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ORIGINAL RESEARCH

Targeting Delivery of Dexamethasone to Inflamed Joints by Albumin-Binding Peptide Modified Liposomes for Rheumatoid Arthritis Therapy

Huan Wang[1,*], Rui Gou[2,*], Wei Li[2], Zhoujiang Chen[2], Chun Gu[3], Sanjun Shi[4], Liang Zou[2], Hanmei Li[2]

[1]Sichuan Industrial Institute of Antibiotics, School of Pharmacy, Chengdu University, Chengdu, 610106, People's Republic of China; [2]Key Laboratory of Coarse Cereal Processing (Ministry of Agriculture and Rural Affairs), School of Food and Biological Engineering, Chengdu University, Chengdu, 610106, People's Republic of China; [3]Department of Hepatobiliary & Pancreatic Center, Sichuan Provincial People's Hospital, University of Electronic Science and Technology of China, Chengdu, 610072, People's Republic of China; [4]State Key Laboratory of Southwestern Chinese Medicine Resources, School of Pharmacy, Chengdu University of Traditional Chinese Medicine, Chengdu, 611137, People's Republic of China

*These authors contributed equally to this work

Correspondence: Hanmei Li; Liang Zou, Email lihanmei@cdu.edu.cn; zouliangcdu@126.com

Introduction: Delivering the anti-inflammatory dexamethasone in nanoformulations is important for reducing off-target effects when treating rheumatoid arthritis. Nanoformulations can be targeted to sites of inflammation by modifying the nanoparticles with albumin before administration, but such particles can be unstable in vivo.

Methods: Here, we have developed and validated an alternative targeting in which dexamethasone-loaded liposomes were modified with a 46-residue peptide called "albumin-binding domain", such that the liposomes would adsorb endogenous albumin after administration.

Results: The resulting liposomes were small (90 nm diameter) and uniformly dispersed, and both X-ray diffraction and differential scanning calorimetry confirmed efficient dexamethasone encapsulation. Functionalizing the liposomes with albumin-binding peptide strongly increased not only their binding to albumin in vitro but also their uptake by RAW264.7 cells in culture. After injection into rats with adjuvant-induced arthritis, the liposomes accumulated and persisted at sites of inflammation, effectively inhibiting joint swelling and reducing the level of the inflammatory factors TNF-α and IL-1β in joints. The liposomes decorated with the albumin-binding peptide did not display obvious hepatotoxicity and did not reduce red and white blood cells number.

Discussion: Our results validate modifying liposomes with albumin-binding domain as a way to target them to sites of inflammation for efficient drug delivery against rheumatoid arthritis.

Keywords: endogenous albumin, rheumatoid arthritis, liposomes, targeting therapy, dexamethasone

Introduction

Rheumatoid arthritis is a chronic, systemic inflammatory disease involving persistent synovitis and irreversible damage to large and small joints.[1] The inflammation in rheumatoid arthritis is driven by various cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, as well as by reactive oxygen intermediates and prostaglandins.[2] Glucocorticoids such as dexamethasone (Dex) inhibit the secretion of pro-inflammatory cytokines and thereby mitigate the symptoms of the disease.[3] While Dex presents the advantages of easy oral absorption and a half-life of approximately 190 min in the blood circulation, its long-term use at high doses is associated with elevated risks of serious side effects, including glaucoma, cataracts, muscle wasting, impaired kidney function and osteoporosis.[4,5] Such off-target effects can be reduced by encapsulating Dex into nanomaterials such as liposomes.[6–8] The liposomes accumulate to some extent at sites of inflammation, presumably through passive targeting involving the "Extravasation via Leaky Vasculature and Inflammatory Cell-mediated Sequestration" (ELVIS) effect.[9] Dex-loaded liposomes have demonstrated their efficacy

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against lung inflammation.[10] However, the passive targeting of liposomes to sites of inflammation may be less effective than free Dex in the treatment of inflammatory diseases.

An alternative approach to targeting Dex to inflamed joints is to deliver it in nanoparticles modified with albumin. Albumin, naturally abundant in the blood, is quite biocompatible and persists in blood with a half-life of 19 days;[11] it can protect drug cargo from metabolic elimination in the body;[12] it can actively target inflammatory tissues, in part through binding to SPARC and perhaps other receptors that are overexpressed by the tissues;[13–17] and albumin can also promote retention of the drug at inflammatory sites.[18,19] However, we considered formulating Dex with albumin to be too risky as a treatment for rheumatoid arthritis, given the fact that the formulation of the anti-cancer drug paclitaxel into nanoparticles of albumin, although approved by the US Food and Drug Administration, proved unstable in vivo. Paclitaxel dissociates rapidly from the exogenous albumin of the formulation, resulting in poor pharmacokinetics.[20,21]

As an alternative, we have been inspired by previous efforts to improve the nanoformulations of cancer drugs, in which the nanoparticles spontaneously bind endogenous albumin in the blood circulation.[17,22,23] In those studies, endogenous albumin binding protected the drug cargo from being eliminated from the blood stream or metabolized into inactive forms. We hypothesized that by conferring albumin-binding ability to Dex-loaded liposomes, we could target them actively to sites of inflammation and thereby improve efficacy and minimize off-target effects.

With the aim, we have encapsulated Dex into liposomes decorated with the albumin-binding domain (ABD), which has a 46-residue sequence of LAEAKVLANRELDKYGVSDFYKRLINKAKTVEGVEALKLHILAALP-cys. This peptide derived from natural streptococci G protein binds strongly to serum albumin from humans and several other species.[11,24–26] Modifying nanocarriers with ABD enables them to interact strongly with endogenous albumin in vivo, it significantly prolongs their half-life in blood, and it targets drugs effectively to the sites of inflammation.[22] Zhang et al had prepared ABD035-modified micelles that can specifically bind albumin in vivo. This ABD035-modified micelles can increase the accumulation of paclitaxel in tumor tissue and significantly improve their therapeutic effect on triple negative breast cancer.[15] Li et al designed trimer albumin-binding domain (ABD-Tri) by fusing ABD with high specificity and affinity for albumin to a self-trimerizing domain (Tri) with an additional cysteine residue. This ABD-Tri can rapidly and specifically form a stable complex with albumin under physiological conditions without obviously changing its receptor- and cell-binding and tumor-homing properties.[27]

In the present study, we constructed ABD-modified liposomes (ABD-Lip) that can specifically bind to endogenous albumin in vivo. The uptake and targeting accumulation of ABD-Lip in inflammatory cells and inflamed joints as well as the anti-inflammatory efficacy of Dex loaded ABD-Lip (ABD-Lip/Dex) was studied throughoutly.

