mice were injected with PC-Exo, PC-Anti-BCMA-Exo, and CL-Anti-BCMA-Exo, PC-Anti-BCMA-Exo-Btz, CL-Anti-BCMA-Exo-Btz, Btz and PBS as control, three times a week for two weeks. The treatment doses for each group were 2.23mg/kg exosomes in PC-Exo, PC-Anti-BCMA-Exo, and CL-Anti-BCMA-Exo group, 2.23mg/kg exosomes loaded with 0.29 mg/kg Btz in PC-Anti-BCMA-Exo-Btz and CL-Anti-BCMA-Exo-Btz group, and 0.29 mg/kg Btz in Btz group. Four-to six-week-old C57BL/KaLwRij mice were housed at the Laboratory animal center of Suzhou medical college. A total of 1×10^6 luciferase labeled 5TGM1 cells were injected via the lateral tail vein. Tumors were allowed to grow for a week, and then mice were injected with CL-Exo (2.23mg/kg) and PBS as control, three times a week for two weeks.

Mice were imaged after injection of 75 mg/kg of D-luciferin (Promega, Madison, WI) using IVIS Lumina II optical imaging system (Caliper Life Sciences, Hopkinton, MA). Tumor burden was

assessed by serial bioluminescence imaging every three days. After injection of DiR-labeled exosomes for 6 h, the biodistribution of DiR-labeled exosomes in mice and organs was monitored by the IVIS Lumina II optical imaging system at the excitation wavelength of 740 nm and the emission wavelength of 780 nm.

Micro-CT imaging

Tomography scans were performed in a SkyScan 1172 micro-CT system (Bruker, Kontich, Belgium). The spectrum was filtered with a 1.0 mm Aluminum filter. After scanning, CTAn and Mimics software were used for micro-CT analysis and 3D reconstruction of the scapula.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 8.0. All data were expressed as the mean ± standard deviation. For normally distributed data, the significance of mean differences was determined using unpaired Student's t-test between two groups or ANOVA followed by Newman-Keuls multiple comparison test among multiple groups. For all tests, a p-value < 0.05 was considered to be statistically significant.

Ethical Approval

The animal experimentation acquired permission from the ethics committee of Soochow university. All human primary monocytes were collected from the Second Affiliated Hospital of Soochow University after receiving permission from the ethics committee of the Second Affiliated Hospital of Soochow University. Informed written consent was obtained from each subject or each subject's guardian. The study was conducted in accordance with the Declaration of Helsinki.

Other detailed assays are available in the supplemental methods.

Results

Engineering exosomes for targeted drug delivery in MM

This approach aimed to utilize the natural targeting properties of exosomes derived from monocytes to specifically deliver therapeutic cargo to myeloma cells expressing BCMA. Monocytes were obtained from either the human monocyte cell line THP-1 (CL-monocytes) or primary monocytes derived from PBMC (PC-monocytes). The myeloma-targeting capability of exosomes was conferred by engineering the monocytes to express anti-BCMA. We introduced a lentiviral vector containing the anti-BCMA construct into either CL-monocytes or PC-monocytes and then employed flow cytometry-based sorting to identify and isolate the significant fraction of cells expressing anti-BCMA (Figure 1A). We then collected exosomes secreted by the engineered monocytes and characterized them using Dynamic Light Scattering analysis (DLS), Western blotting, and transmission electron microscopy (TEM) to confirm their size, presence of exosome markers, and morphology (Figure 1B-1D). Anti-BCMA was strongly expressed in engineered monocytes, incorporated into the monocyte-derived exosomes, and identified on the external surface of the exosomes based on flow cytometry (Figure 1E).

Based on the properties of Btz, we employed co-incubation to load Btz into anti-BCMA-Exos. This loading process did not alter the exosomes' physical attributes, as confirmed through DLS, Western blotting, and TEM analyses (Figure 1B-1D). Meanwhile, the zeta potential of the anti-BCMA-Exo was determined to be -6.25 mV and -7.18 mV, whereas the anti-BCMA-Exo- Btz exhibited a zeta potential of -9.23 mV and -8.12 mV, with no significant alterations observed (Figure 1B). The retention of anti-BCMA on the exosomal surface was evidenced by colloidal gold immunoelectron microscopy (Figure 1F). Furthermore, infrared spectroscopy detected the presence of hydroxyl

groups and benzene ring frameworks characteristic of Btz within the anti-BCMA-Exo-Btz (Figure 1G). Loading efficiency and encapsulation efficiency are commonly used parameters to evaluate the efficiency of drug loading into exosomes or other drug delivery systems. The anti-BCMA-modified exosome-loaded Btz (anti-BCMA-Exo-Btz) achieved a loading efficiency of 13.24 ± 1.2% and an encapsulation efficiency of 12.05 ± 0.50% when the dosage was 400 ng/ml, as measured by High Performance Liquid Chromatography (HPLC) (Figure 1H). Collectively, these outcomes solidify the confirmation of Btz's effective encapsulation within anti-BCMA-Exo.

Evaluation of anti-BCMA-Exo-Btz for myeloma cell uptake and controlled drug release

We assessed the BCMA expression levels of ARH-77, U266, and LP-1 cell lines, with 293T cells as a control. U266 and LP-1 cells exhibited significantly higher BCMA expression levels compared to ARH-77 cells (Supplementary figure S1A). Therefore, U266 and LP-1 cell lines were selected for further experiments. To assess the potential uptake of anti-BCMA-Exo-Btz by myeloma cells, we introduced PKH67-labeled anti-BCMA-Exo-Btz to LP-1 and U266 cells. The myeloma cells' ability to internalize anti-BCMA-Exo-Btz was confirmed via flow cytometry (Figure 2A) and immunofluorescence assays (Figure 2B). Cells expressing high levels of BCMA demonstrated significantly greater uptake of exosomes, underscoring the specificity of BCMA targeting (Supplementary figure S1B and S1C). For a comprehensive assessment of cellular uptake and drug delivery, we conducted a quantitative comparison of drug accumulation within cells, both for the free drug and the exosome-loaded drug, using HPLC. In LP-1 cells (Figure 2C), at 12 hours, the intracellular bortezomib concentration was notably higher for CL-anti-BCMA-Exo-Btz (11.62-fold) and even more so for PC-anti-BCMA-Exo-Btz (16.82-fold), compared to the free Btz group (1.36-fold). This trend persisted and intensified after 24 hours for the anti-BCMA-Exo-loaded Btz.

Similar results were observed in U266 cells. These findings indicated that bortezomib, when encapsulated within anti-BCMA-Exo, exhibited enhanced accessibility to MM cells and sustained intracellular accumulation over an extended duration.

Assessing drug release from exosomes provides valuable information about drug delivery kinetics and the potential for sustained drug release, which is essential for the development of effective drug delivery systems[14]. In our study, we placed Btz-loaded exosomes in a dialysis membrane using filtration techniques to separate the exosomes from the release medium. The quantification of Btz released over time was achieved by periodically collecting and analyzing the release medium. We employed pH values of 7.35 and 7.45 to simulate physiological human body pH levels. As depicted in Figure 2D, both pH conditions exhibited no significant influence on the release of bortezomib. The release pattern of bortezomib from anti-BCMA-Exo was time-dependent. At 6 hours, approximately 50% of Btz was released, and this figure approached nearly 100% release after 17 hours. Therefore, the release experiments indicated that the anti-BCMA-Exo-Btz construct developed in this study can achieve a gradual, time-dependent release of bortezomib.

