# Supporting Information

## Neutrophils Enable Local and Non-Invasive Liposome Delivery to Inflamed Skeletal Muscle and Ischemic Heart

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## Supplementary Experimental Section

*Isolation and characterisation of murine neutrophils from mouse bone marrow*

Mouse neutrophils were isolated from bone marrow by negative selection using a neutrophil isolation kit (Miltenyi Bio). Briefly, bones from mouse legs were immersed in RPMI 1640 medium after muscle removal. Mouse bone marrow cells were flushed out with buffer solution (0.5% (w/v) bovine albumin and 2 mM EDTA), filtered through the cell strainer, centrifuged at 400 g for 10 min and resuspended in the buffer solution. A cocktail of biotin-conjugated monoclonal antibodies was added to bind to non-target cells, followed by multiple washing steps. The secondary labelling reagent, anti-biotin monoclonal antibodies conjugated to Microbeads, was added and magnetically labelled non-target cells were depleted by retaining

them within a MACS Column in the magnetic field of a MACS Separator, allowing unlabelled neutrophils to run through the column. The yield was determined by using a haemocytometer and the cell viability was calculated by trypan blue exclusion. The purity of isolated neutrophils was measured using immunofluorescence antibodies staining with PE anti-mouse CD11b (250 ng $mL^{-1}$, BioLegend) and APC anti-mouse Ly6G/Ly6C (250 ng $mL^{-1}$, BioLegend). Then, the morphology of neutrophils was visualised under the widefield microscope (Zeiss Axio Observer) using Giemsa-Wright stain.

*Preparation and characterisation of MTX-liposome/neutrophils*

MTX loaded liposomes composed of 16:0-18:1 PC (POPC), 18:0 TAP (Avanti Polar Lipid) and cholesterol (Sigma) (44:16:40, w:w:w) were prepared using a thin-film hydration method.[1] MTX (Sigma) solutions at 5 mg $mL^{-1}$ and 10 mg $mL^{-1}$ in PBS were added to hydrate the film, followed by extrusion through membrane filters with a pore size of 100 nm. Free MTX was removed using two sequential size exclusion chromatography (PD-10 desalting columns, GE Healthcare) and purified MTX-loaded liposomes were used for all the subsequent studies. Dynamic light scattering (DLS) was used to measure the hydrodynamic diameter and zeta potential of liposomes (Malvern, Zetasizer). The size of MTX-liposomes was further confirmed by transmission electron microscopy (TEM). MTX-liposomes were dropped onto a cooper grid (CF400-Cu, Electron Microscopy Science) and stained with 2% fresh ammonium molybdate. After air-dry, the sample was imaged by TEM (JEOL 2100F).[2] The release of MTX from liposomes was measured using a dialysis tube (Spectra-Por, 5 mL, MWCO 100 kDa) in PBS pH 7.4 at room temperature. To mimic the release of MTX from liposomes in the extracellular environment at the inflammatory site, MTX-liposomes were incubated in 90% FBS (v/v) for 8 h and then transferred to a dialysis tube (Spectra-Por, 1 mL, MWCO 300 kDa). After an additional 12 h and 44 h dialysis in PBS, the amount of retained MTX inside liposomes in the dialysis tube was quantified using Methotrexate ELISA kit (Enzo). The liposome solution

inside the tube was collected at different time points and the retained amount of MTX in liposomes was measured by LC-MS. The LC-MS system consisted of an Agilent Technologies 1260 Infinity, coupled to an Agilent 6130 Series Quadrupole spectrometer (electrospray ionization mode, ESI+). All the analysis were carried out using a Gemini NX column (5 micron pore size, 150 x 4 mm). A flow rate of 1 mL $min^{-1}$ and a gradient of (10-90)% B over 10 min were used. Eluent A: water/0.1% $NH_3.H_2O$; eluent B: acetonitrile/0.1% $NH_3.H_2O$. UV detection was performed at 272 nm in a scan mode ranging from 100 to 1000 m/z. MTX eluted at t = 2.01 min (m/z = 455$[M+H]^+$). The corresponding UV peak at λ=272 nm was integrated and used for quantification based on established standard curve.

