Supporting Data

Leutusome: A biomimetic nanoplatform integrating plasma membrane components of leukocytes and tumor cells for remarkably enhanced solid tumor homing

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EXPERIMENTAL SECTION

Materials

Paclitaxel (PTX), cremophor EL® and cell proliferation reagent WST-1 were purchased from Sigma-Aldrich (St. Louis, MO). Vybrant™ Multicolor Cell-Labeling Kit (DiO, DiI, DiD solutions), near infrared lipophilic fluorescent tracer DiR, eBioscience™ Annexin V-FITC Apoptosis Detection Kit were purchased from Thermo Scientific (Rockford, IL). Hydro egg phosphatidylcholine (EPC), mini extruder and polycarbonate porous membranes with different pore sizes were obtained from Avanti Polar Lipids (Alabaster, AL). Dulbecco's modified Eagle medium (DMEM), trypsin-EDTA (0.25%), and penicillin-streptomycin (10,000 U/ml) were purchased from Life Technologies (CA, USA). Antibodies against lamin B (sc-6216, Santa Cruz), PDI (sc-136230, Santa Cruz) and cytochrome c (sc-13156, Santa Cruz), Na+/K+ ATPase α-1 (clone C464.6, Millipore), E-Cadherin (Clone 36/E-Cadherin, BD Biosciences), CD45 (ab10558, Abcam), CD47 (ab108415, Abcam), SR-A (sc-20444, Santa Cruz), folate receptor (PA5-24186, Thermo Scientific) and mannose receptor (ab8918, Abcam) were used in western blot analysis. Unless noted otherwise, all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture

Murine J774A.1 cells (ATCC, Rockville, MD) were used as the source of leukocyte membrane, and HN12 cells were used as the source of tumor cell membrane. Unless noted otherwise, both cell lines were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), streptomycin (100 U/mL) and penicillin (100 U/mL) at 37 °C in 95% air/5% CO_2.

Cell membrane extraction and characterization

Cell membrane extraction and purification. Cell membrane extraction followed the reported protocol with minor modifications.[1-3] Briefly, 2.5×10^9 cells (J774A.1 or HN12) were suspended in 10 mL of pH 7.4 homogenization buffer (225 mM mannitol, 75 mM sucrose, 30 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM KCl, 2 mM phenylmethylsulfonyl fluoride, EDTA-free protease inhibitor, DNase 10 μg/mL and RNase 10 μg/mL). The cells were gently homogenized on ice using a Potter-Elvehjem homogenizer (1500 rpm and 20-30 passes) and centrifuged at 500 g for 10 min. The supernatant containing the crude cell membrane fraction was collected. The pellet containing the unbroken cells was subjected to extra rounds of homogenization and centrifugation as needed until the most cells were free of intact membrane structure observed under the light microscope. All the supernatants were pooled and centrifuged at 10,000 g for 30 min. The pellet containing the mitochondrial contamination was discarded, and the supernatant was further centrifuged at 25,000 g for 20 min to collect the pellet. The pellet containing crude cell membrane was suspended in resuspension buffer (5mM Bis-Tris and 0.2 mM EDTA, pH 6.0) and layered on top of the 11-mL discontinuous sucrose gradient, composed of 55 (w/v), 43 (w/v) and 38% (w/v) sucrose in resuspension buffer. The discontinuous gradients were centrifuged in a Sorvall™ TH-641 swinging bucket rotor at 95,000 g for 2 h. The plasma membrane-rich region (interface between 43/55%) was collected, diluted 10-fold with resuspension buffer, centrifuged at 10,000 g for 10 min to eliminate any carryover mitochondrial contamination (present as a pellet that can be discarded), and finally the supernatant was centrifuged to the pellet of pure cell membrane at 95,000 g for 1 h. All centrifugations were performed at 4 °C. The collected cell membrane was re-suspended in saline solution and stored at -80 °C for future use.

Western blot analysis. Extracted cell membranes were subjected to Western blot analysis for characterization of representative cell membrane proteins including E-cadherin,

CD47, CD45, folate receptor, Na+/K+ ATPase α-1, SR-A, and mannose receptor. Intracellular proteins lamin B, PDI and cytochrome c were also characterized to confirm the complete removal of intracellular components. Proteins were transferred onto the Immobilon-P PVDF membrane (Millipore), probed with a series of antibodies and corresponding secondary antibodies labeled with IRDye™ (LI-COR Biosciences, Lincoln, NE) and detected using the Odyssey® CLx imaging system. The whole cell lysates were subjected to western blot for comparison.

Cell membrane proteins and phospholipids quantification. Extracted cell membranes were further analyzed and quantified by the membrane-associated proteins and membrane-associated phospholipids, respectively. Leukocyte membrane proteins (LMP) and tumor cell membrane proteins (TMP) were quantified using a Pierce™ BCA Protein Assay Kit, and liposomal formulations were prepared based on membrane protein concentrations. In addition, membrane-associated phospholipids were also assayed using a Phospholipid C kit (Wako Pure Chemical Industries, Ltd.). Generally, one batch of purified leukocyte membrane (from 2.5×10^9 J774A.1 cells) contained approximately total 1.2 mg proteins with 0.6 mg phospholipids, and one batch of the purified tumor cell membrane (from 2.5×10^9 HN12 cells) contained approximately total 1.8 mg proteins with 0.85 mg phospholipids. The extracted cell membranes were stored at -80 °C and used within one month to ensure stability.

Verification of the presence of both cell membranes in leutusome. To check whether the cell membranes extracted from both cell types could be incorporated during the extrusion-derived vesicle formation, two highly lipophilic fluorescent dyes DiO (green) and DiD (red) were used to label leukocytes and tumor cells, respectively, following the manufacturer's protocol. The labeled cell membranes were extracted following the method described above,

mixed, sonicated briefly in a Branson ultrasonic bath, and subjected to a series of polycarbonate membrane-based extrusion. The incorporation of cell membrane from the two cell types was monitored by confocal microscopy (Zeiss LSM 710) and transmission electron microscopy (TEM) (JEOL JEM-3010). To further ascertain the incorporation of the two cell membranes, HN12 cells (80% confluence) were incubated with the resulting vesicles (50 μg of total cell membrane proteins/mL) for 6 h, rinsed and then imaged by confocal microscopy for intracellular colocalization. Cell membrane-derived vesicles (before and after extrusion) were analyzed by western blotting as well.

Preparation and characterization of liposomal nanoparticles

Preparation. Liposomal nanoparticles were prepared by using the thin-film hydration method.[4, 5] EPC was used as the only type of external phospholipid to form drug-loaded liposomes. Every batch was prepared on the basis of 100 mg EPC. For instance, to prepare one batch of PTX-loaded leutusome (LTM-PTXL), PTX and EPC at the drug-to-lipid mass ratio of 1:20 (w/w) (or drug-to-lipid molar ratio of 4.3%) were dissolved in 10 mL of methanol/chloroform (1/1, v/v) and rotary evaporated to form a thin film. The film was further dried in an oven at 40 °C overnight. The dry film was hydrated with 5 mL of saline solution or saline solution containing cell membranes at 37 °C for 1 h under nitrogen flushing, and then sonicated (60 kHz and 150 W) on ice for 3 min. The quantities of L membrane and T membrane were adjusted such that both carried an equal amount of membrane proteins. The mass ratio of LMP: TMP: PTX: EPC was 1:1:30:600 (w/w/w). The resulting leutusome was extruded sequentially through polycarbonate membranes with pore sizes from large to small (800, 400 and 200 nm).