Identifying the function of monocyte-exosome

To evaluate the safety of exosomes as drug carriers and their cellular impact, we employed a Liquid chromatograph mass spectrometer (LC-MS) to identify and characterize exosomal proteins, resulting in the identification of 400 proteins. Protein interaction analysis was then conducted using the GeneMANIA database (Figure 3A). The involvement of exosomal proteins in apoptosis and immune response pathways was revealed by KEGG pathway enrichment analysis (Figure 3B).

We next proceeded to experimentally validate these essential functions. Firstly, monocyte exosomes inhibited myeloma cell proliferation (Figure 4A). Analysis of 7-AAD/Annexin V-PE double staining

indicated an elevated proportion of apoptotic myeloma cells following treatment with monocyte exosomes (Figure 4B). Additionally, monocyte exosomes induced G0/G1 cell cycle arrest in myeloma cells (Figure 4C).

Next, we assessed the impact of monocyte exosomes on macrophage polarization. Monocyte exosomes were found to enhance the expression of the M1 marker, IL-1β, while concurrently diminishing the expression of the M2 marker CD206 in macrophages. This shift suggested that monocyte exosomes have the potential to repolarize M2 macrophages into an active M1 phenotype.

Furthermore, we investigated the influence of monocyte exosomes on NK cells. Monocyte exosomes promoted NK cell proliferation (Figure 4E). To assess the impact of monocyte exosomes on NK cell cytotoxicity, we co-cultured NK cells with luciferase-labeled LP-1 cells. As depicted in Figure 4F, monocyte exosomes significantly enhanced NK cell cytotoxic activity. Moreover, we established a syngeneic tumor model of multiple myeloma in immunocompetent mice to examine the impact of CL-Exo on immune cells in vivo (Supplementary figure S1D). Following CL-Exo treatment, we analyzed macrophages and NK cells isolated from the bone marrow. Our findings revealed that CL-Exo treatment resulted in elevated levels of CD107a+ NK cells and decreased proportion of M2-type macrophages. The CL-Exo group showed an increase in the M1/M2 ratio compared to the control groups. These results highlight the immunomodulatory role of CL-Exo in MM treatments (Supplementary figure S1E).

MM is characterized by the impaired osteogenic differentiation of mesenchymal stromal cells (MSCs)[15]. We found that monocyte exosomes led to enhanced calcium deposition in MSCs, as assessed by Von Kossa staining on days 7 and 14 (Figure 4G). Furthermore, the expression of osteogenic differentiation markers, including Runx2, OPN, OCN, ALP, and Col-I, was increased

following monocyte exosomes treatment in MSCs. Consequently, it could be inferred that monocyte exosomes increased the osteogenic potential of MSCs.

In vitro and in vivo anti-myeloma efficacy of anti-BCMA-Exo-Btz

We next evaluated the ability of anti-BCMA-Exo-Btz to inhibit myeloma cell proliferation. Myeloma cell lines LP-1 and U266 were treated with Exo, anti-BCMA-Exo-Btz, and an equivalent dose of free Btz (Figure 5A). Anti-BCMA-Exo-loaded Btz exhibited a more potent myeloma-suppressive effect when compared to free Btz. Exosomes alone also displayed a certain level of myeloma inhibition. Furthermore, we evaluated the anti-tumor effect of anti-BCMA-Exo-Btz on CD138+ cells derived from MM patients. The results revealed that CL-Exo and CL-Anti-BCMA-Exo-Btz significantly inhibited the cell viability of CD138+ cells, consistent with the observations in MM cell lines (Supplementary figure S1F).

To evaluate the efficacy of anti-BCMA-Exo-Btz in vivo, orthotopic MM xenograft models were established. Luciferase-labeled myeloma cell line LP-1 was injected via the lateral tail vein in NOD-Prkdcscid Il2rgtm1/Bcgen (NSG) mice. Mice bearing established myelomas were randomly sorted into eight groups and the groups were treated as follows: PBS, PC-Exo, PC-Exo-Btz, PC-anti-BCMA-Exo, PC-anti-BCMA-Exo-Btz, CL-anti-BCMA-Exo-Btz, CL-anti-BCMA-Exo, and an equivalent dose of free Btz. Tumor burden was assessed by serial bioluminescence imaging (Figure 5B).

The fluorescence intensity of tumor burden demonstrated that monocyte exosomes had a certain anti-tumor activity in MM mice. Loading Btz into exosomes enhanced the anti-myeloma effect compared to free Btz treatment. Importantly, anti-BCMA-Exo-Btz exhibited the most robust anti-myeloma effect, surpassing the efficacy of free Btz, Exo, Exo-Btz, and anti-BCMA-Exo (Figure

5C), leading to extended overall survival (Figure 5D). This pattern was observed in drug-loaded anti-BCMA-Exo derived from both a human monocyte cell line and human primary monocytes. Furthermore, this pattern was consistent with the in vitro anti-myeloma effect. Moreover, the pharmacokinetic studies indicated that anti-BCMA-Exo-Btz exhibited higher concentrations and extended blood circulation time compared to free bortezomib (Supplementary figure S1G). These results suggested that anti-BCMA-Exo-Btz had a significant anti-tumor effect in MM mice.

To assess the biodistribution and targeting capability of anti-BCMA-Exo-Btz, exosomes were labeled with VivoTrack DiR (Fluorescence). Fluorescence signals at the tumor site were observed for anti-BCMA-Exo-Btz, while Exo-Btz exhibited systemic distribution (Figure 6A). Six hours post-injection, organ removal revealed weaker fluorescence signals in the heart and kidney for anti-BCMA-Exo-Btz, confirming its specific targeting of Btz to myeloma in vivo (Figure 6B). These data suggested that anti-BCMA-Exo-Btz specifically targeted Btz to myeloma in vivo.

Since the above results suggested that monocyte exosomes enhanced the osteogenic potential of MSCs, we proceeded to investigate the impact of anti-BCMA-Exo-Btz on myeloma-related bone lesions in mouse models. Micro-CT scanning and three-dimensional reconstruction were employed to analyze bone destruction in the scapula and knee joints. In the PBS treatment group, there was evident and extensive bone destruction, with significant cavities appearing inside the scapula and pathological fractures occurring at the scapular spine. Severe bone damage was also observed around the knee joint, with tibial plateau collapse and pathological fractures around the femoral condyles. The anti-BCMA-Exo group showed a tendency to alleviate bone destruction compared to the PBS control group. The Btz group showed superior treatment outcomes compared to the previous two groups. Furthermore, in comparison to the PBS group, anti-BCMA-Exo group, and Btz group, mice

in the anti-BCMA-Exo-Btz group displayed the mildest bone destruction. This consistent pattern was observed in two groups where anti-BCMA-Exo-Btz was used, one derived from a human monocyte cell line and the other from human primary monocytes (Figure 6C, 6D).

Safety assessment of anti-BCMA-Exo-Btz

As with any therapeutic, safety assessments are necessary before exosome-based drug delivery systems can be widely used. In our research, PC-anti-BCMA-Exo-Btz and CL-anti-BCMA-Exo-Btz had a slight effect on the cell viability of red blood cells (RBC), peripheral blood mononuclear cells (PBMC), monocytes and PBMC except monocytes (PBMC-monocyte) (Figure 7A). However, the concentrations used in the experiment were significantly higher than those used in treating MM cells. To assess potential toxicity, we conducted histological examinations of vital organs, including the heart, liver, spleen, lung, and kidney tissues. Gratifyingly, no toxicity or significant histological damage was observed in any of these organs in the anti-BCMA-Exo-Btz treated group (Figure 7B). These results demonstrated that anti-BCMA-Exo-Btz caused no adverse effects on blood cells or vital organs, suggesting its safety for potential clinical applications.