Materials and Methods

Materials

Soybean lecithin was purchased from Beijing Haifuda Technology (Beijing, China); $mPEG_{2000}$-DSPE and Mal-PEG_{2000}-DSPE, Tanshtech (Guangzhou, China); cholesterol, Kelon Chemical Reagent Factory (Chengdu, China); Dex, Meilun Biotechnology (Dalian, China); HEPES, Aid Science (Chengdu, China); and DiD, Chengdu Fulkangbei Biotechnology (Chengdu, China). The ABD peptide was synthesized by China Gill Biochemical (Shanghai, China).

High-glucose Dulbecco's modified Eagle medium, trypsin and fetal bovine serum were purchased from Gibco (Shanghai, China). Lipopolysaccharide was purchased from Biosharp Biotechnology (Guangzhou, China). DAPI solution was purchased from Solarbio Science & Technology (Beijing, China). Calcein/PI Cell Viability/Cytotoxicity Assay Kit was obtained from Beyotime Biotechnology (Beijing, China). Complete Freund's adjuvant was obtained from Chondrex (Beijing, China), while hematoxylin and eosin were purchased from Beyotime Biotechnology (Beijing, China). Chloral hydrate was obtained from Chengdu Chron Chemical (Chengdu, China), while ultrafiltration microcolumns with a molecular weight cut-off of 100 kDa were obtained from Millipore (Shanghai, China). Dialysis strips with a molecular weight cut-off of 3500 Da were purchased from Shanghai Yuanye Biotechnology (Shanghai, China). Enzyme-linked immunosorbent assays for TNF-α and IL-1β were obtained from China Elite Biotechnology (Chengdu, China). Other reagents were obtained in analytical purity from commercial sources.

Cells and Animals

Murine RAW264.7 monocyte-macrophage cells were purchased from Beyotime Biotechnology. Male Sprague-Dawley rats 5–6 weeks old were purchased from Si Pei Fu (Beijing) Biotechnology (Beijing, China). All animal experiments had been performed in accordance with the guidelines for the care and use of experimental animals and were approved by the Experimental Animal Administrative Committee of Chengdu University.

Preparation of Liposomes

Liposomes were prepared using the film dispersion method as described, with soy lecithin (S100), cholesterol (Chol), $mPEG_{2000}$-DSPE, the maleimide-containing phospholipid Mal-PEG_{2000}-DSPE, and dichloromethane as the solvent.[28–30] The resulting liposomes were stirred at 25 °C for 8 h with ABD in HEPES to prepare ABD-modified liposomes. Then, the mass ratio of the various components as well as the power and duration of ultrasonication for preparing ABD-modified liposomes were optimized using single-factor analysis. ABD-unmodified and ABD-modified liposomes that have been prepared using the optimized procedure were loaded with Dex by mixing Dex with the above materials for preparing liposomes (Lip/Dex, ABD-Lip/Dex). The same procedure was used to prepare ABD-unmodified and ABD-modified liposomes containing the fluorescent dye DiD instead of Dex (Lip/DiD, ABD-Lip/DiD).

The Characterization of Liposomes

The size distribution and zeta potential of Lip/Dex and ABD-Lip/Dex were determined by dynamic light scattering (ZEN3600 Particle Size and Zeta Potential Analyzer, Malvern, UK). The morphology of Lip/Dex and ABD-Lip/Dex was observed under transmission electron microscopy (H-600, Hitachi, Tokyo, Japan). Briefly, liposomes were diluted into deionized water and then placed onto a copper grid. Then, the samples were stained with phosphotungstic acid (1%) for 20s and observed under transmission electron microscopy.

The Encapsulation Efficiency and Drug Loading of the Liposomes

Drug loading (DL%) and encapsulation efficiency (EE%) of Dex in liposomes were determined by using ultrafiltration method. Lip/Dex and ABD-Lip/Dex were passed through an ultrafiltration tube (300 kD, 3000 g, 15 min) to remove the free Dex. The encapsulated Dex in the liposomes was detected by using high-performance liquid chromatography (LC-2010A, SHIMADZU, Kyoto, Japan).[31] The chromatographic conditions are as follows: octadecylsilane bonded silica gel was used as filler; acetonitrile-water (40:60) was used as mobile phase; the detection wavelength was 254 nm and the sample volume was 20 μL. The DL% and EE% were calculated according to the following equations (1) and (2), respectively:

$$DL\% = (W_1/W_2) \times 100\% \quad (1)$$

$$EE\% = (W_1/W_3) \times 100\% \quad (2)$$

W_1 represents the weight of drug in liposomes; W_2 represents the total weight of liposomes; W_3 represents the weight of drug added;

Stability of the Liposomes

To assess the stability of liposomes, Lip/Dex and ABD-Lip/Dex were stored at 4 or 37 °C for one week. The particle size and polydispersity index of the liposomes were detected by dynamic light scattering every day.

Fourier Transform Infrared Spectroscopy, x-Ray Diffraction and Differential Scanning Calorimetry of the Liposomes

Lip and ABD-Lip were prepared and freeze-dried for Fourier transform infrared spectrometer (PerkinElmer, MA, USA) detection. The recording range was 4000 ~ 400 cm^{-1}, and the resolution was 4 cm^{-1}. Lyophilized powders of free Dex, Lip/Dex, or ABD-Lip/Dex (1 mg) were pressed into x-ray diffraction molds and analyzed using X-ray diffractometer (DX-2700B, Dandng Haoyuan Instrument, China).

Lyophilized powders of the same three samples as above, in addition to powder of a simple physical mixture of Dex and ABD-Lip (1 mg) were analyzed by differential scanning calorimetry (DSC 3500 Sirius, NETZSCH, Germany). The amount of sample in each calorimetry run was 4–5 mg, the temperature ranged from 30 to 300 °C, and the N_2 flow rate was 20 mL/min.

In vitro Release of the Liposomes

The in vitro release of Dex from liposomes in PBS solution[32] or PBS contain 10% (v/v) fetal bovine serum[33] was analyzed using a dialysis method. Liposomes containing 333 μg of Dex were added into dialysis tubes, which were immersed in 50 mL of PBS or PBS contain 10% (v/v) fetal bovine serum and incubated at 37 °C with stirring at 70 rpm. At predetermined time points, 500 μL of the release medium was removed and replaced with the same volume of fresh PBS or PBS contain 10% (v/v) fetal bovine serum. The concentration of Dex in the aliquot was determined by high-performance liquid chromatography described in The Encapsulation Efficiency and Drug Loading of the Liposomes.