Following the same method, empty leutusome (LTM-L; LMP: TMP: EPC at 1:1:600, w/w/w), leukocyte membrane-camouflaged PTX-loaded liposomal nanoparticles (LM-PTXL; LMP:PTX:EPC at 1:30:600, w/w/w) and leukocyte membrane-camouflaged empty liposomal nanoparticles (LM-L; LMP:EPC at 1:600, w/w), tumor cell membrane-camouflaged PTX-loaded liposomal nanoparticles (TM-PTXL; TMP:PTX:EPC at 1:30:600, w/w/w) and tumor cell membrane-camouflaged empty liposomal nanoparticles (TM-L; TMP: EPC at 1:600, w/w), and blank plain liposomes (L; EPC only), PTX-loaded plain liposomal nanoparticles (PTXL; PTX:EPC at 30:600, w/w) were prepared, respectively. For imaging purpose, fluorescent liposomal nanoparticles containing lipophilic fluorescent dye (DiI or DiR) were inserted into bilayers to replace the PTX under the same condition. The mass ratio of the dye to EPC was 1:20 (w/w). Taxol (6 mg PTX, 527 mg of purified Cremophor EL and 49.7% (v/v) dehydrated ethanol per mL) was prepared and included as a standard formulation for comparison studies. Cell membrane proteins of the LTM-L and LTM-PTXL were analyzed using western blot following the method described above.

Particle size and zeta potential measurements. Liposomal nanoparticles were diluted with PBS (1:20) and filtered through a 0.22 μm PVDF syringe filter to remove undispersed aggregates. The diluted liposomal nanoparticles solution was transferred to 1 mL disposable plastic cuvette. Size and size distributions of liposomal nanoparticles were analyzed by dynamic light scattering (DLS) analysis using the Malvern Zetasizer Nano ZS90 series instrument (Malvern, Worcestershire, UK). Water (Milli-Q) was used to dilute liposomes in zeta potential measurements. Following equilibration for 2 min, measurements were performed at 25 °C using a 633 nm laser at a scattering angle of 90°. The solution refractive indices were 1.33 and 1.332 for water PBS, respectively. Several measurements were taken in a row on each sample, with up

to 50 individual scans done in each measurement. The number of scans was determined by the DLS software based on the quality of the sample. The formulations after 1 week-storage at 4 °C were tested again for colloidal stability assessment.

The morphologies of liposomal nanoparticles were imaged under transmission electron microscope (TEM). Briefly, liposomal nanoparticles were first diluted to 0.4 mg/mL (phospholipids concentration) with Milli-Q water, deposited onto a glow discharged carbon-coated 300 square mesh copper grid and imaged under a JEOL 1400Plus TEM operating at 120 kV. Real time images were recorded at a frame rate of 12 frames per second with a 500 ms exposure time.

PTX encapsulation efficiency and loading content assessment. PTX encapsulation enfficiency (EE%) for the formulations prepared at various drug-to-lipid mass ratio were estimated. Briefly, the PTX-containing liposomal nanoparticles were subjected to ultrafiltration (100 kDa MWCO, 20000 g for 10 min) to remove loosely bound PTX and then analyzed with high-performance liquid chromatography (HPLC) for drug loading content quantification (reported as drug-to-lipid molar ratio (mole %)). EE% was determined as encapsulated PTX amounts in the final formulations relative to the intitial drug amounts. PTX loading content was determined as encapsulated PTX amounts in the final formulations relative to the total lipid amounts. The HPLC system (SHIMADZU LC-20AD, Columbia, MD, USA) included a Force C18 LC column maintained at 40 °C and a UV detector. The mobile phase was composed of deionized water and HPLC grade methanol (25:75, v/v). The flow rate was set at 1.0 mL/min. PTX in the eluent was detected at the wavelength of 227 nm.

***In vitro* studies**

Drug release studies. *In vitro* PTX release from liposomal nanoparticles was studied by using the dialysis method. Briefly, 0.2 mL of freshly prepared PTX liposomal nanoparticles was loaded into a dialysis bag with molecular weight cutoff of 7,000 Da. The dialysis bag was immersed in 5.0 mL PBS (supplemented with 0.1% Tween 20 that acted as surfactant to maintain the sink condition in the release medium) agitated gently (150 rpm) at 37 °C . At predetermined time intervals, 0.5 mL of the release medium was taken out for HPLC analysis. Immediately after each sampling, an equal volume of fresh medium (preequilibrated at 37 °C) was added to the release medium to keep the overall volume unchanged.

Cell-specific uptake studies using a co-culture model. To examine whether the compositions of the liposomal formulations affect their uptake, we established a co-culture model by co-culturing J774A.1 with YFP-expressing HN12 cells as described before.[6] The co-cultured cells at 80% confluence were incubated with DiI-labeled liposomal nanoparticles (DiIL, LM-DiIL, TM-DiIL or LTM-DiIL) carrying equal fluorescence intensity for 6 h, rinsed with PBS 3 times, and then subjected to confocal microscopy and LSRFortessa X20 SORP flow cytometer (BD Biosciences) for cellular uptake comparison, respectively.

***In vitro* antitumor effect.** Cells after 24 h incubation with the different empty liposomal nanoparticles (without PTX) were assessed to show the biocompatibility of those nanocarriers. The antitumor effect of the PTX-loaded liposomal nanoparticles on HN12 cells was then studied. Briefly, 5×10^4 HN12 cells were seeded in 48-well plates and cultured overnight. The cells were incubated with different doses of liposomal nanoparticles for 24 h, washed with PBS three times, and then subjected to the WST-1 assay. The inhibitory concentration (IC50) of each formulation was calculated on the basis of PTX doses.

***In vivo* studies**

Animal model. Six-week-old athymic nude mice (both male and female) were obtained from Envigo (Indianapolis, IN). A xenograft tumor model of head and neck squamous cell carcinomas (HNSCC) was established by subcutaneously injected YFP-expressing HN12 cells or HN12 cells (2×10^6 cells) to the flank of athymic nude mice.[7] Biodistribution studies were conducted on mice when the tumors had grown to a volume of ~60 mm^3 (length × $width^2/2$) on average 12 days post-inoculation. The procedures conducted on mice were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Tolerance test of blank liposomal nanoparticles. The tolerance of empty liposomal nanoparticles was tested. Briefly, a high dose of empty liposomal nanoparticles (L, LM-L, TM-L or LTM-L) (320 mg phospholipids/kg) was administered to the six-week-old athymic nude mice (n=4) via tail vein injection. YFP-expressing HN12 tumor-bearing mice (n=4) via tail vein injection. Mice receiving plain saline were used as a control. The mice were sacrificed at 48 h post-injection. Blood and major organs were collected and analyzed.