Discussion

Multiple myeloma (MM), the second most common hematologic malignancy, is characterized by the uncontrolled proliferation of monoclonal plasma cells within the bone marrow, leading to the excessive synthesis of nonfunctional intact immunoglobulins or immunoglobulin chains [16,17]. Recent advancements in the treatment of MM have expanded therapeutic options, incorporating both single-agent and combination regimens involving chemotherapy drugs and immunomodulatory agents, significantly improving the prognosis for MM patients [1,17]. Despite these strides, it is important to note that MM remains an incurable condition.

Bortezomib (Btz) is the first FDA-approved proteasome inhibitor, holding significance as a pivotal anti-cancer drug widely employed in the treatment of various cancer types, including multiple myeloma, non-small-cell lung cancer, and breast cancer. [18-20]. The clinical efficacy of Btz is, however, limited by its poor stability, quick clearance, and low selectivity [21,22]. Additionally, bortezomib induces dose-limiting side effects such as peripheral neuropathy, gastrointestinal toxicity, and viral infections, reducing the quality of life of MM patients and potentially leading to dose reduction or treatment interruption [23,24]. Therefore, an effective drug delivery method is needed to overcome these obstacles associated with chemotherapy based on Btz.

In this context, a biomimetic nanomaterial-based drug delivery system, such as exosomes, emerges as a highly promising strategy[25]. Exosomes, with a diameter ranging from 30 to 150 nm, are small vesicles released by cells, consisting of a bilayer of phospholipid membranes and internal biomolecules[26]. The internal cavity of exosomes facilitates the encapsulation of drugs [25]. Additionally, the membrane provides a protective shield for the drugs they carry, slowing enzymatic hydrolysis or degradation in the circulation. This protective effect enhances drug stability and prolongs its half-life

293 in the blood, extending the duration that the drug remains effective in circulation [27,28]. Furthermore,
294 exosomes can be engineered with specific ligands to facilitate selective receptor binding, enabling
295 targeted therapeutic interventions [6]. Owing to their circulating stability, low immunogenicity, and
296 modifiable properties, exosomes have emerged as promising candidates for drug delivery vectors[29].
297 Based on the above, we selected monocytes as the maternal cells as the source of exosomes, which
298 exhibit low immunogenicity, making them relatively resistant to active clearance by the immune
299 system. In our study, monocyte-derived exosomes demonstrated remarkable efficacy in reshaping the
300 tumor microenvironment and displaying anti-tumor effects. Monocyte-derived exosomes enhanced
301 the cytotoxic effect of NK cells on MM cells, induced the polarization of macrophages to the
302 anti-tumor M1 subtype, and promoted the osteogenic differentiation of mesenchymal stromal cells
303 (MSCs). Moreover, monocyte-derived exosomes induced apoptosis and cell cycle arrest in MM cells.
304 These results collectively indicate that monocyte-derived exosomes are an ideal drug delivery
305 vehicle for MM treatment.
306 B-cell maturation antigen (BCMA) is reported to be overexpressed in MM cells, making it a
307 prominent target for MM-targeted therapy and immunotherapy[12,30]. Thus, anti-BCMA was selected
308 to increase the selectivity of exosomes to MM cells. We developed a targeted drug delivery system
309 for Btz utilizing monocyte-derived exosomes and evaluated its anti-tumor effect in MM. To
310 specifically deliver Btz-loaded exosomal carriers to MM cells, the monocyte-derived exosome was
311 first modified with anti-BCMA before being incubated with Btz to achieve anti-BCMA-Exo-Btz.
312 Subsequently, the morphology, particle size, encapsulation efficiency, and stability of
313 anti-BCMA-Exo-Btz were characterized. The zeta potential, considered to be a characteristic
314 property of the exosomes, reflects the surface charge of the exosome and its stability in solution[31].

315 The results suggested that the surface morphology, particle size and the zeta potential of exosomes
316 did not significantly change after modification and Btz encapsulation, indicating that the processes of
317 modification and loading did not have adverse effects on the morphological properties and stability
318 of natural exosomes. In addition, the encapsulation efficiency of Btz achieved 12.05 ± 0.50%,
319 indicating that the exosomes provided an efficient capacity for Btz loading. Assessing drug release
320 from exosomes and drug accumulation within cells provides valuable information about drug
321 delivery kinetics and the potential for sustained drug release. Anti-BCMA-Exo-Btz exhibited
322 enhanced accessibility to MM cells and sustained intracellular accumulation over an extended
323 duration, compared to free Btz. Besides, the release pattern of Btz from anti-BCMA-Exo exhibited a
324 time-dependent pattern, with 50% of Btz released at 6 hours, and nearly 100% released after 17
325 hours.
326 The in vitro antitumor results demonstrated that anti-BCMA-Exo-Btz effectively inhibited the
327 proliferation of MM cells, exhibiting a superior inhibitory effect compared to free Btz. This
328 enhanced efficacy could be attributed to the effective fusion of exosomes with cells, promoting drug
329 absorption, and accumulation, and subsequently exerting their therapeutic effects. In cytotoxicity
330 experiments involving normal cells, anti-BCMA-Exo-Btz had a slight effect on the cell viability of
331 red blood cells (RBC), peripheral blood mononuclear cells (PBMC), monocytes and PBMC except
332 monocytes (PBMC-monocyte) derived from healthy donor. However, it's important to note that the
333 concentrations used in the experiment were significantly higher than those used in treating MM cells.
334 This finding suggests that anti-BCMA-Exo-Btz is safe for potential clinical applications.
335 In view of the significant efficacy of the above in vitro cell efficacy, we proceeded to investigate the
336 in vivo targeted properties and treatment efficacy of anti-BCMA-Exo-Btz. The administration of

anti-BCMA-Exo-Btz significantly inhibited tumor growth and prolonged the overall survival of the animals. Importantly, treatment with anti-BCMA-Exo-Btz did not induce significant histological damage to vital organs, including the heart, liver, spleen, lung, and kidney tissues, indicating that anti-BCMA-Exo-Btz exhibits good safety for injection and does not induce toxic or side effects in animals. In vivo imaging results showed that fluorescence signals of VivoTrack DiR-labeled exosomes at the tumor site were observed for anti-BCMA-Exo-Btz, while Exo-Btz exhibited systemic distribution. This suggests that anti-BCMA-Exo-Btz reaches the tumor site by actively targeting BCMA in MM cells, facilitating better targeting of Btz to the tumor site and exerting a therapeutic effect.

In brief, our findings demonstrate that anti-BCMA-Exo-Btz actively and selectively targets MM cells, efficiently inhibiting tumor proliferation without inducing toxic or side effects. We strongly believe that our research provides a reference for the further development of targeted delivery drugs for the treatment of MM.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Bingzong Li and Wenzhuo Zhuang designed the research, and contributed reagents and other essential materials. Shushu Yuan and Qi Li performed research and wrote the paper, Hao Xu, Xinyun Zhang, Yuchen Zhang and Zhiming Wang analyzed data. Meifang Zhao and Yali Chai modified the paper.

Funding

The research leading to these results has received funding from National Natural Science Foundation of China (82270197; 82270211); Suzhou City Basic Research Program-Key Clinical Technology Research (SKY2023010); The Project of State Key Laboratory of Radiation Medicine and Protection, Soochow University (GZK12023020); The special project of “Technological innovation” project of CNNC Medical Industry Co. Ltd (ZHYLYB2021002); Nantong University Research Fund Clinical Medicine Special Funding Project (2023JY016).

References

1. Cowan AJ, Green DJ, Kwok M, et al. Diagnosis and Management of Multiple Myeloma: A Review. *JAMA*. 2022;327(5):464-477.