Albumin Binding Ability of the Liposomes

Bovine serum albumin was labeled with fluorescein isothiocyanate as described,[34] and 1 mL of the resulting FITC-BSA was added to the same volume of Lip/Dex or ABD-Lip/Dex. The mixtures were incubated at 37 °C for 1 h, then centrifuged at 15000 rpm for 1 h. The pellet was washed three times with ultrapure water to remove unbound albumin, then resuspended in PBS. The amount of albumin in the resuspended material was quantified based on fluorescence (excitation wavelength, 495 nm; emission wavelength, 525 nm) and a calibration curve prepared using solutions of FITC-BSA.[35]

Cytotoxicity of the Liposomes in vitro

RAW264.7 cells were seeded into 24-well plates (3×10^5 cells per well) and cultured for 24 h. Then, cells were exposed various concentration (25–400 μg/mL) of Lip or ABD-Lip for 24 h. The medium was discarded, and cells were washed with PBS for three times. After that, 250 μL Calcein-AM/PI solution was added to each well and incubated at 37 °C for 30 min in dark. The stained cell was then observed under a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). In addition, MTT assay was used to evaluate cell viability. RAW264.7 cells were seeded into 96-well plates (1×10^4 cells per well) after treatment with the same administration method. The medium was discarded, and each well received 100 μg MTT in 200 μL of fresh medium for 4 h. The medium was discarded, and each well received 150 μL DMSO. The plate was incubated at 37 °C for 15 min with shaking. Finally, absorbance of the wells at 490 nm was measured using a microplate reader, and cell viability was calculated as the ratio of absorbance of sample wells to absorbance of blank wells without liposomes.

Cellular Uptake of the Liposomes

Briefly, RAW264.7 cells were seeded into confocal dishes (2×10^5 cells per dish), incubated for 24 h, then exposed to 1 μg/mL lipopolysaccharide for 12 h. Lip/DiD and ABD-Lip/DiD were first incubated with serum for 2 h, then added to each dish. After 2 h of incubation, the medium was removed, and the cells were washed twice with PBS, fixed with 4% paraformaldehyde and stained with DAPI solution. The cells were then observed under a confocal laser scanning microscope (MICA, Leica Microsystems, Germany). In addition, a quantitative evaluation of uptake was also performed using flow cytometry. RAW264.7 cells were seeded into 6-well plates (5×10^5 cells per well) after treatment with the same administration method. After 2 h of incubation, the cells were trypsinized, harvested, pelleted at 15000 rpm for 5 min, resuspended in 500 μL of PBS and analyzed by flow cytometer (Cytomics FC 500, Beckman Coulter, CA, USA).

Biodistribution of the Liposomes in a Rat Model of Arthritis

Rats (180 ± 20 g) were injected with 50 μL of complete Freund's adjuvant subcutaneously into the left and right hind feet in order to induce arthritis.[36–38] The adjuvant suspension was shaken well immediately before injection. Only animals showing the expected swelling of hind limbs were used in experiments.

To examine the biodistribution of liposomes, arthritic rats were randomly divided into two groups (n = 6). They were injected via the tail vein with Lip/DiD or ABD-Lip/DiD (4 μg DiD per rat). Fluorescence intensity was measured in vivo at 1, 10 and 24 h after the injection by using an IVIS spectral system (QuickView3000, Bio-real, Sweden). After the last measurement at 24 h, rats were killed; then, key organs and joints were analyzed ex vivo by using the same IVIS system.

Therapeutic Efficacy of the Liposomes in a Rat Model of Arthritis

To assess the therapeutic efficacy of the nanoformulations, on day 14 after the induced arthritis model. Arthritic rats (140 ± 20 g) were randomly divided into four groups (n = 6), they were injected via the tail vein with PBS, Dex solution, Lip/Dex or ABD-Lip/Dex. All rats that received a Dex dose of 0.8 mg/kg.[4] In parallel with these treatments of arthritic rats, healthy rats (n = 6) were injected with the same volume of saline.

Rats received the first injection on day 14 after induction of arthritis, followed by a single daily injection for the next six days. Starting on day 14, we measured body weight, paw thickness and the severity of arthritis symptoms in hind limb joints on a previously reported scale[37] daily for one week. We also photographed the hind limb joints on day 14 and day 21.

On day 21 after induction of arthritis, ankle joint tissue from three animals per condition was cleaned of blood using pre-chilled PBS, fixed in 4% paraformaldehyde for 48 h, decalcified in 15% EDTA for five weeks, embedded in paraffin and cut into thin sections. The sections were stained with hematoxylin and eosin, then analyzed under an inverted microscope (DSZ2000X, Aopu, China).

TNF-α and IL-1β Levels of the Rat Joint Tissues

On day 21 after induction of arthritis, all rats were killed, and joint tissues were removed, homogenized in PBS and centrifuged. Levels of TNF-α and IL-1β in the supernatant were determined using commercial assays according to the ELISA kits manufacturer's instructions.

Safety of the Liposomes in Healthy Rats

Healthy rats (140 ± 10 g) were randomly divided into four groups (n = 5), they were injected via the tail vein with PBS, Dex solution, Lip/Dex or ABD-Lip/Dex. All rats that received Dex received a dose of 0.8 mg/kg. Rats were injected once daily for one week. On day 8, their serum was assayed for levels of alanine aminotransferase and aspartate aminotransferase, while numbers of red and white blood cells in the whole blood were determined. These analyses were performed using a fully automatic biochemical analyzer (BK-200, Boke Biotechnology, China).

Statistical Analysis

Data were reported as mean ± standard deviation (SD), and inter-group differences were assessed for significance using a one-way analysis of variance. Significance was defined at three levels: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. All statistical analyses were performed within GraphPad Prism 8.0.

Results and Discussion

Preparation and Characterization of ABD-Modified Liposomes Loaded With Dex

The optimal preparation of liposomes was found to involve the following mass ratios of components: soy lecithin, 3; cholesterol, 1; $mPEG_{2000}$-DSPE, 1.5; and Mal-PEG_{2000}-DSPE, 0.45. The optimal ultrasonication regime was power of 260 W and duration of 5 min. Liposomes loaded with Dex, whether ABD-modified or not, had an average diameter of 90 nm and a polydispersity index below 0.30 (Figure 1A). Consistent with these results, the transmission electron microscopy image showed that ABD-modified liposomes loaded with Dex had a diameter around 100 nm, zeta potential of −20 mV, and uniformly spherical shape (Figure 1A and B). The encapsulation efficiency of ABD-Lip/Dex was about 80.04%, and the drug loading was 2.18%. The size of these liposomes did not change significantly during one week of storage at 4 °C or 37 °C (Figure 1C and D), indicating no substantial aggregation, sedimentation or degradation. The

A

Sample	Size (nm)	PDI	Zeta (mV)
Lip/Dex	98.72 ± 2.75	0.270 ± 0.001	- 42.8 ± 4.4
ABD-Lip/Dex	92.85 ± 0.39	0.250 ± 0.011	- 20.3 ± 1.43