Clearance by phagocytes in the blood. The mice were intravenously given DiI-labeled lipid nanocarriers (DiIL, LM-DiIL, TM-DiIL, and LTM-DiIL)(n=4). All the formulations were adjusted to carry an equal fluorescent intensity (2.5 mg DiI/kg). A small amount of peripheral blood was collected at 2, 6 and 24 h post-administration. Accumulations of DiI-labeled nanocarriers within $CD11b^+Ly6G^-Ly6C^{high}$ monocytes and $CD11b^+Ly6G^+$ neutrophils in the peripheral blood were analyzed by flow cytometry.

Biodistribution studies. DiR-labeled empty liposomes (DiRL, LM-DiRL, TM-DiRL or LTM-DiRL) were tested in mice bearing an YFP-expressing HN12 tumor. All the nanoparticles

were adjusted to carry an equal fluorescent intensity and administered to the mice (n=4) via tail vein injection (5 mg DiR/kg). At pre-determined time points, the mice were anesthetized and imaged non-invasively using a Pearl Trilogy small animal imaging system (LI-COR Biotechnology, Lincoln, NE, USA).[7] The acquisition was set "auto" for each image, with f/stop fixed at F1, and medium binning. The fluorescence image was collected in the 800 nm channel and analyzed by Image Studio Ver. 5.0 software. A small amount of blood (8 μL) was collected at 2 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h and 48 h post-administration for fluorescence signal quantification. Briefly, the blood (8 μL) was diluted in 200 μL of PBS containing heparin (16 U/mL) as anti-coagulant. Blood cells were removed by spinning at 300 g for 5 min, and 180 μL of the supernatant was added into ProxiPlate-96 black microplate (Perkin Elmer, MA, USA) and quantified by for fluorescence intensity measurement with Infinite® M1000 PRO (TECAN). All the mice were euthanized at 48 h post-administration. Tumor and major organs (brain, heart, liver, spleen, lung, and kidney) were harvested and subjected to *ex vivo* imaging using Pearl Trilogy. One tumor sample of each group was cryosectioned for confocal imaging. The collected tumors and organs were homogenized for fluorescence intensity quantification with Infinite® M1000 PRO. A second tumor model, i.e., B16 xenograft model was established using C57BL/6 mice.[8] Following the same fabrication method, LTM-DiRL was prepared from membrane fractions of B16 melanoma cells and leukocytes (J774A.1). Its tissue distribution at 48-h post-i.v. administration was also investigated following the method mentioned above.

Antitumor efficacy study. A xenograft tumor model was established by subcutaneously injected tumor cells (2×10^6 HN12 cells) to the flank of mice. HN12 tumor-bearing mice with similar tumor size (~35 mm^3) were sorted out and randomly divided into six groups (n=6). The mice were intravenously injected with various PTX-loaded formulations, including Taxol, PTXL,

LM-PTXL, TM-PTXL or LTM-PTXL (10 mg of PTX/kg) every 4 days for 6 doses. Saline was also included as nontherapeutic control. During the treatment, tumor volume and body weight were recorded every four days. The mice were euthanized 30 days after tumor innoculation. The tumors were collected, weighed, and then processed for TUNEL apoptotic assay. Serum levels of hepatic function indicators (ALT and AST), renal function indicators (BUN and CREA) and inflammatory cytokines (IL-1β and TNF-α) were assayed.[9, 10]

Statistical analysis

The data are expressed as mean ± SD. The data were analyzed by one-way analysis of variance (ANOVA) followed by Student's t test for pairwise comparison of subgroups. P values <0.05 were considered statistically significant.

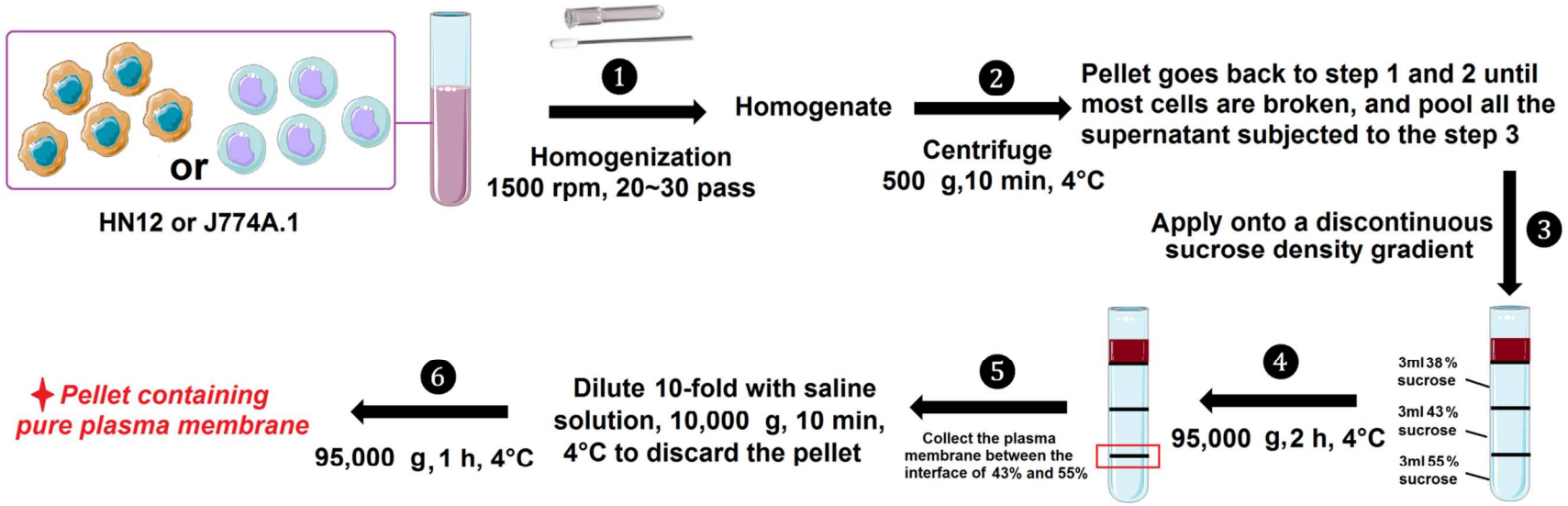

Figure S1. Schematic illustration of the procedure used for cell membrane extraction.