2. Scott K, Hayden PJ, Will A, Wheatley K, Coyne I. Bortezomib for the treatment of multiple myeloma. *Cochrane Database Syst Rev*. 2016;4(4):CD010816.

3. Goldberg AL. Development of proteasome inhibitors as research tools and cancer drugs. *J Cell Biol*. 2012;199(4):583-588.

4. Liu J, Zhao R, Jiang X, Li Z, Zhang B. Progress on the Application of Bortezomib and Bortezomib-Based Nanoformulations. *Biomolecules*. 2021;12(1).

5. Lee SM, Backenroth D, Cheung YK, et al. Case Example of Dose Optimization Using Data From Bortezomib Dose-Finding Clinical Trials. *J Clin Oncol*. 2016;34(12):1395-1401.

6. Liang Y, Duan L, Lu J, Xia J. Engineering exosomes for targeted drug delivery. *Theranostics*. 2021;11(7):3183-3195.

7. Familtseva A, Jeremic N, Tyagi SC. Exosomes: cell-created drug delivery systems. *Mol Cell Biochem*. 2019;459(1-2):1-6.

8. Vader P, Mol EA, Pasterkamp G, Schiffelers RM. Extracellular vesicles for drug delivery. *Adv Drug Deliv Rev*. 2016;106(Pt A):148-156.

9. Wang S, Shi Y. Exosomes Derived from Immune Cells: The New Role of Tumor Immune Microenvironment and Tumor Therapy. *Int J Nanomedicine*. 2022;17:6527-6550.

10. Pisano S, Pierini I, Gu J, et al. Immune (Cell) Derived Exosome Mimetics (IDEM) as a Treatment for Ovarian Cancer. *Front Cell Dev Biol*. 2020;8:553576.

11. Zhang Y, Bi J, Huang J, Tang Y, Du S, Li P. Exosome: A Review of Its Classification, Isolation Techniques,

386 Storage, Diagnostic and Targeted Therapy Applications. *Int J Nanomedicine*. 2020;15:6917-6934.

387 12. Yu B, Jiang T, Liu D. BCMA-targeted immunotherapy for multiple myeloma. *J Hematol Oncol*.

388 2020;13(1):125.

389 13. Yang J, Zhou W, Li D, Niu T, Wang W. BCMA-targeting chimeric antigen receptor T-cell therapy for

390 multiple myeloma. *Cancer Lett*. 2023;553:215949.

391 14. Sawaftah NA, Paul V, Awad N, Husseini GA. Modeling of Anti-Cancer Drug Release Kinetics From

392 Liposomes and Micelles: A Review. *IEEE Trans Nanobioscience*. 2021;20(4):565-576.

393 15. Giuliani N, Mangoni M, Rizzoli V. Osteogenic differentiation of mesenchymal stem cells in multiple

394 myeloma: identification of potential therapeutic targets. *Exp Hematol*. 2009;37(8):879-886.

395 16. Boise LH, Kaufman JL, Bahlis NJ, Lonial S, Lee KP. The Tao of myeloma. *Blood*.

396 2014;124(12):1873-1879.

397 17. Joshua DE, Bryant C, Dix C, Gibson J, Ho J. Biology and therapy of multiple myeloma. *Med J Aust*.

398 2019;210(8):375-380.

399 18. Fanucchi MP, Fossella FV, Belt R, et al. Randomized phase II study of bortezomib alone and bortezomib

400 in combination with docetaxel in previously treated advanced non-small-cell lung cancer. *J Clin Oncol*.

401 2006;24(31):5025-5033.

402 19. Yang CH, Gonzalez-Angulo AM, Reuben JM, et al. Bortezomib (VELCADE) in metastatic breast cancer:

403 pharmacodynamics, biological effects, and prediction of clinical benefits. *Ann Oncol*. 2006;17(5):813-817.

404 20. Palumbo A, Chanan-Khan A, Weisel K, et al. Daratumumab, Bortezomib, and Dexamethasone for Multiple

405 Myeloma. *New England Journal of Medicine*. 2016;375(8):754-766.

406 21. Chen M, Juengpanich S, Li S, et al. Bortezomib-Encapsulated Dual Responsive Copolymeric

407 Nanoparticles for Gallbladder Cancer Targeted Therapy. *Adv Sci (Weinh)*. 2022;9(7):e2103895.

22. Gu Z, Wang X, Cheng R, Cheng L, Zhong Z. Hyaluronic acid shell and disulfide-crosslinked core micelles for in vivo targeted delivery of bortezomib for the treatment of multiple myeloma. *Acta Biomater*. 2018;80:288-295.

23. Cengiz Seval G, Beksac M. The safety of bortezomib for the treatment of multiple myeloma. *Expert Opin Drug Saf*. 2018;17(9):953-962.

24. Geisler S. Vincristine- and bortezomib-induced neuropathies - from bedside to bench and back. *Exp Neurol*. 2021;336:113519.

25. Shao J, Zaro J, Shen Y. Advances in Exosome-Based Drug Delivery and Tumor Targeting: From Tissue Distribution to Intracellular Fate. *Int J Nanomedicine*. 2020;15:9355-9371.

26. Kalluri R, LeBleu VS. The biology, function, and biomedical applications of exosomes. *Science*. 2020;367(6478).

27. Duan L, Xu L, Xu X, et al. Exosome-mediated delivery of gene vectors for gene therapy. *Nanoscale*. 2021;13(3):1387-1397.

28. Zeng H, Guo S, Ren X, Wu Z, Liu S, Yao X. Current Strategies for Exosome Cargo Loading and Targeting Delivery. *Cells*. 2023;12(10).

29. Sadeghi S, Tehrani FR, Tahmasebi S, Shafiee A, Hashemi SM. Exosome engineering in cell therapy and drug delivery. *Inflammopharmacology*. 2023;31(1):145-169.

30. Shah N, Chari A, Scott E, Mezzi K, Usmani SZ. B-cell maturation antigen (BCMA) in multiple myeloma: rationale for targeting and current therapeutic approaches. *Leukemia*. 2020;34(4):985-1005.

31. Helwa I, Cai J, Drewry MD, et al. A Comparative Study of Serum Exosome Isolation Using Differential Ultracentrifugation and Three Commercial Reagents. *PLoS One*. 2017;12(1):e0170628.

Figure legend

Figure 1. Characterization of anti-BCMA-Exo-Btz.

A Flow sorting of anti-BCMA positive monocytes. **B** Size distribution of anti-BCMA-Exo and anti-BCMA-Exo-Btz determined by dynamic light scattering (DLS). **C** CD81 and CD63 expressions of anti-BCMA-Exo and anti-BCMA-Exo-Btz by western blot. **D** Representative transmission electron microscopy (TEM) image of anti-BCMA-Exo and anti-BCMA-Exo-Btz. The expression of anti-BCMA on the surface of anti-BCMA-EXO-Btz membrane was detected by nano-flow cytometry (**E**) and colloidal gold immunoelectron microscopy (**F**). Scale bar, 100 nm. **G** The loading of Btz into exosomes was detected by fourier transform infrared spectrometer (FTIR). **H** The loading efficiency and encapsulation efficiency of Btz into exosomes.

Figure 2. The uptake, Btz intracellular accumulation and release of anti-BCMA-Exo-Btz in vitro.

U266 cells and LP-1 cells were treated with anti-BCMA-Exo-Btz. Internalization was measured by and flow cytometry (**A**) and fluorescence microscopy (**B**). Scale bar, 20 μm. **C** Intracellular Btz accumulation of U266 cells and LP-1 cells treated with free Btz and anti-BCMA-Exo-Btz. **D** In vitro release of Btz from anti-BCMA-Exo-Btz in pH 7.45 and pH 7.35 by HPLC. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$.