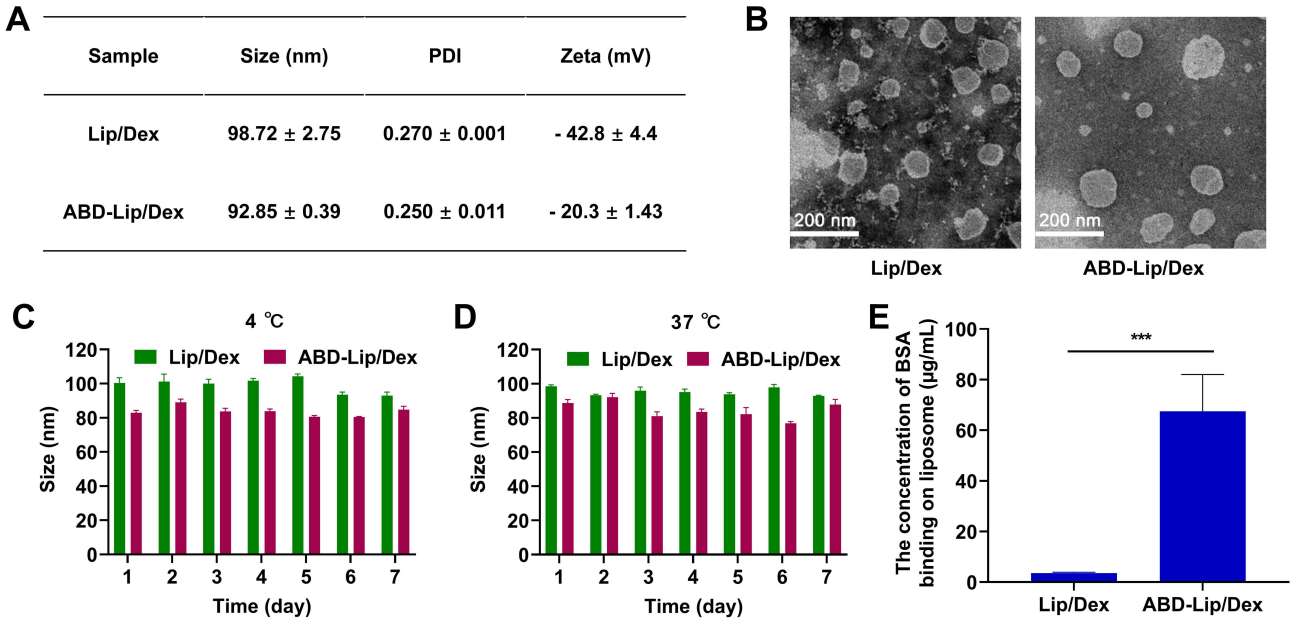

Figure 1 Characterization of liposomes loaded with Dex and either unmodified (Lip/Dex) or modified with the "albumin-binding domain" peptide (ABD-Lip/Dex). (**A**) Diameter, polydispersity index and zeta potential, based on dynamic light scattering. Results are the mean ± SD of three independent batches. (**B**) Transmission electron micrographs. (**C**) Liposome diameter during storage at 4 °C. (**D**) Liposome diameter during storage at 37 °C. (**E**) Quantitation of the binding of FITC-conjugated bovine serum albumin to the surface of liposomes in vitro. Results are the mean ± SD of three independent batches. ***, $P < 0.001$.
Abbreviations: Lip, liposomes; Dex, dexamethasone; ABD, albumin-binding domain; FITC, fluorescein isothiocyanate.

ABD functionalization substantially increased the ability of Lip/Dex to bind FITC-conjugated bovine serum albumin in vitro: each 1 mg of ABD-Lip bound 14.76 μg of albumin, compared to only 0.78 μg per 1 mg of Lip (Figure 1E).

Characterization of the Structure of Liposomes

As shown in Figure 2A, the 1736 cm^{-1} was the C=C stretching vibration peak of maleimide group in liposomes, the 3286 cm^{-1} was the characteristic peak of sulfhydryl group in ABD peptide. In the infrared spectrum of ABD-Lip, characteristic peaks of sulfhydryl and maleimide group were disappear or weakened. In addition, at 635 cm^{-1}, an obvious stretching vibration peak was generated, which may be the peak of thioether bond generated by the addition reaction between sulfhydryl group and C=C. These indicating the successful conjugation between ABD peptide and liposomes.

X-ray diffraction analysis detected the same peak forLip/Dex or ABD-Lip/Dex, but not a peak for the drug itself (Figure 2B), implying successful encapsulation and intact liposome structure. Consistently, differential scanning calorimetry detected a heat absorption peak at 268 °C that is characteristic of Dex[39] when the drug was free in solution, but not

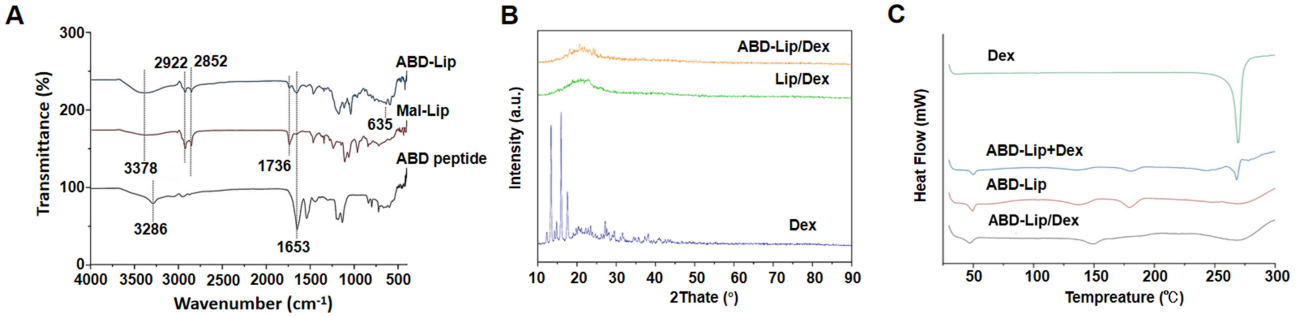

Figure 2 Characterization for the structure of liposomes. (**A**) Fourier transform infrared spectroscopy. (**B**) X-ray diffraction analysis. (**C**) Differential scanning calorimetry. Results are the mean ± SD of three independent batches.
Abbreviations: ABD, albumin-binding domain; Dex, dexamethasone; PBS, phosphate buffered saline.

when it was encapsulated within ABD-modified liposomes (Figure 2C). This result implies that the drug exists in an amorphous phase within the liposomes.[40]

Drug Release of the Liposomes in vitro

When the release medium was PBS, free Dex showed cumulative in vitro release of 90% by 8 h, compared to 60% by 72 h for Dex in Lip/Dex or ABD-Lip/Dex (Figure 3A). When the medium was PBS containing 10% (v/v) fetal bovine serum, free Dex showed nearly complete cumulative release by 8 h, compared to 80% by the same time point for Dex in liposomes (Figure 3B). Thus, encapsulation of Dex into liposomes, whether modified with ABD or not, substantially delays its release. All results indicated that ABD-Lip/Dex with suitable particle size, uniform size, good stability, sustained drug release and strong binding ability to albumin were successfully prepared. The liposomes can be further used for pharmacokinetic and pharmacodynamic studies of inflammation-related diseases.