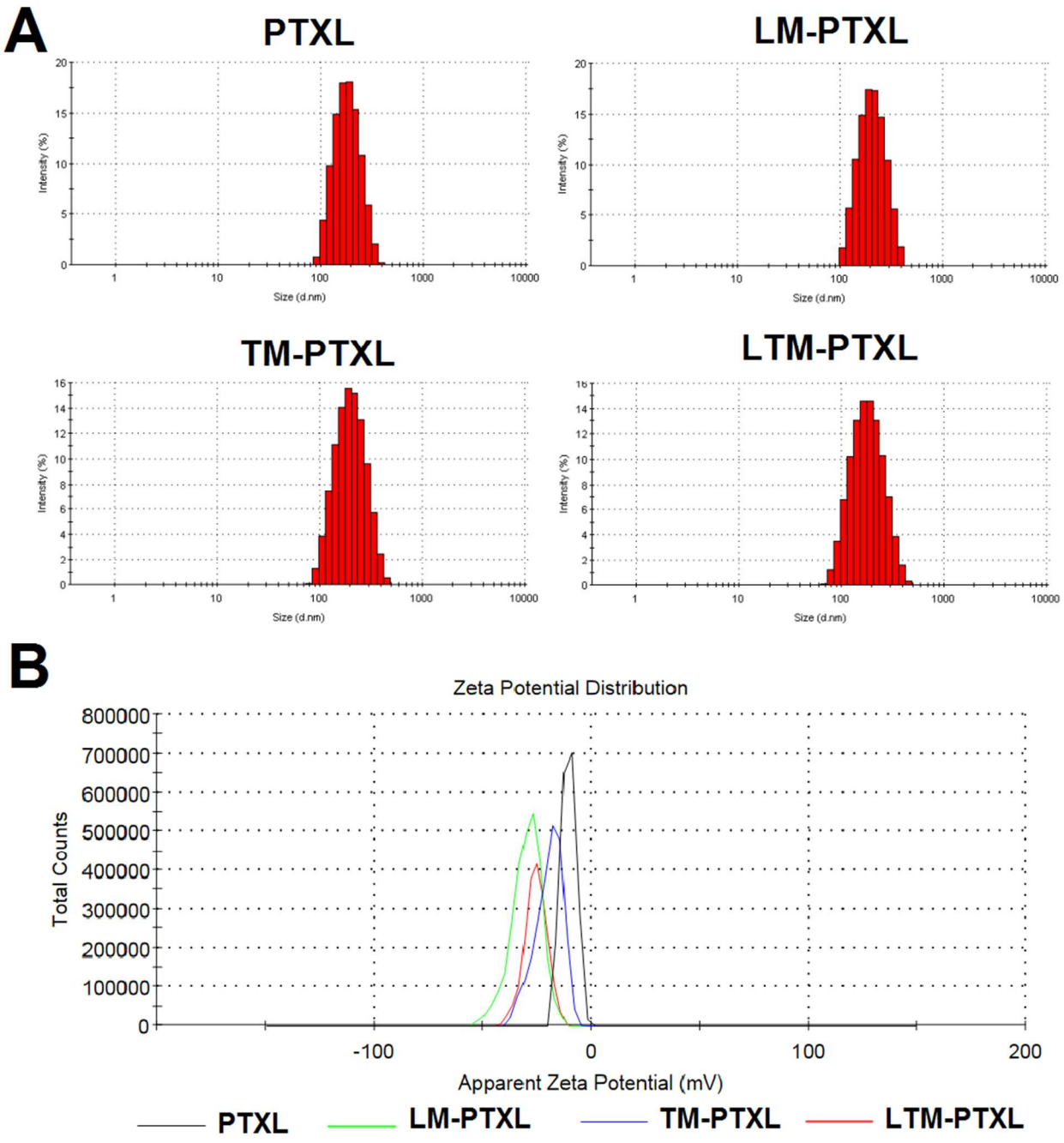

Figure S2. The size distribution and zeta potential of PTX-loaded liposomal nanoparticles.

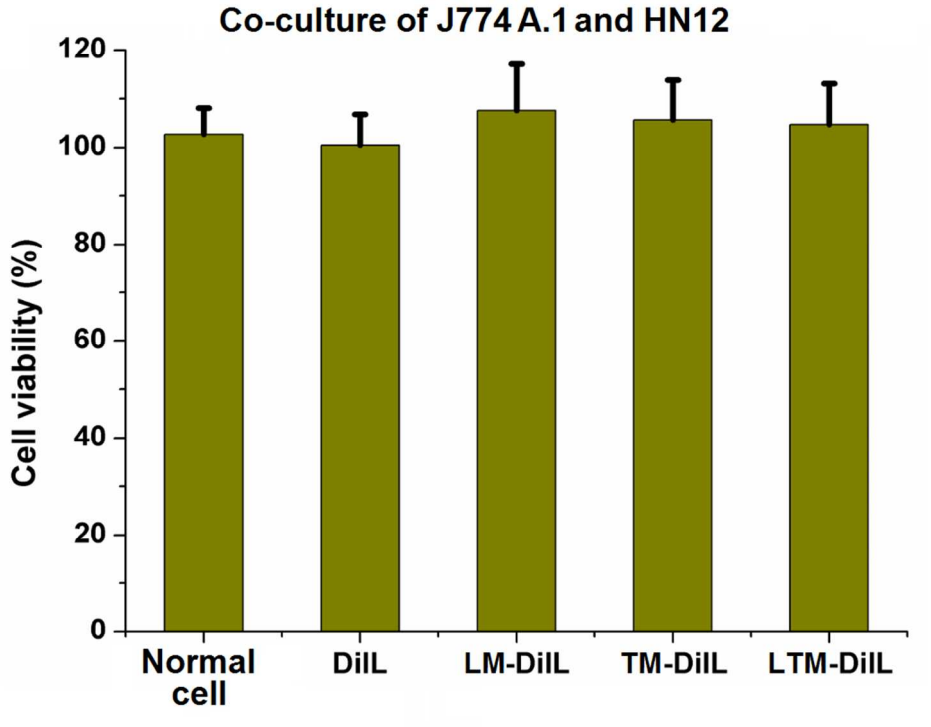

Figure S3. The cells remained 100% viable in the co-culture model.

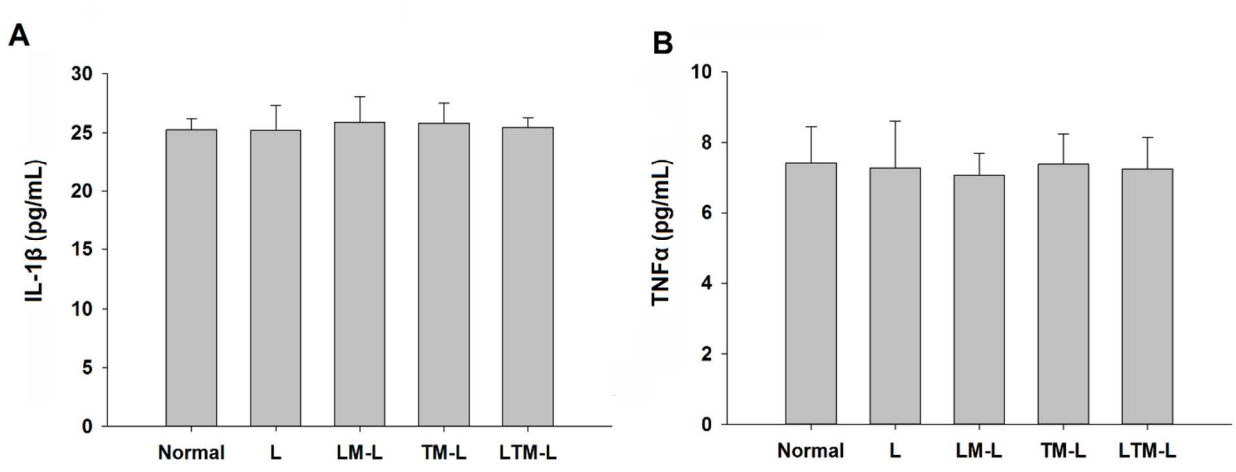

Figure S4. Serum levels of cytokines IL-1β (A) and TNFα (B) in the mice administered with different empty liposomal nanoparticles.