Figure 3. Proteomic analysis of monocyte-derived exosomes.

A The GeneMANIA database was adopted for protein interaction analysis of the protein in monocyte-derived exosomes. **B** The protein in monocyte-derived exosomes were enriched in pathways.

Figure 4. The function of monocyte-exosome.

A Cell viability of MM cells in response to treatment with CL-Exo. B The CL-Exo treatment at the IC50 concentration induced apoptosis (**B**) and cell cycle arrest (**C**) in MM cells. **D** The relative mRNA expression level of IL-1β and CD206 in macrophage after treated with 100μg/ml CL-Exo. **E** Cell viability of NK-92 cells in response to treatment with 100μg/ml CL-Exo. **F** The CL-Exo treatment at a concentration of 100μg/ml enhanced anti-tumor activity of NK-92 cells to MM cells. **G** The calcium deposition and ALP activity was determined after 7-days and 14-days treatment of 100μg/ml CL-Exo in MSCs. Scale bar, 100 μm. **H** The relative mRNA expression levels of Runx2, OPN, OCN, ALP and Col-l were determined by RT-PCR after 7-days and 14-days treatment of 100μg/ml CL-Exo in MSCs. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$.

Figure 5. The in vitro and in vivo anti-tumor effect of anti-BCMA-Exo-Btz in MM.

A Cell viability of MM cells in response to treatment with free Btz, CL-Exo and CL-anti-BCMA-Exo-Btz. In situ myeloma models were constructed by injecting LP-1 cells in the tail vein of NSG mice, and measured once every 3 days after treatment. The fluorescence intensity (**B**) and tumor growth curve (**C**) of the mice are shown. (**D**) Kaplan–Meier analysis revealed describe the survival rate of the mice in experiment. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$.

Figure 6. The distribution of DiR-labeled exosomes in vivo and its effect on bone lesion repair.

A Biodistribution of DiR-labeled exosomes in LP-1 tumor bearing NSG mice. **B** Biodistribution of DiR-labeled exosomes in the heart, kidney, lung, liver and spleen of the mice. **C** Micro-CT image shown the bone destruction of the scapular. **D** The percentage of scapular bone destruction area in different treatment groups. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$.

Figure 7. Safety assessment of anti-BCMA-Exo-Btz.

A Red blood cells (RBC), peripheral blood mononuclear cells (PBMC), monocytes and PBMC

except monocytes (PBMC-monocyte) were exposed to varying concentrations of 1, 2, 4, and 8mg/ml anti-BCMA-Exo loaded with Btz for 48 and 72 hours, and the cell viability were detected. **B** H&E staining of the tissue of heart, kidney, lung, liver and spleen. Scale bar, 200 μm.