Cytotoxicity and Cellular Uptake of ABD-Modified Liposomes Containing Dexamethasone

The cytotoxicity of the liposome was assayed by Calcein-AM/PI staining and MTT. Fluorescence microscope observation showed ABD-Lip and Lip had comparable cell viability at each concentration. As the concentration of liposome increases, the number of dead cells increases slightly (Figure 4A and C). The MTT detection results were consistent with the cytotoxicity measured by Calcein-AM/PI staining (Figure 4B and D). Even at lipid concentration up to 200 μg/mL, the cell viability of Lip and ABD-Lip treated groups was above 80%, suggesting that the ABD-modified drug delivery material had low cell toxicity and good biocompatibility. Cell uptake results showed that cultures of inflammatory RAW264.7 cells internalized modified liposomes to a significantly greater extent than unmodified liposomes in the presence of the serum, suggesting that the ABD peptide would be able to target the drug to sites of inflammation in vivo (Figure 4E and F).

Biodistribution of the Liposomes in a Rat Model of Arthritis

At 1 h after injection of Lip/DiD or ABD-Lip/DiD into rats with adjuvant-induced arthritis, in vivo fluorescence analysis showed that the liposomes accumulated in inflamed joints in a time-dependent manner, although only the ABD-Lip remained abundant in inflamed joints at longer times (Figure 5A). Ex vivo measurement of fluorescence at 24 h after injection showed substantially greater abundance of modified than unmodified liposomes in joints (Figure 5B and C). These results indicated that ABD-modified liposomes could bind to endogenous albumin at sites of inflammation in vivo, thereby lingering at sites for a long time, and ABD-modified liposomes could target drugs to the inflammatory joint tissues.

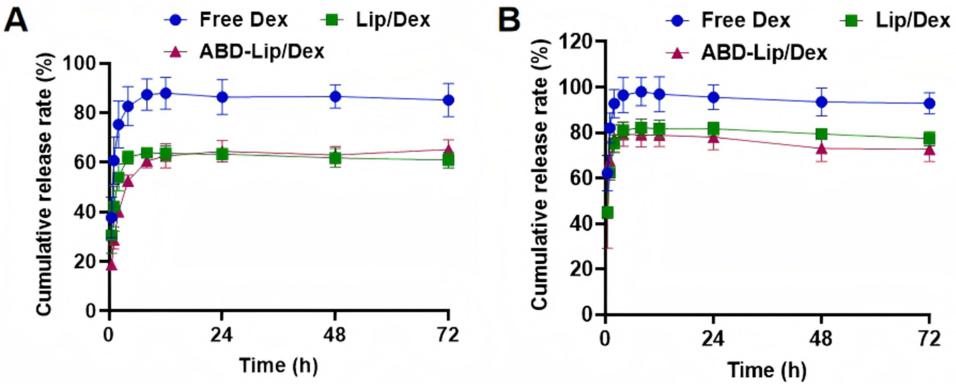

Figure 3 Drug release of the liposomes in vitro. (**A**) Dialysis-based assays of drug release in vitro in PBS. (**B**) Dialysis-based assays of drug release in vitro in PBS containing 10% (v/v) fetal bovine serum. Results are the mean ± SD of three independent batches.
Abbreviations: ABD, albumin-binding domain; Dex, dexamethasone; PBS, phosphate buffered saline.

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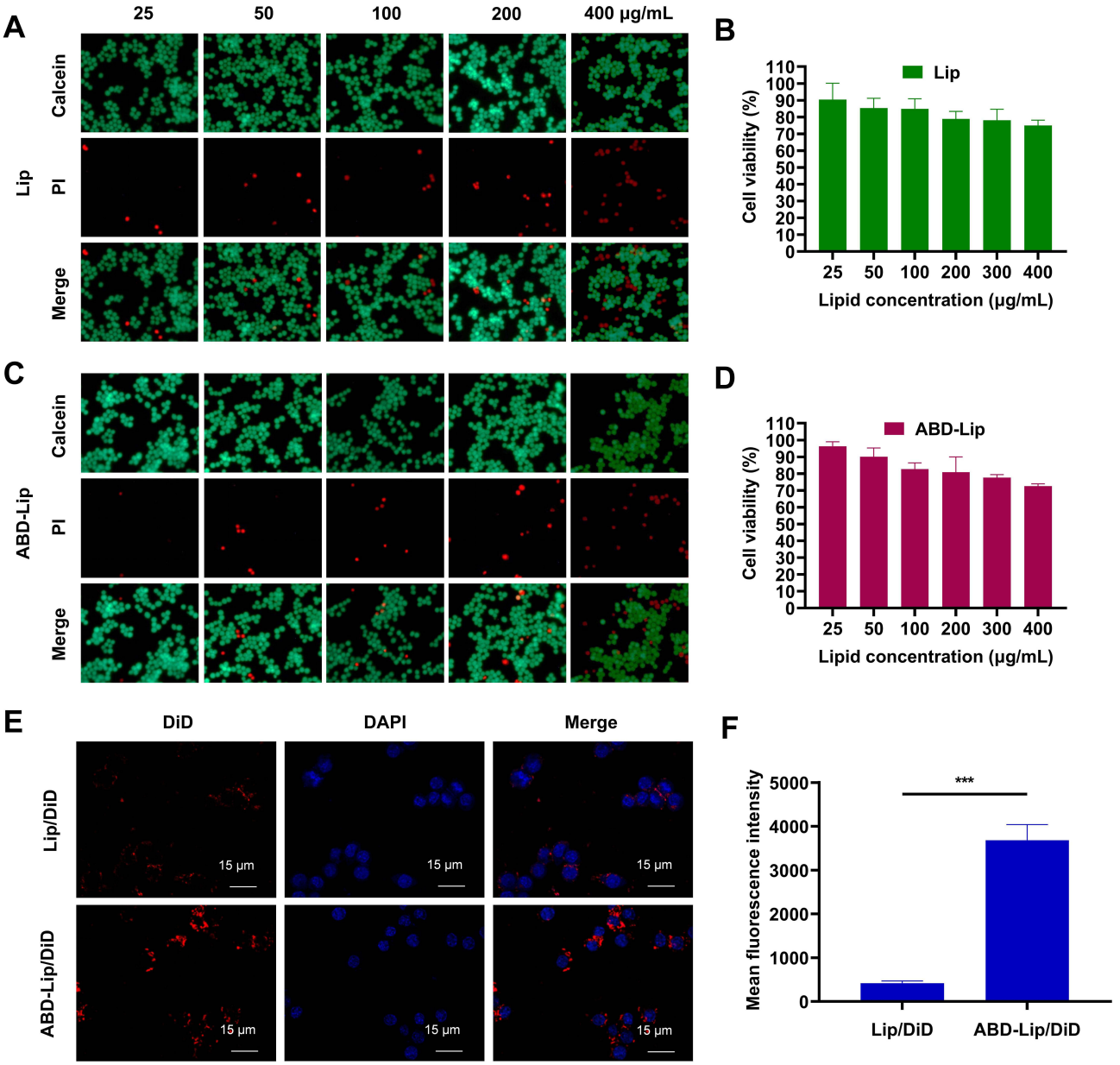