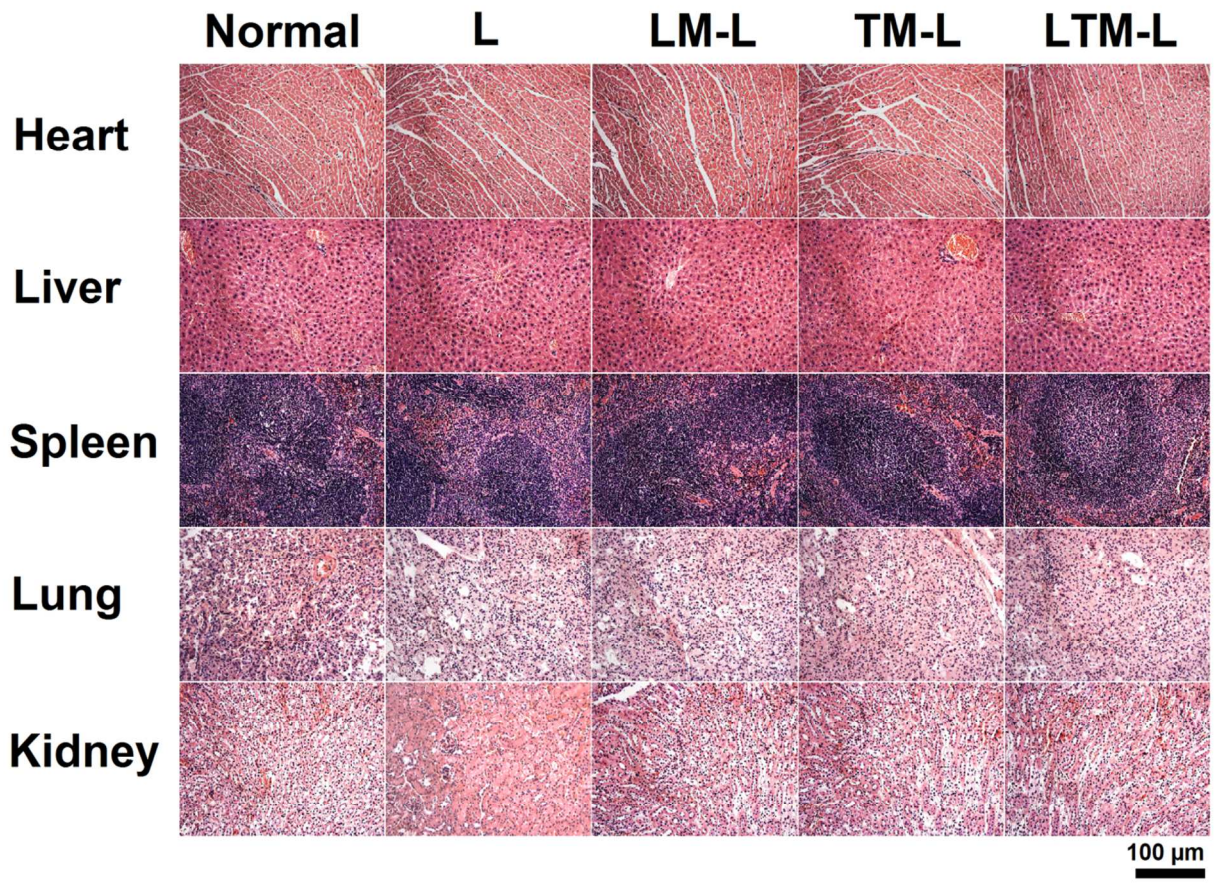

Figure S5. Histological examination of major organs of the mice administered with empty liposomal nanoparticles.

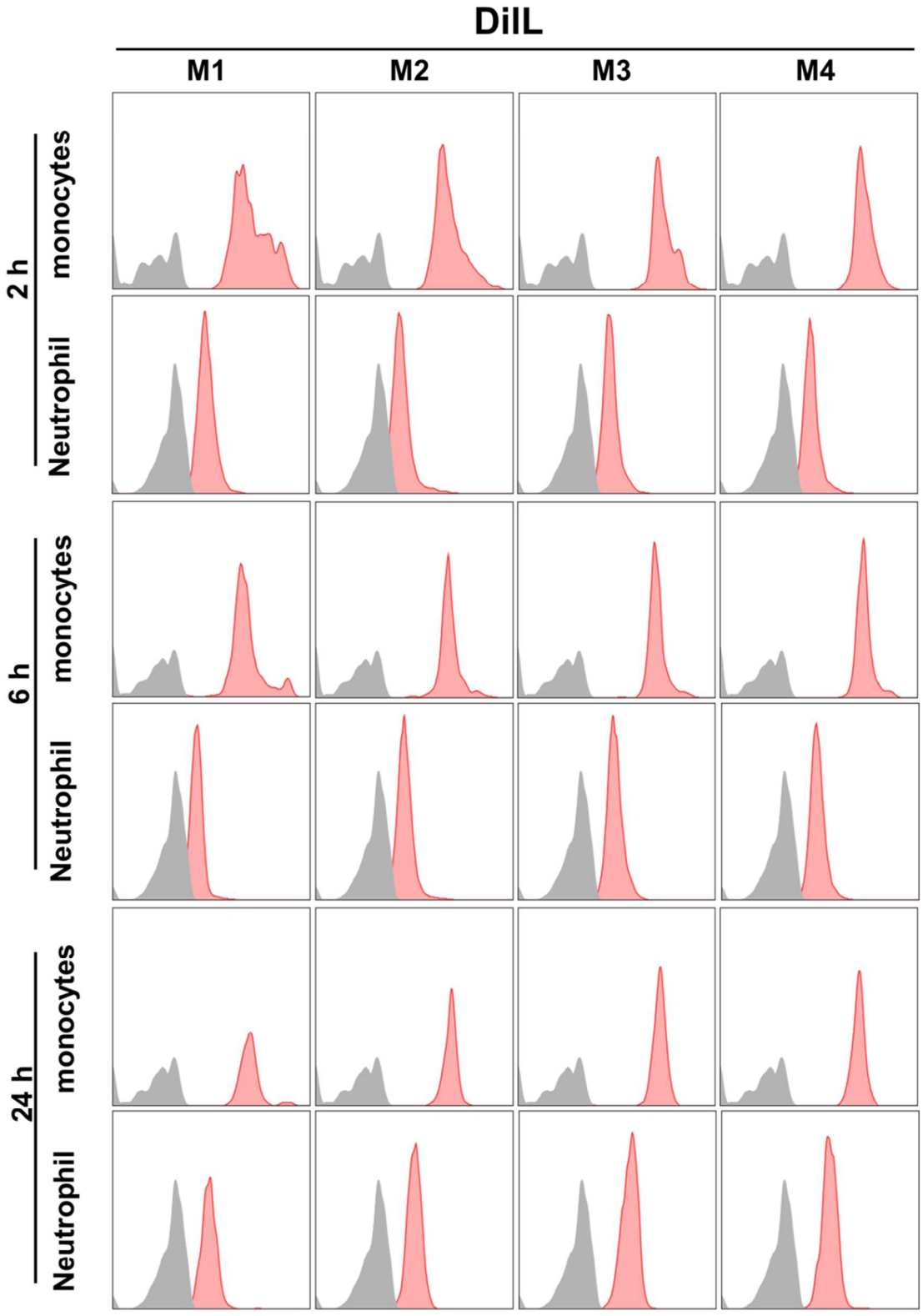

Figure S6. Flow data of the uptake of DiIL by monocytes and neutrophils at 2, 6, and 24 h post-administration. M# means mouse number.