Figure 1

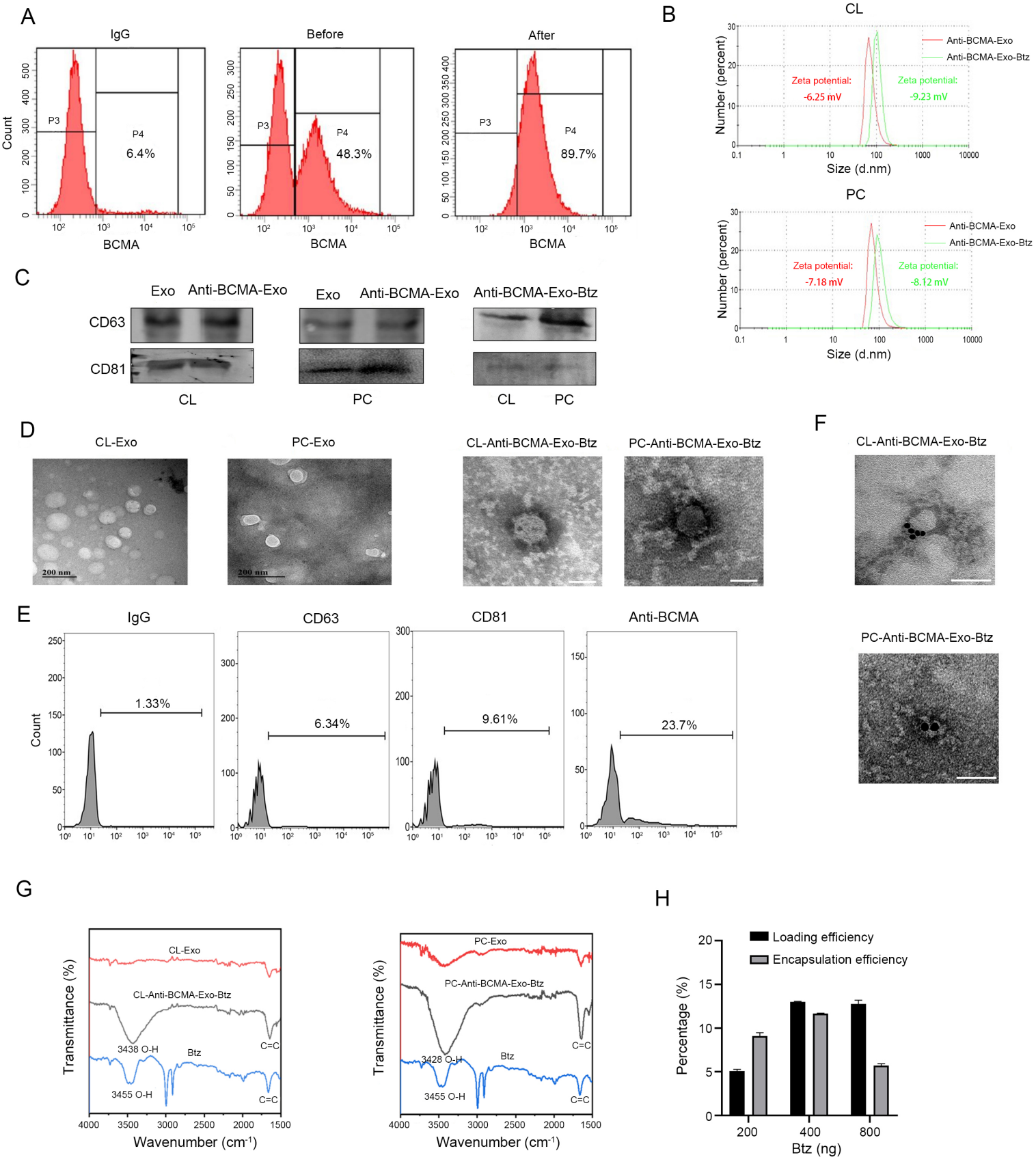


Figure 2

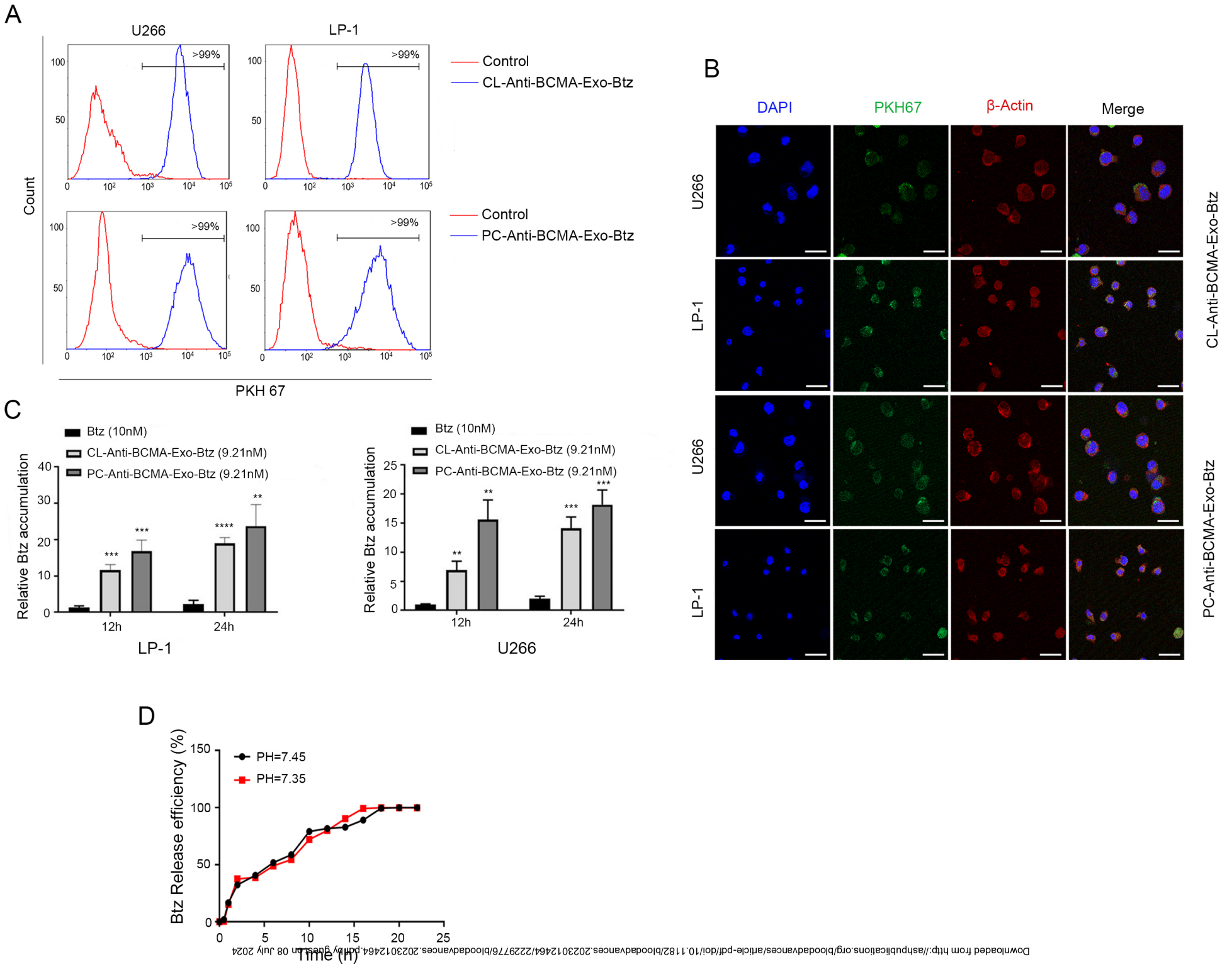
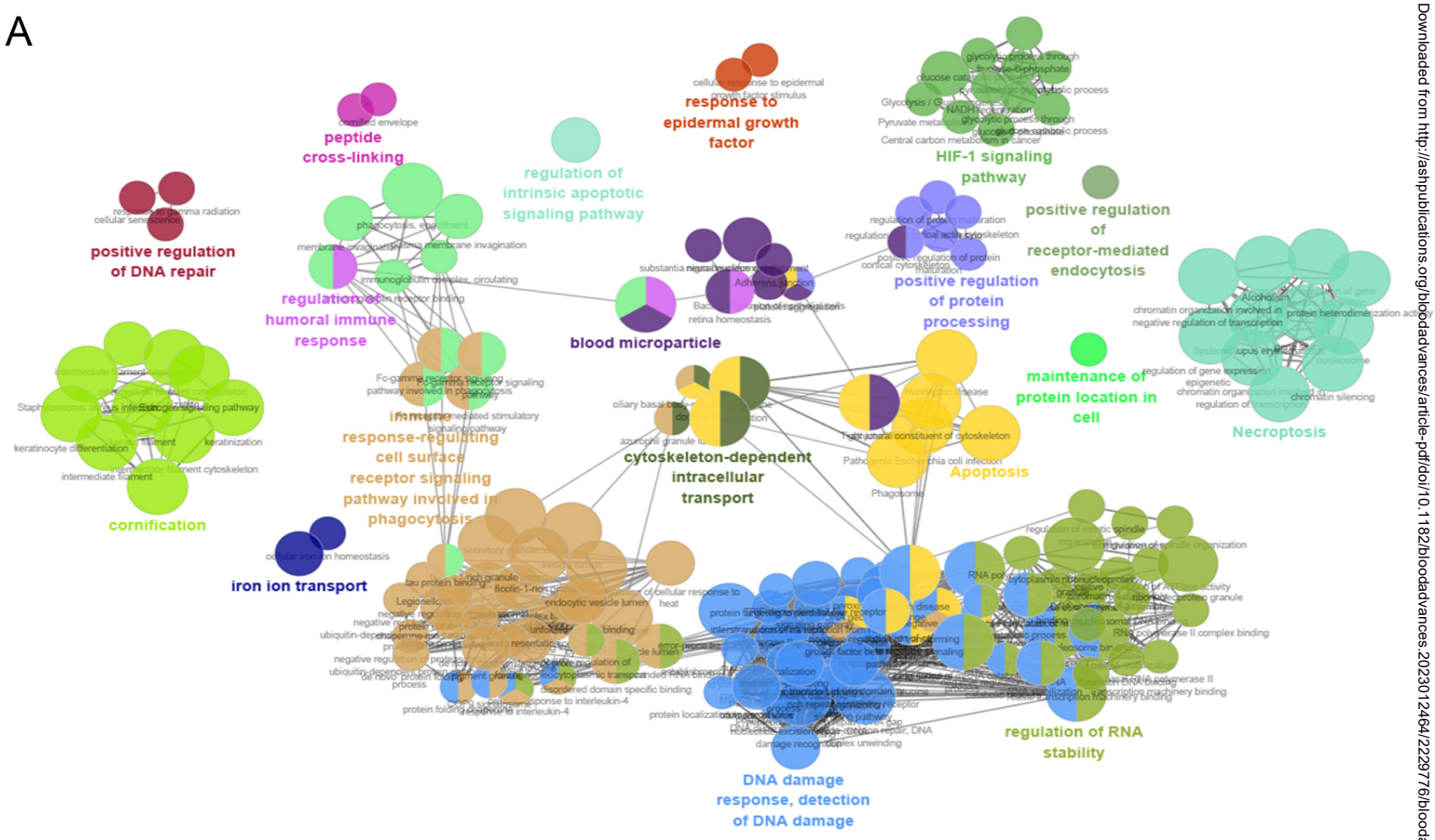
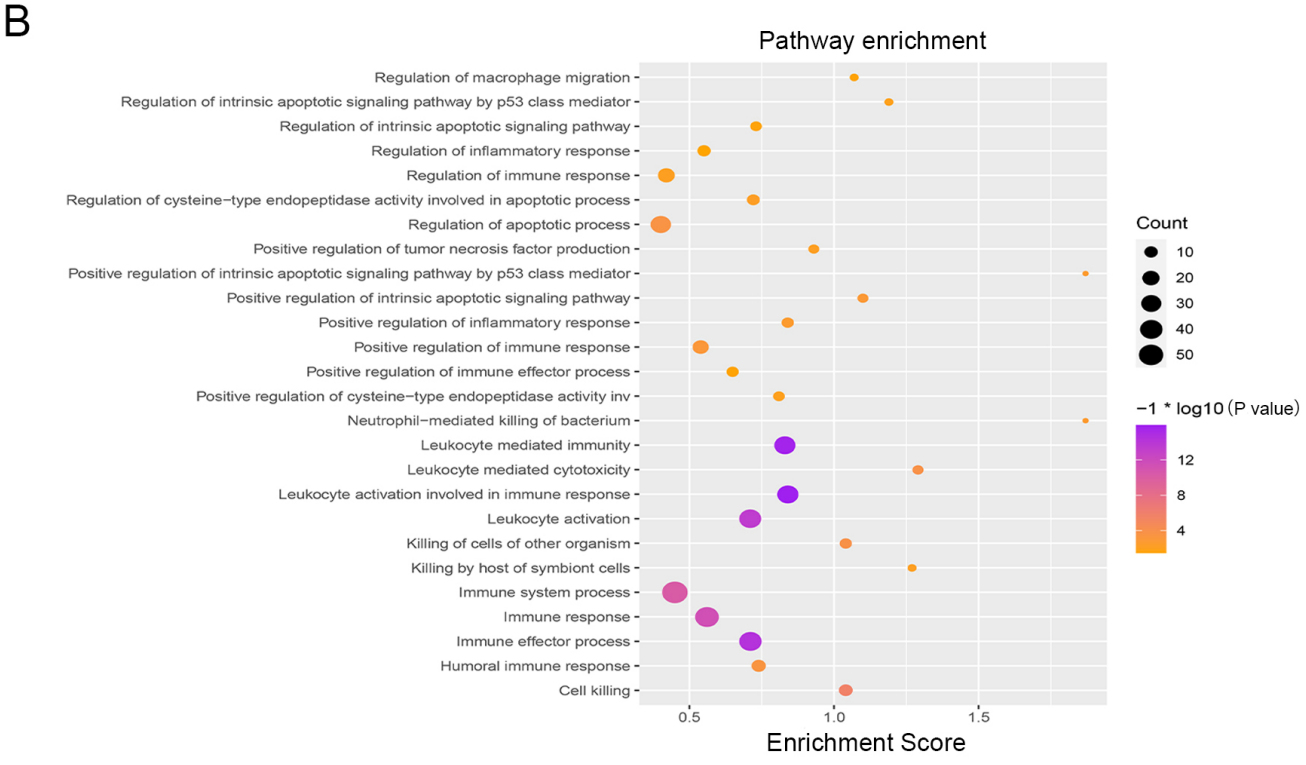