Figure 4 Cytotoxicity and cellular uptake of liposomes in cultures of RAW264.7 cells. (**A**) Live/dead staining images of RAW264.7 cells treated with Lip at different concentrations. (**B**) Cell viability of RAW264.7 cells treated with Lip was assessed using the MTT assay. (**C**) Live/dead staining images of RAW264.7 cells treated with ABD-Lip at different concentrations. (**D**) Cell viability of RAW264.7 cells treated with ABD-Lip was assessed using the MTT assay. (**E** and **F**) Cellular uptake of liposomes in RAW264.7. Cultures were exposed for 2 h to unmodified liposomes loaded with the fluorescent dye DiD (Lip/DiD) or ABD-modified liposomes loaded with the dye (ABD-Lip/DiD), and cellular internalization of the dye was observed using (**E**) confocal laser scanning microscope (magnification of ×63, scale bar = 15 μm) and (**F**) flow cytometry. Results are the mean ± SD of three independent batches. ***, $P < 0.001$.

Abbreviations: Calcein, calcein acetoxymethyl ester, green fluorescence; PI, propidium iodide, red fluorescence; Lip, liposomes; ABD, albumin-binding domain; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide; DiD, 1.1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine, red fluorescence; DAPI, 4′,6-diamidino-2-phenylindole, blue fluorescence.

Therapeutic Efficacy of Nanoformulations in a Rat Model of Arthritis

Next, we injected arthritic rats with free Dex, Lip/Dex or ABD-Lip/Dex. All three formulations relieved paw swelling to a certain extent in a time-dependent manner, based on joint score (Figure 6A), paw thickness (Figure 6B), visual inspection of paws (Figure 6C) and hematoxylin–eosin staining of joint sections (Figure 6D). Joints from animals given PBS showed obvious cartilage layer destruction, pannus formation and infiltration by inflammatory cells, while this pathology was milder in joints from animals treated with free Dex or Lip/Dex. However, joints from animals treated with

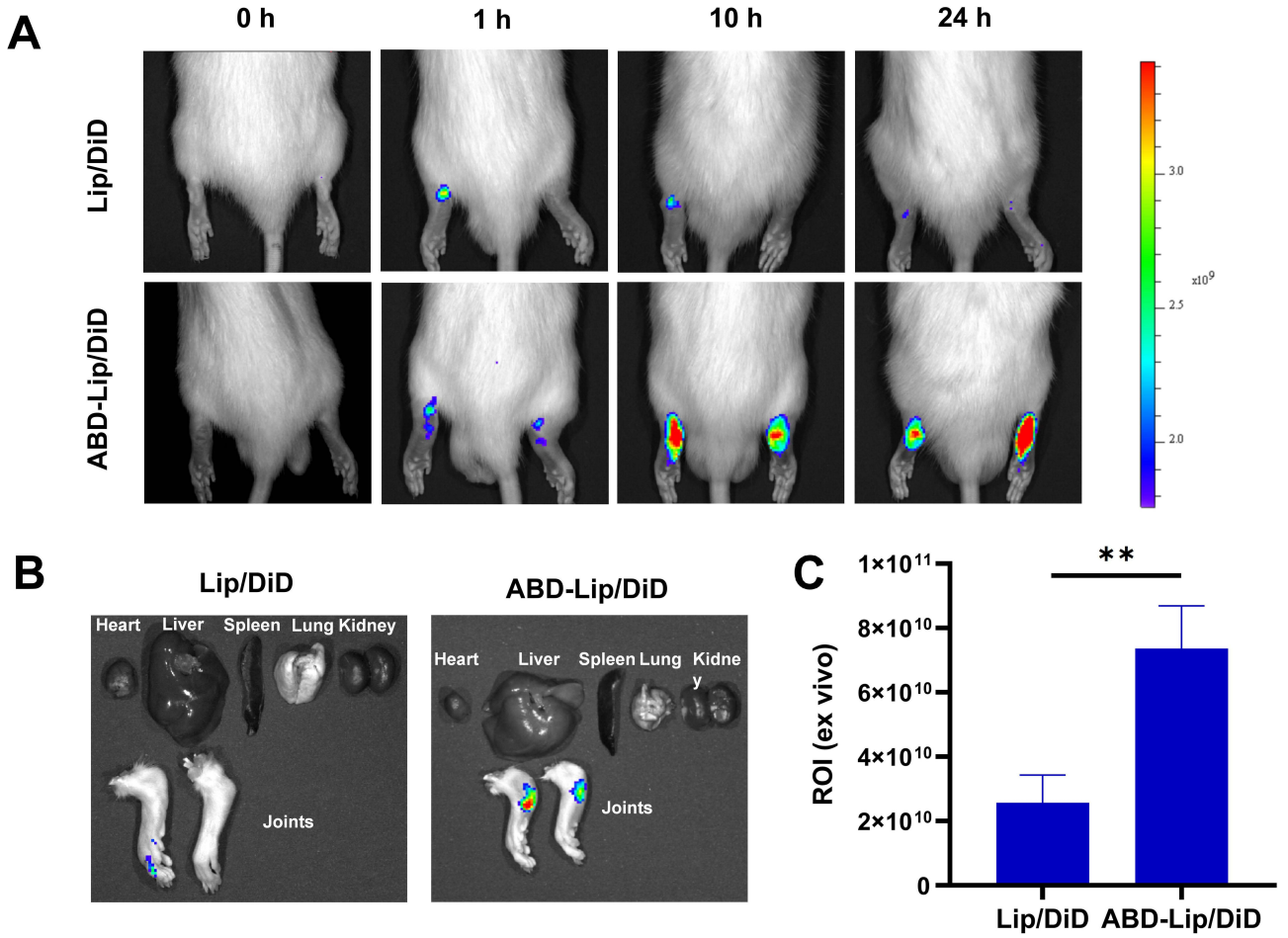

Figure 5 Biodistribution of unmodified liposomes containing the fluorescent dye DiD (Lip/DiD) or liposomes modified with the "albumin-binding domain" peptide and containing DiD (ABD-Lip/DiD) in rats with adjuvant-induced arthritis. Liposomes were injected intravenously. (**A**) Analysis of in vivo fluorescence at the indicated times after injection. (**B**) Analysis of ex vivo fluorescence of joints and key organs at 24 h after injection. (**C**) Fluorescence quantification of joints ex vivo. Results are the mean ± SD of six animals per condition. **, $P < 0.01$.