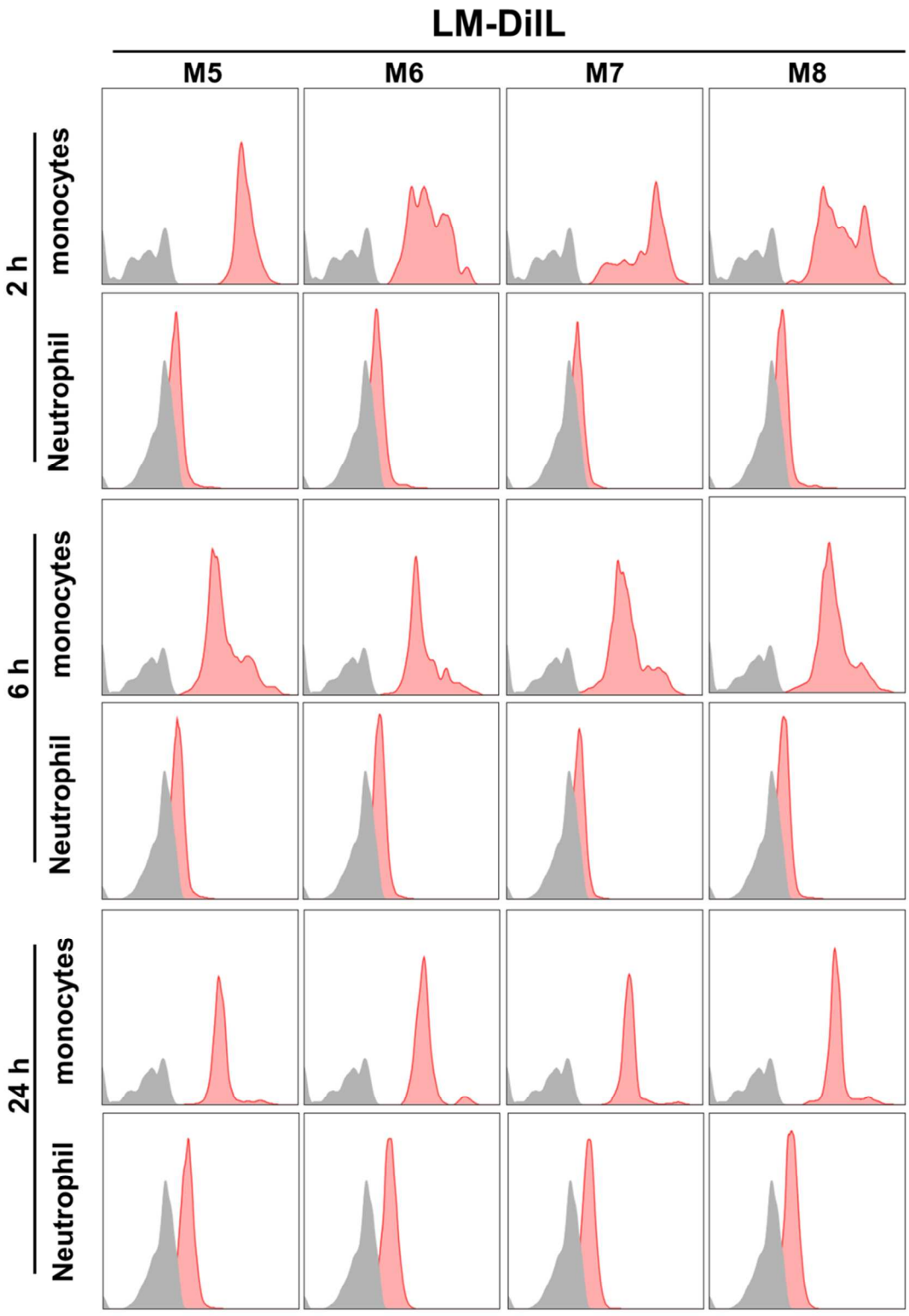

Figure S7. Flow data of the uptake of LM-DiIL by monocytes and neutrophils at 2, 6, and 24 h post-administration. M# means mouse number.