MTX-liposome/neutrophils were prepared by incubating neutrophils with MTX-liposomes. Briefly, isolated neutrophils were placed in a DNA low bind tube, followed by incubating with MTX-liposomes (MTX concentration used to hydrate lipid film: 5 mg $mL^{-1}$ and 10 mg $mL^{-1}$; final lipid concentration: 1 mg $mL^{-1}$ and 2 mg $mL^{-1}$) for 1h. After centrifugation and washing with PBS three times, MTX-liposome/neutrophils were resuspended in PBS and ready for subsequent experiments. Neutrophils uptake efficiency of MTX-liposomes was measured by using flow cytometry (BD FACSCanto, BD Biosciences) and the location of MTX-liposomes (liposomes were labelled with DiD) was observed using confocal laser scanning microscopy (CLSM, Leica SP5/MP) and Zeiss PS1 Elyra microscope. Multi-channel 3D SIM images were reconstructed from five rotational widefield acquisitions at each imaging plane using Zen software. Neutrophil viability after 4 h and 8 h incubation with MTX-liposomes was measured using Zombie Green Fixable Viability Kit (Biolegend) by flow cytometry. In order to quantify the loading amount of MTX, MTX-liposome/neutrophils were sonicated in buffer solution and the MTX amount was quantified by using Methotrexate ELISA kit (Enzo).

*Evaluation of physiological functions of neutrophils after loading with MTX-liposomes*

Three different physiological functions of neutrophils were evaluated before and after loading with liposomes, including CD11b protein expression on the neutrophil cell surface, superoxide-anion generation, and cell migration. Blank neutrophils and MTX-liposome/neutrophils were treated with different concentrations of fMLP at 37 °C for 30 min. After centrifugation and washing with PBS three times, PE anti-mouse CD11b (250 ng $mL^{-1}$, BioLegend) was added to conjugate to protein for 30 min and then washed with PBS three times. 4% of PFA was added to fix cells. The fluorescence intensity was measured using flow cytometry (BD FACSCanto, BD Bioscience).

Blank neutrophils and MTX-liposome/neutrophils were treated with fMLP (1 μM) at 37 °C for 30 min. After washing with PBS three times, the cells were incubated with dihydroethidium (10 μM, MedChemExpress) at 37 °C for 30 min. After centrifugation and washing with PBS three times, the fluorescence intensity was measured using flow cytometry to determine the superoxide-generation ability of neutrophils after loading with MTX-liposomes.

The migration ability of MTX-liposome/neutrophils was determined using a transwell cell migration method. Cell culture inserts were put into the cell plate. Blank neutrophils and MTX-liposome/neutrophils were added into cell culture inserts (polycarbonate membrane, 3.0 μm pore size, 6.5 mm membrane diameter, 0.33 $cm^2$ surface area) (upper chambers) and meanwhile, wells of the plate (lower chambers) were filled with different concentrations of fMLP (1 nM and 100 nM). After 3 h, cell culture inserts were taken out and cells on the upper side of the membrane were removed. The cells on the bottom side of the membrane were stained with DAPI, imaged and counted under bright field. CLSM was used to observe the MTX-liposomes (liposomes were labelled with DiD) in the migrated neutrophils.

*Stimulated release of MTX-liposomes from neutrophils*

NETs formation of neutrophils after treating with PMA was imaged using CLSM. Liposomes were labelled with DiD, followed by incubation with neutrophils to form liposome loaded neutrophils. Formulated liposome loaded neutrophils were incubated in the presence of fMLP or PMA for 0 h and 8 h. Released DNA fragments were stained with PI. 4% PFA was used to fix samples and DAPI was used later to stain for cell nuclei. CLSM was used to image the samples.