Abbreviations: Lip, liposomes; ABD, albumin-binding domain; DiD, 1.1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine, red fluorescence.

free Dex still showed chondrocyte proliferation, disordered cartilage arrangement, and hyperplasia of surface fibers. Joints from animals treated with Lip/Dex showed chondrocyte proliferation but not the other types of pathology observed with free drug. Joints from animals treated with ABD-Lip/Dex showed a nearly intact joint cavity, minimal synovial hyperplasia and minimal infiltration by inflammatory cells. In other words, Lip/Dex was marginally more effective than free Dex, while ABD-Lip/Dex was by far the most efficient of all three treatments. We found the same trend in efficacy when we compared levels of TNF-α and IL-1β in joints: ABD-Lip/Dex reduced the levels significantly more than the other two formulations (Figure 6E and F). Thus, ABD-modified liposomes significantly improved the anti-inflammatory effect of Dex in vivo.

Safety of the Liposomes in Healthy Rats

Injecting free Dex or Lip/Dex or ABD-Lip/Dex did not significantly alter levels of aspartate transaminase or alanine aminotransferase in serum (Figure 7A and B) or numbers of white blood cells (Figure 7C). These results suggest minimal impact on function of the liver or leukocytes in the blood. However, all three formulations significantly increased the number of red blood cells (Figure 7D), which may reflect the ability of Dex to stimulate hematopoiesis in bone marrow.[41] These data suggest that ABD-modified liposomes containing Dex may show acceptable safety in vivo.

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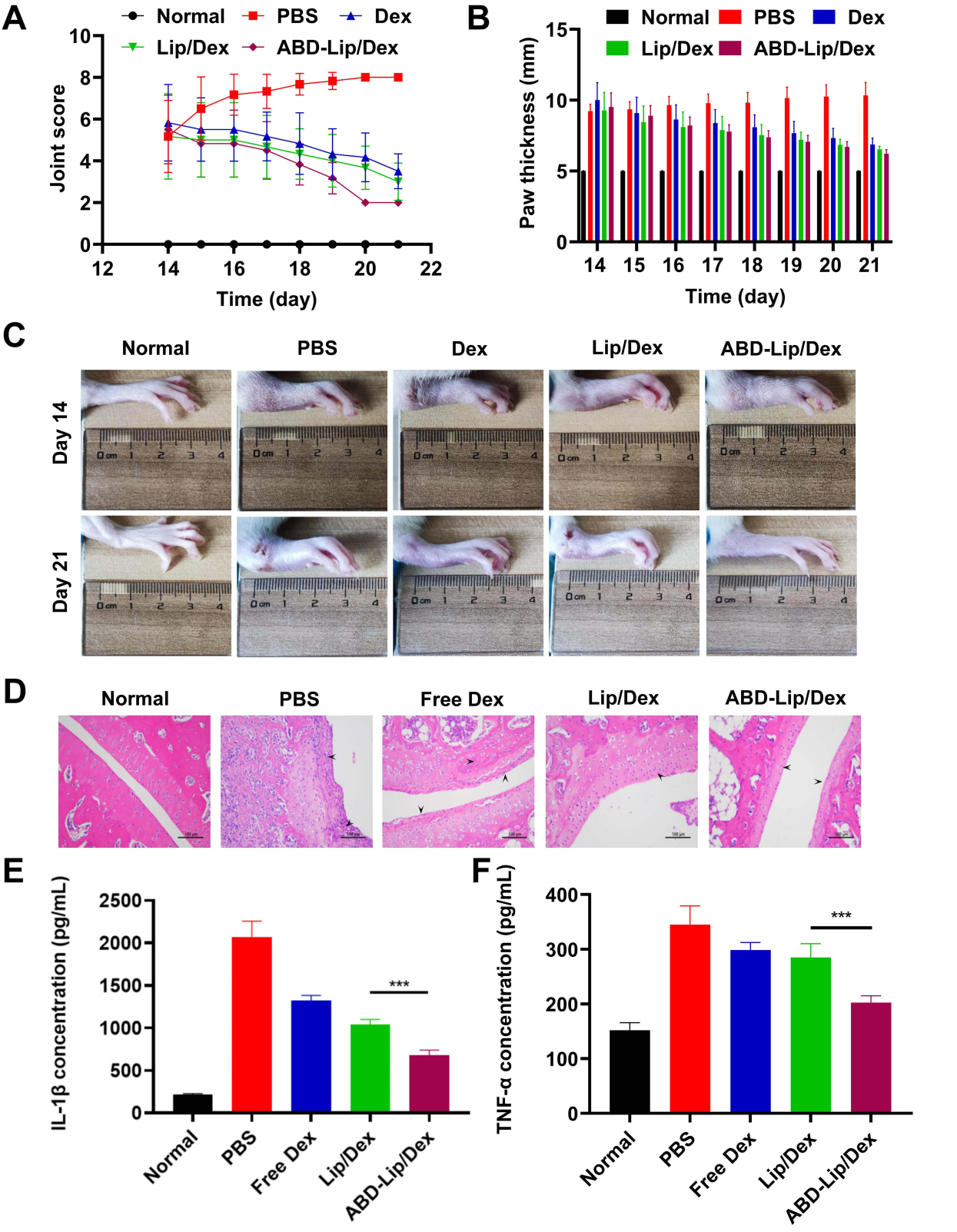

Figure 6 Therapeutic efficacy of free Dex, unmodified liposomes containing Dex (Lip/Dex) or liposomes modified with the "albumin-binding domain" peptide and containing Dex (ABD-Lip/Dex) in rats with adjuvant-induced arthritis. Liposomes were injected intravenously. As negative controls, arthritic rats were injected with phosphate-buffered saline (PBS), and healthy rats were injected with saline (Normal). (**A**) Severity of arthritic symptoms in hind limb joints as a function of days after injection. (**B**) Thickness of hind limb paws as a function of days after injection. (**C**) Representative photographs of hind limbs. (**D**) Histopathology of ankle joint sections after staining with hematoxylin-eosin. (**E** and **F**) The level of (**E**) TNF-α and (**F**) IL-1β in the joint tissues. Animals were treated as described in that figure legend, and joint tissues were homogenized at 21 days after injection of arthritis and assayed for TNF-α and IL-1β. Results are the mean ± SD of six animals per condition. ***, $P < 0.001$.
Abbreviations: Dex, dexamethasone; Lip, liposomes; ABD, albumin-binding domain; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β.