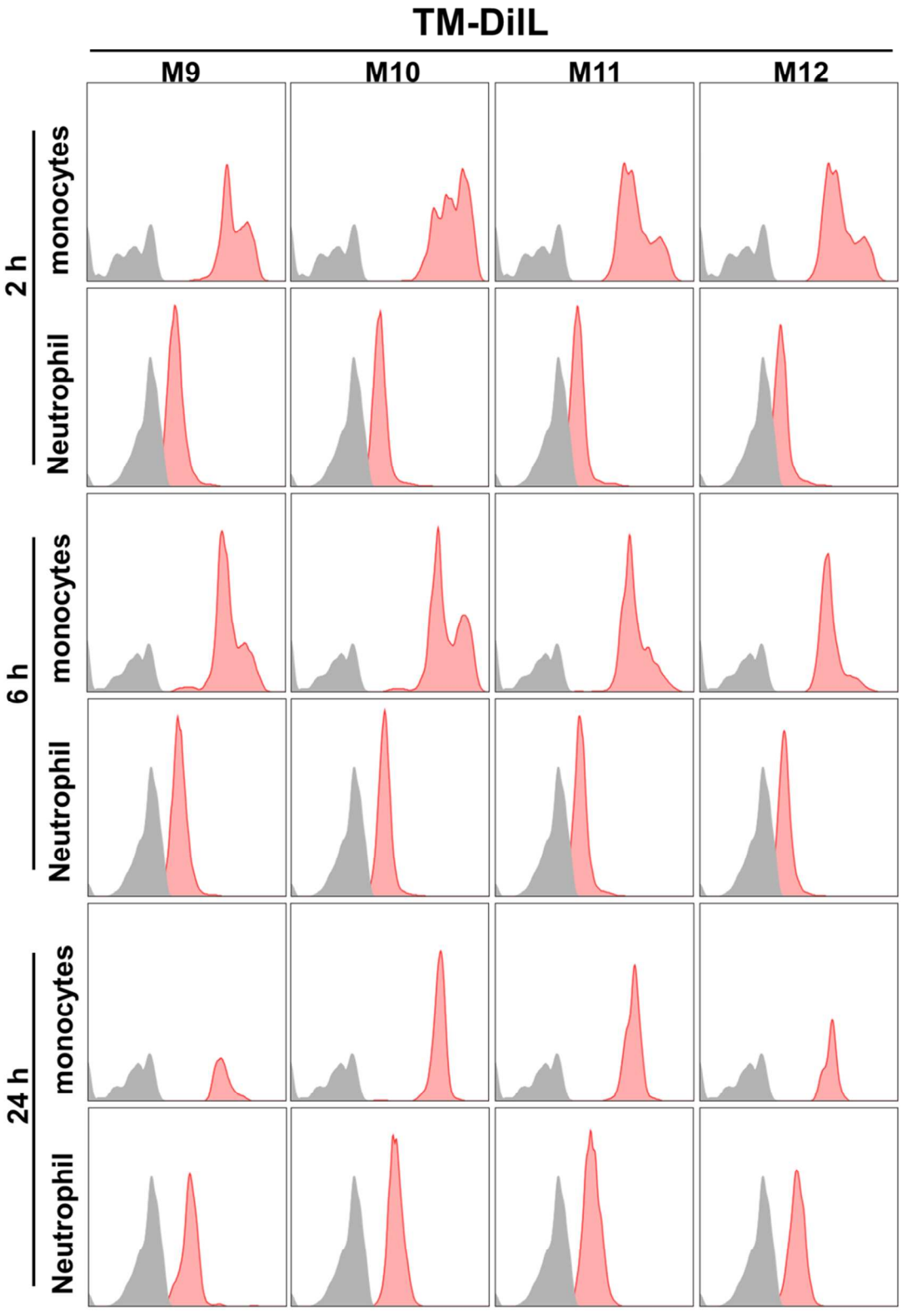

Figure S8. Flow data of the uptake of TM-DiIL by monocytes and neutrophils at 2, 6, and 24 h post-administration. M# means mouse number.

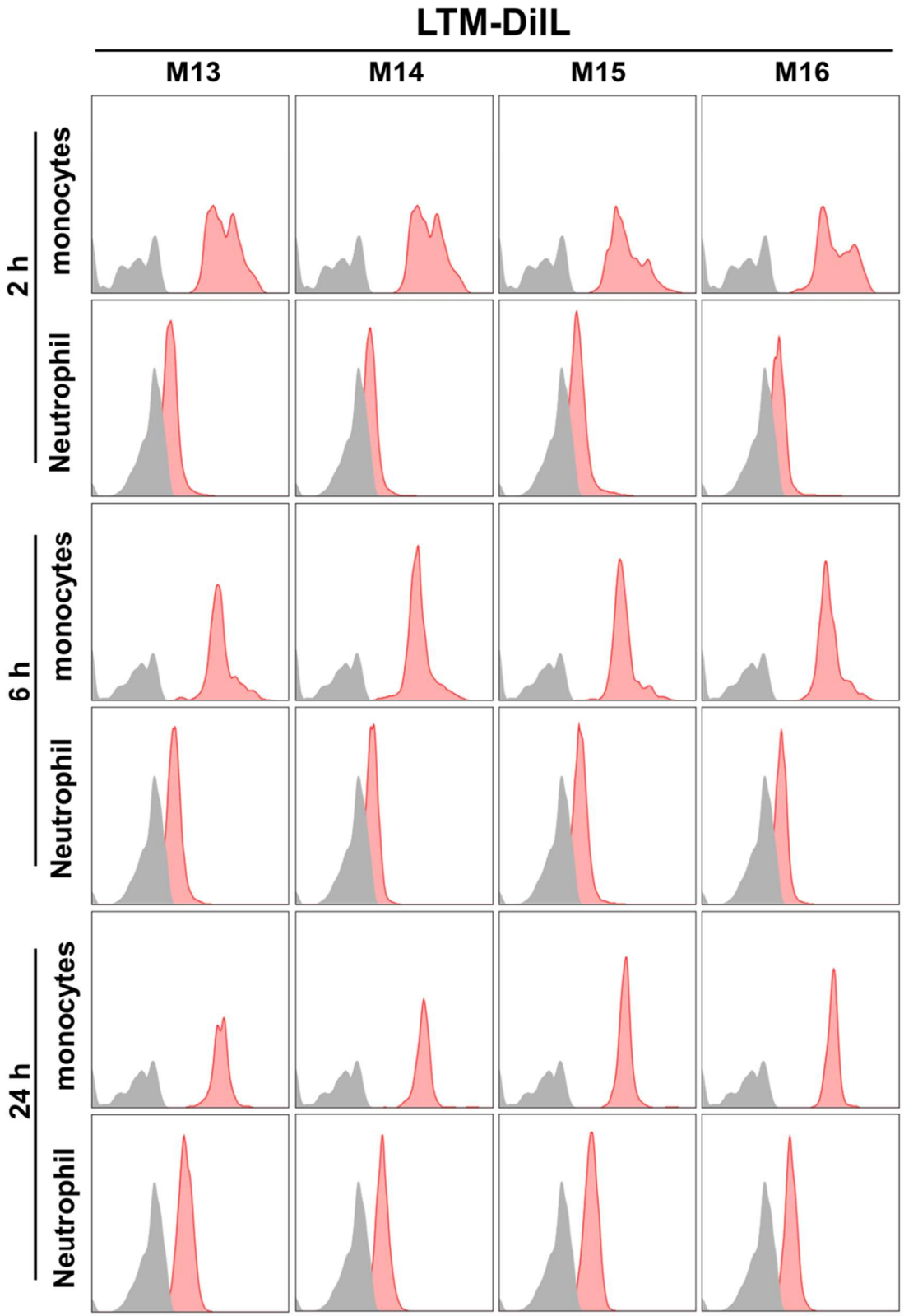

Figure S9. Flow data of the uptake of LTM-DiIL by monocytes and neutrophils at 2, 6, and 24 h post-administration. M# means mouse number.