The stimulated release of MTX-liposomes from neutrophils was determined using Fluorescence correlation spectroscopy (FCS). MTX-liposome/neutrophils (liposomes were labelled with DiD) were treated with or without PMA for 8 h, followed by centrifugation to collect the supernatant. FCS measurements were run to detect the amount and properties of liposomes after release from neutrophils. FCS was performed on a commercial LSM 880 (Carl Zeiss, Jena, Germany) equipped with an incubation chamber set to 37°C. A HeNe laser at 633nm was used as excitation source combined with an appropriate filter set to detect the fluctuating fluorescence signal. As objective, we used a 40x C-Apochromat water immersion objective (numeric aperture of 1.2). Glass-bottom ibidi 8-well plates (80827, ibidi, Germany) were used to place 5 µl sample droplets and measurements were conducted 200 µm above the glass plate. Alexa647 in PBS was used as a standard to calibrate the beam waist ($D$ = 3.3 x $10^{-6}$ $cm^2/s$ (Alexa647) at 25°C was corrected for the higher temperature used: $D$ = 4.42 x $10^{-6}$ $cm^2/s$ at 37°C).[3] Intensity traces of 30 x 5s were recorded per sample. In the figures, we always plotted the full intensity curve and the average autocorrelation curves across the whole measurement (both 150 s). Autocorrelation was performed on ZEN software (Carl Zeiss, Jena, Germany) and data was exported for fitting and analysis using PyCorrfit program 1.1.6.[4] using one component fits:

$$G_{1comp}(\tau) = \left(1 + \frac{T}{1-T} e^{\frac{-\tau}{\tau_{trip}}}\right) * \frac{1}{N * \left(1 + \frac{\tau}{\tau_D}\right) * \sqrt{1 + \frac{\tau}{SP^2 \tau_D}}}$$

$N$ refers to the effective number of diffusing species in the confocal volume with a height to weight ratio (structural parameter $SP$) fixed to 5. $\tau_{trip}$ is the triplet time with corresponding triplet fraction $T$, and $\tau_D$ is the diffusion time.

The x-y dimension of the confocal volume ($\omega_{xy}^2$) was calibrated using the calibration solution of Alexa647 in PBS. Diffusion coefficients ($D$) for the sample measurements were then obtained by plugging in the calculated diffusion times ($\tau_D$) from above:

$$D = \frac{\omega_{xy}^2}{4\tau_D}$$

Einstein-Stokes equation was subsequently used with the obtained diffusion coefficients ($D$) to calculate hydrodynamic radii ($R_h$).

The amount of MTX in the released particle solution after incubation of loaded neutrophils in the presence of PMA was quantified using Methotrexate ELISA kit (Enzo). Formulated MTX-liposome/neutrophils were incubated with and without PMA for 8 h. The supernatants were collected at 0 h, 4 h and 8 h time points and the amount of MTX was measured by Methotrexate ELISA kit. The percentage of released MTX was calculated by MTX amount (at different time points)/MTX amount (initial loading).

*Co-culture of MTX-liposome/neutrophils with RAW 264.7 cells*

To further demonstrate the transport of liposomes from neutrophils to target cells, RAW 264.7 cells were used as the target cell line to co-culture with liposome loaded neutrophils. In order to distinguish RAW 264.7 cells from neutrophils, CellTracker Green CMFDA Dye (ThermoFisher) was used to label the cytoplasm of RAW 264.7 cells. Neutrophils were incubated with liposomes for 1 h and free liposomes were washed away by centrifugation. Labelled RAW 264.7 cells were then added to co-culture with formulated liposome loaded

neutrophils in FBS free medium containing PMA (100 nM). After 8 h incubation, cell samples were fixed and measured by flow cytometry.

To image the release of liposomes from neutrophils in responding to PMA-induced NETs formation and the re-uptake of released liposomes by RAW 264.7 cells, liposome loaded neutrophils and RAW 264.7 cells were cultured together in the presence of PMA and observed by CLSM. RAW 264.7 cells were seeded in the ibidi 8-well plate. After overnight incubation, DiD-liposome/neutrophils ($0.3x10^6$ neutrophils) were added to RAW 264.7 cells and incubated in the medium with or without PMA (100 nM). At 4 h and 8 h incubation time point, RAW 264.7 cells were washed with PBS to remove suspended neutrophils in the medium and stained with propidium iodide (PI, 5 μg $mL^{-1}$) for 30 min. DAPI was used to stain for cell nuclei.

To investigate the biological effect of MTX-liposome/neutrophils system on target cells, cell proliferation was chosen as the outcome to measure after incubating with MTX-liposome/neutrophils containing different inflammatory cytokines. RAW 264.6 cells were chosen as the targeted cells and were seeded in the 96-well plate ($2x10^4$ cells/well). After 12 h incubation, MTX-liposome/neutrophils were added into each well with growth medium containing LPS and PMA. In this case, LPS (100 ng $mL^{-1}$) was used to mimic the inflammatory environment and PMA was used to induce neutrophil forming NETs to release encapsulated MTX-liposomes. After 24 h incubation, neutrophils were washed away with PBS and the viability of RAW 264.7 cells was measured using Cell Counting Kit-8 (Sigma Aldrich).