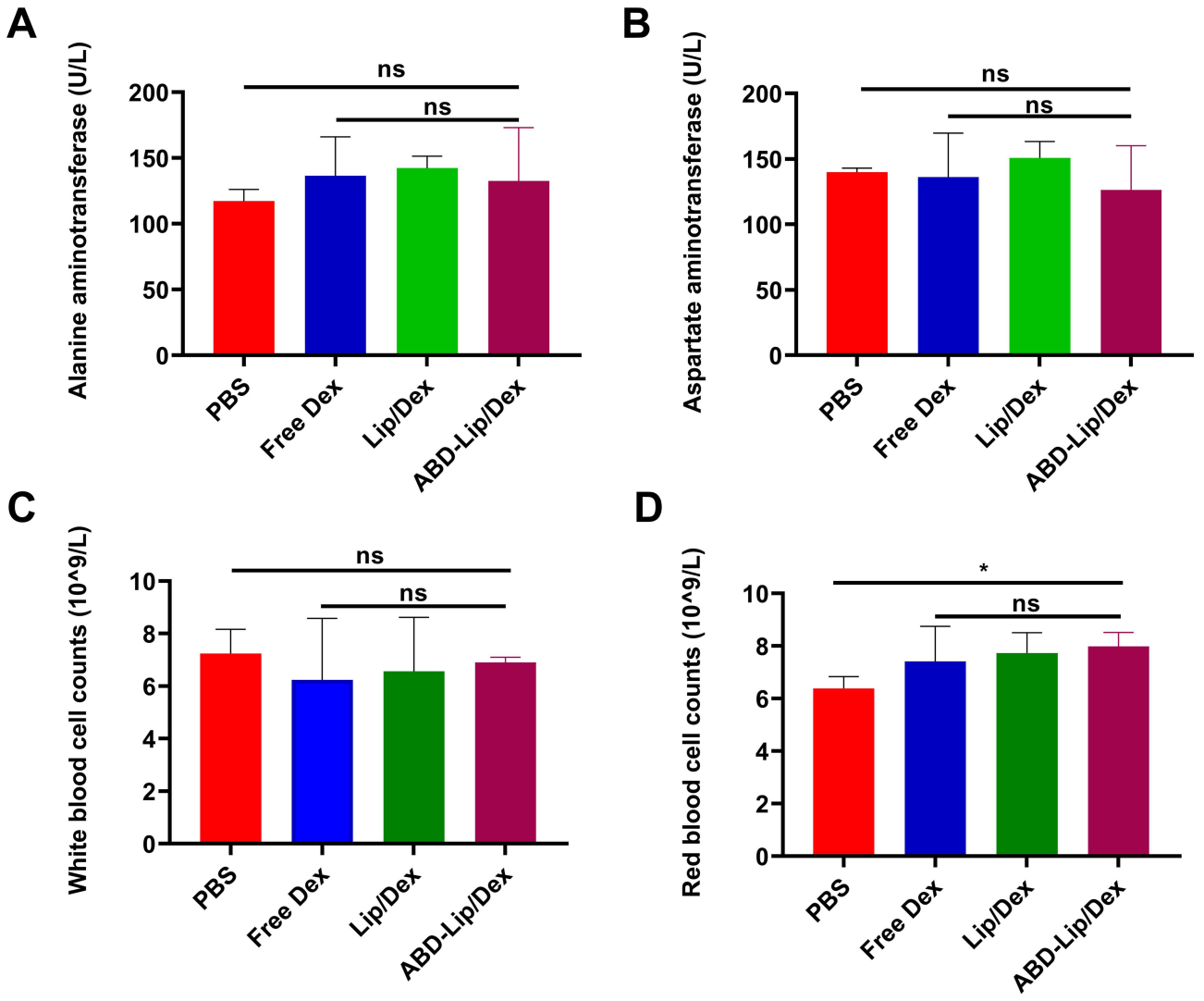

Figure 7 Effects of the formulations on the liver function indicators (**A**) alanine aminotransferase and (**B**) aspartate aminotransferase. The numbers of (**C**) white blood cells and (**D**) red blood cells in healthy rats. Animals were treated as described in that figure legend, and blood was analyzed at 8 days after injection. Results are the mean ± SD of five animals per condition.*, $P < 0.05$.

Abbreviations: ns, no significance; Dex, dexamethasone; Lip, liposomes; ABD, albumin-binding domain domain.

Discussion

Rheumatoid arthritis is a chronic, systemic inflammatory disease characterized by persistent synovitis, which leads to damage to the joints and periarticular structures. Dex is a common used drug for treating rheumatoid arthritis. However, Dex presents non-specific distribution after entering the human body, resulting in systemic side effects and low treatment efficiency. Encapsulating Dex in nanodelivery systems has shown potential for reducing its off-target effects and sustaining its release over longer periods, which make long-term treatment regimes against rheumatoid arthritis more safer. In order to further increase the therapeutic effect, the nanodelivery systems need to be modified with targeted molecules. Nanoparticles can target inflammatory sites by the modification of exogenous albumin before administration. But exogenous albumin has the risk of infection, and its quality control is very difficult. Therefore, here we modified Dex-loaded liposomes with the ABD peptide with high affinity to endogenous albumin. After injection through the tail

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vein, the ABD-modified liposomes can bind to endogenous albumin in vivo, which in turn bind to SPARC receptors that are overexpressed by inflamed tissues.

In this study, ABD-modified liposomes containing Dex (ABD-Lip/Dex) were prepared and characterized, and the results showed that the ABD peptide was successfully attached to the liposomes and Dex was encapsulated into the liposomes successfully. In vitro release showed that the modified liposomes can released Dex sustainably. In vivo fluorescence distribution showed that the ABD peptide-modified liposomes had more longer circulation time and could target the inflamed joint tissues efficiently. In vivo therapeutic efficacy showed that ABD-Lip/Dex could reduce joint swelling and the level of inflammatory factors significantly compared with free Dex and Lip/Dex. Pathologic sections of joint tissues showed that ABD-Lip/Dex group had lower inflammatory cell infiltration and synovial hyperplasia compared with free Dex and Lip/Dex, and more intact joint cavity was observed. In addition, the safety of the liposomes in healthy rats suggesting that the ABD-modified liposomes containing dexamethasone also had acceptable safety in vivo.

Conclusion

Encapsulating Dex in nanodelivery systems has shown potential for reducing its off-target effects and prolonging its therapeutic efficient on rheumatoid arthritis. Ideally, such nanodelivery systems should deliver the drug through active targeting of sites of inflammation, and we modified Dex-loaded liposomes with the ABD peptide, which binds to albumin in vivo and then targeting to inflamed tissues through the overexpressed SPARC receptors. We showed in vitro that the modified liposomes sustainably released Dex, and we found in a rat model of arthritis that they accumulated and persisted in inflamed joints, leading to milder joint swelling and lower local levels of pro-inflammatory cytokines. This therapeutic efficacy did not involve any substantial harm to liver function or to counts of red or white blood cells, suggesting biocompatibility. Our system validates the approach of modifying Dex delivery systems with ABD peptide instead of directly with albumin as a way to target the drug to inflamed tissues, and it merits further investigation in preclinical models of rheumatoid arthritis and potentially other chronic inflammatory diseases.

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Disclosure

The authors declare that they have no conflicts of interest in this work.

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