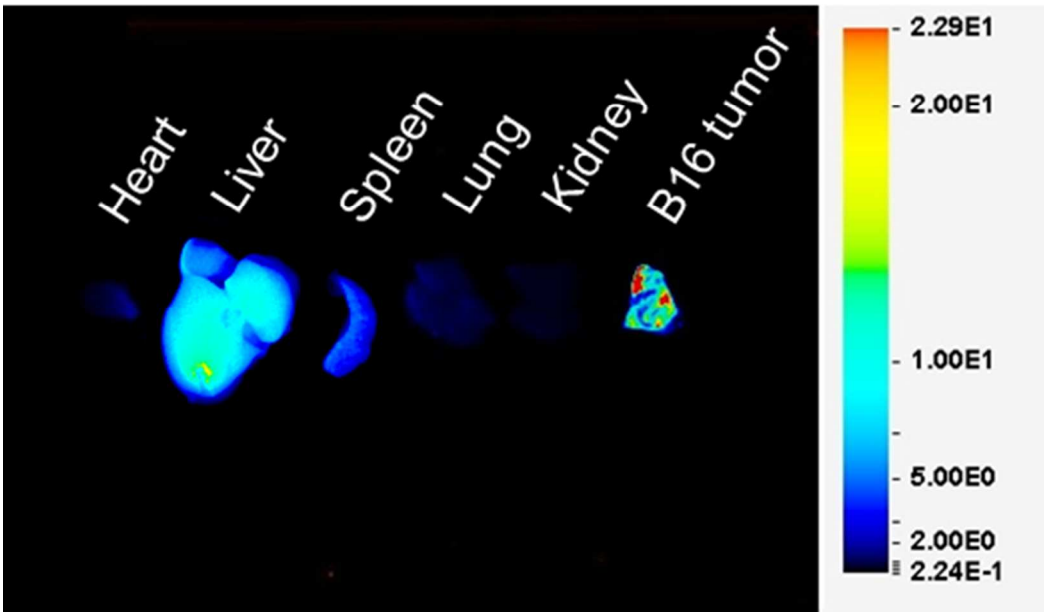

Figure S10. *Ex vivo* tissue distribution of LTM-DiRL composed of membrane fractions of B16 melanoma cells and leukocytes (J774A.1) at 48 h post-i.v. administration in a B16 xenograft model (C57BL/6 mice established s.c. with B16 melanoma, n=4). %ID/g tissue: 5.5±0.9 in heart, 29.2±4.3 in liver, 19.3±3.1 in spleen, 6.6±2.0 in lung, 2.4±0.5 in kidney, and 69.3±2.2 in B16 tumor.

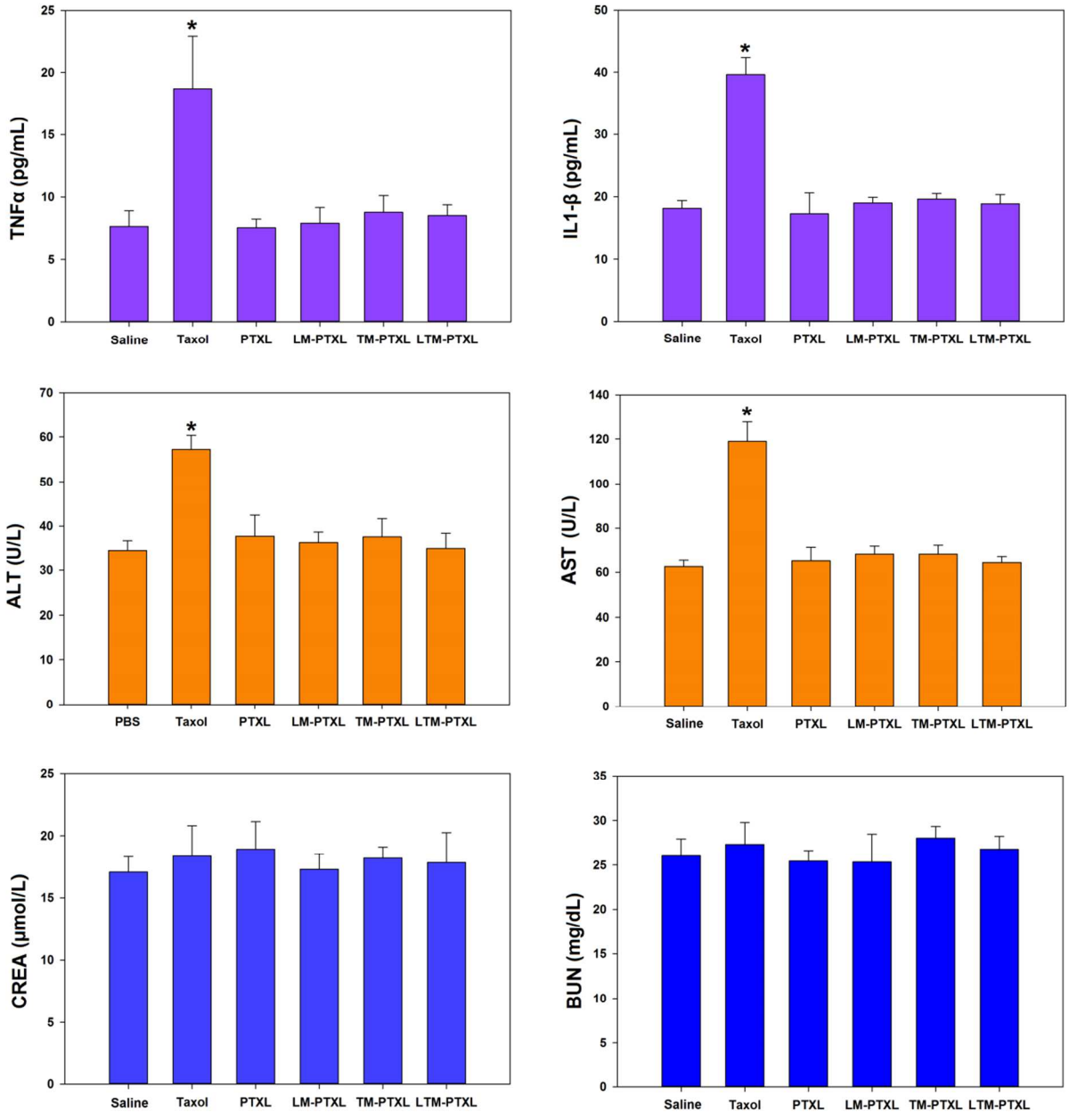

Figure S11. Inflammatory examination, hepatic function and renal function after various treatments. * $p<0.05$ vs. the saline control.

Table S1. Both %ID/tissue and %ID/gram tissue for different liposomes at 48 h post-i.v. administration

	DiRL		LM-DiRL		TM-DiRL		LTM-DiRL	
	%ID /tissue	%ID /g tissue	%ID /tissue	%ID /g tissue	%ID /tissue	%ID /g tissue	%ID /tissue	%ID /g tissue
Heart	1.0±0.2	5.9±1.3	1.1±0.2	6.9±1.2	1.2±0.2	7.8±1.6	1.0±0.1	6.8±1.5
Liver	56.8±2.6	52.6±1.24	23.6±1.8	21.1±2.1	41.0±2.8	34.6±1.7	33.0±1.6$^{*\gamma}$	23.9±3.9$^{*\gamma}$
Spleen	8.9±0.7	46.2±7.6	3.0±0.6	19.3±2.5	4.9±0.6	28.4±1.9	3.5±0.4*	24.5±2.7*
Lung	3.1±0.6	18.6±3.3	2.0±0.6	11.1±2.9	2.6±0.3	14.2±0.4	2.2±0.2	13.3±1.4
Kidney	3.1±0.6	6.8±4.7	1.0±0.3	1.9±0.2	1.4±0.2	2.6±1.6	1.0±0.4	2.1±1.6
Brain	0.8±0.2	3.0±0.6	1.0±0.4	2.6±1.6	1.1±0.5	3.1±0.3	0.9±0.7	2.7±1.3
Tumor	0.6±0.1	8.5±1.2	1.6±0.4	23.0±4.3	2.6±0.4	37.3±6.4	5.2±0.2$^{*\#\&}$	71.9±6.6$^{*\#\&}$

Note: Mean (n=4), $^{*}p<0.01$ for comparison between LTM-DiRL and DiRL; $^{\#}p<0.01$ for comparison between LTM-DiRL and LM-DiRL; $^{\gamma}$ $p<0.05$ and $^{\&}p<0.01$ for comparison between LTM-DiRL and TM-DiRL.

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