Figure 3





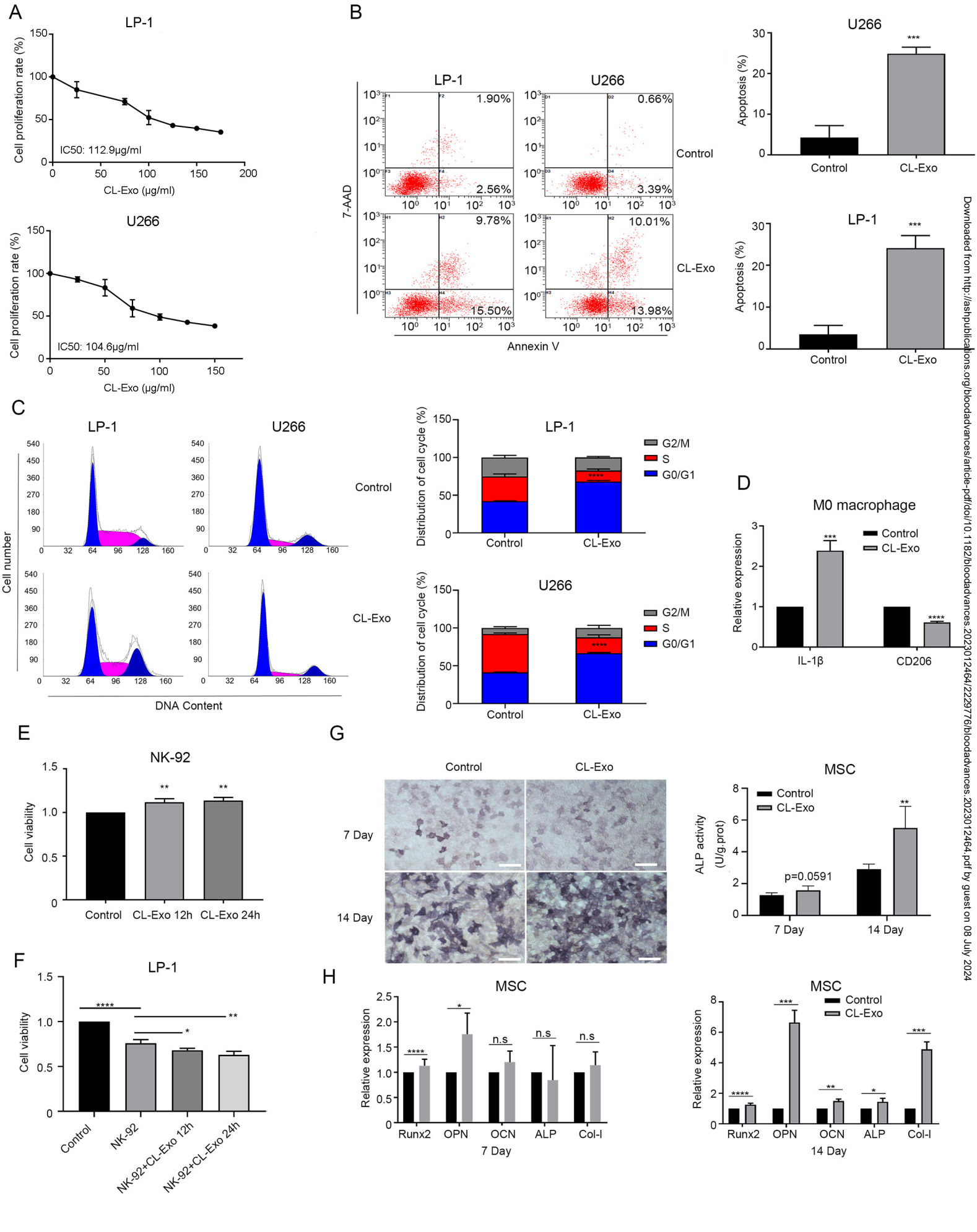
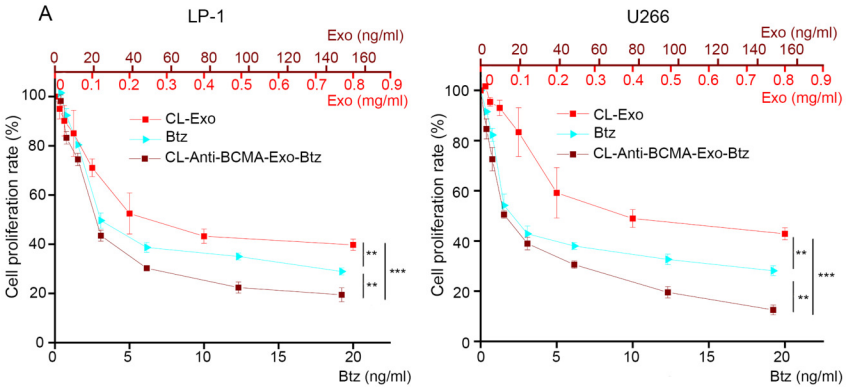
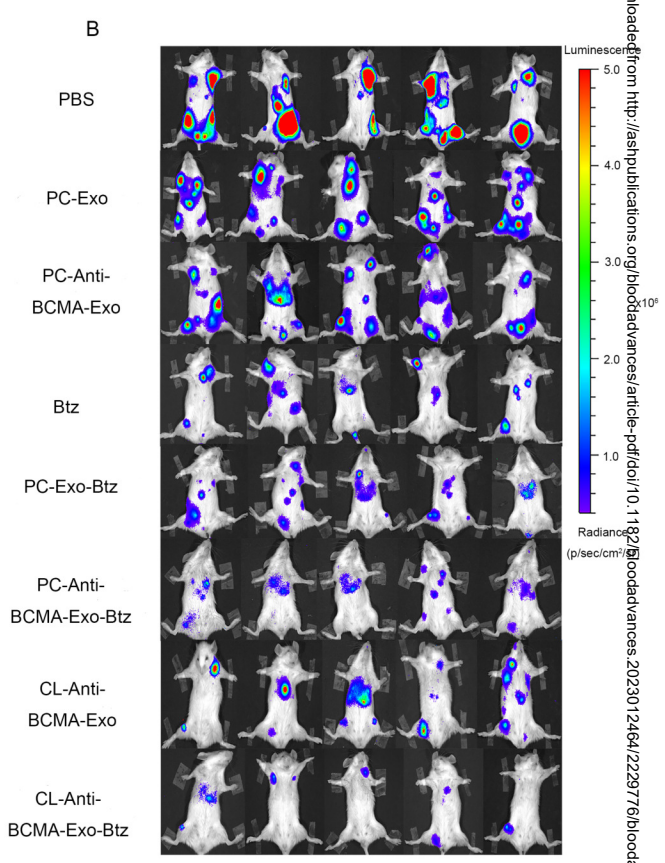
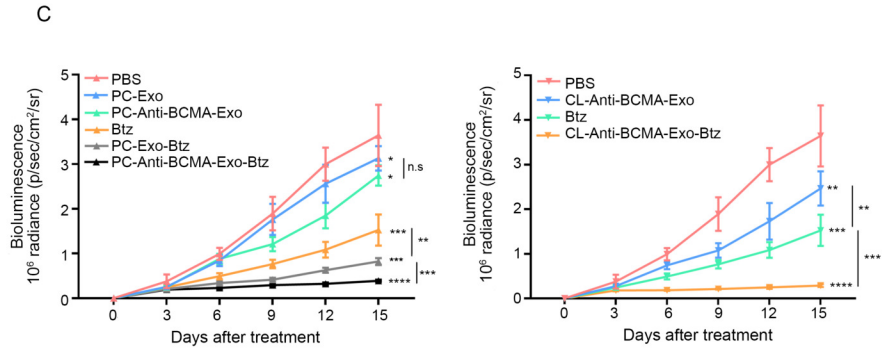


Figure 5







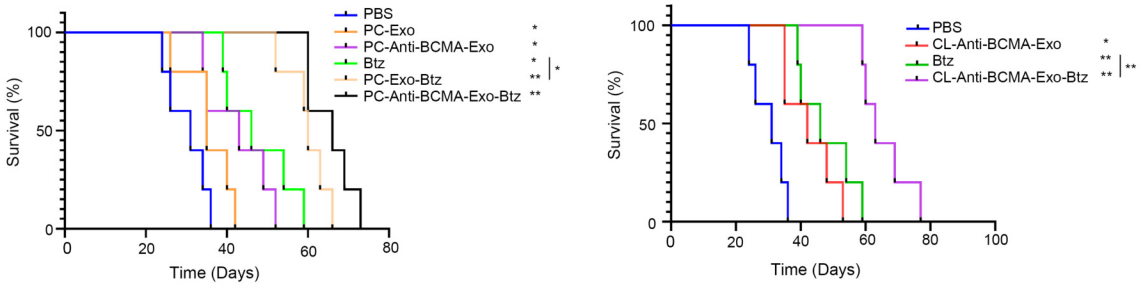


Figure 6

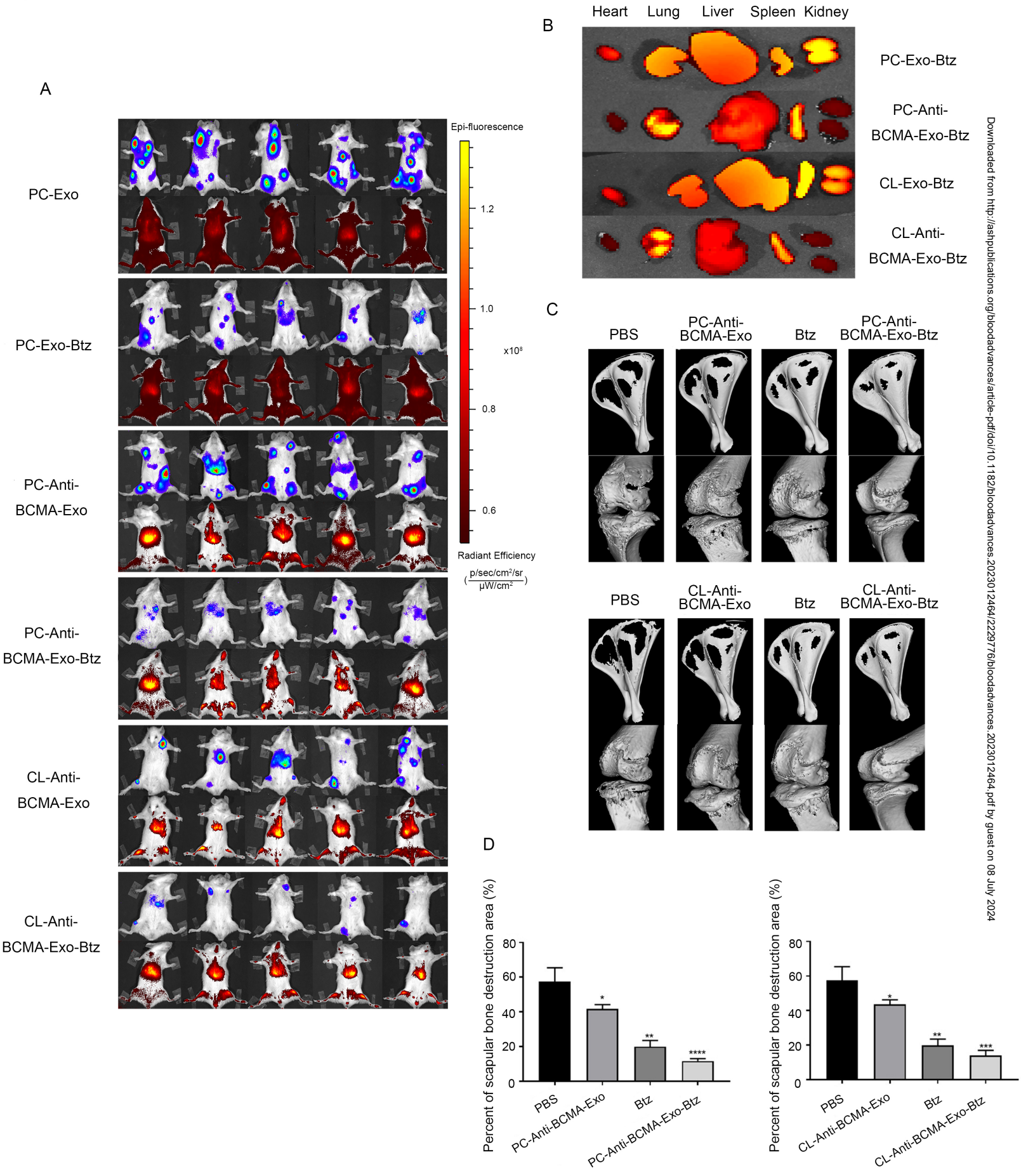


Figure 7