*Animals*

All animals were handled in accordance with the UK Home Office Animals Scientific Procedures Act 1986 and with an internal ethics board and UK government approved project (P63FE629C) and personal license (IC37CBB8F) for LPS-injury skeletal muscle model, project (PD359F318) and personal license (I6D2D5295) for myocardial IRI model. Food and water

were supplied *ad libitum*. Female BALB/c mice (Charles River, UK) 6-8 weeks (treatment mice) or 10-12 weeks (donor mice) of age were placed into groups (n = 5) and housed in a fully acclimatized room, used for LPS-injury skeletal muscle model. Female CD1 mice (Charles River, UK) 4-5 weeks (treatment mice) or 8-10 weeks (donor mice) of age were placed into groups (n = 5) and housed in a fully acclimatized room, used for myocardial IRI model.

*LPS-injury skeletal muscle model and in vivo neutrophil migration and anti-inflammation treatment studies*

LPS-biotin/streptavidin particles were prepared by complexing biotinylated LPS elementary bodies (LPS Biotin-EB, Invivogen, France) with streptavidin from *Streptomyces avidinii* (Sigma, UK) at a ratio of 1:4 biotin:streptavidin (w/w) in PBS. Mice were injected intramuscularly in the right quadriceps with 50 μg of particles (10 μg LPS Biotin EB, 40 μg streptavidin) in 50 μL, while the left quadriceps was not injected to serve as an internal control.

To investigate the migration ability of injected neutrophils to the inflamed quadriceps, 24 h after LPS injection, isolated neutrophils from donor mice (labelled with VivoTrack 680) were intravenously injected to LPS treated mice ($4 \times 10^6$ per mouse). 1 h, 2 h and 4 h post i.v. injection, mice were sacrificed and quadriceps from both legs were taken out. Quadriceps were digested using 2 mL of Dulbecco's Modified Eagle's Medium supplemented with 1 mg $mL^{-1}$ collagenase P (Sigma, UK) and 5 mg $mL^{-1}$ dispase II (Sigma, UK) for 30 min and smashed through the cell strainer to obtain a single cell suspension. The cell suspension was then stained with Fixable Aqua Dead cell stain (Thermo Fisher, UK) diluted 1:400 for 20 min and neutrophil surface markers PE anti-mouse CD11b (250 ng $mL^{-1}$) and Alexa 488 anti-mouse Ly6G/Ly6C (250 ng $mL^{-1}$, Biolegend) for 30 min. Cells were fixed with 3% PFA and measured using flow cytometry.

To investigate the migration ability of neutrophils after loading with liposomes, 24 h after LPS injection, formulated liposome/neutrophils (liposomes were labelled with DiL and neutrophils were labelled with VivoTrack 680) were intravenously injected to LPS injected mice. 1 h post i.v. injection, mice were sacrificed and quadriceps from both legs were taken out. A single cell suspension was obtained as described above and then stained with Aqua Dead cell stain and neutrophil surface markers PerCP anti-mouse CD11b (250 ng $mL^{-1}$) and Alexa 488 anti-mouse Ly6G/Ly6C (250 ng $mL^{-1}$). Cells were fixed with 1.5% PFA and measured using flow cytometry.

To demonstrate the anti-inflammation treatment effect of liposome/neutrophils system, MTX was loaded in liposomes (10 mg $mL^{-1}$ MTX was used during liposome formation) and MTX-liposome/neutrophils were formulated as described above. 24 h after LPS injection, formulated MTX-liposome/neutrophils were intravenously injected (10^7 neutrophils per mouse) to LPS treated mice. Free MTX, MTX-liposomes (an equivalent amount of 2μg of MTX per mouse) and blank neutrophils (10^7 per mouse) were also injected *i.v.* to LPS injected mice as control groups and model mice without any treatment as the untreated control group. An extra 24 h after neutrophil i.v. injection, mice from five groups were sacrificed and the quadriceps from left healthy legs and right LPS injected legs were taken out. All the quadriceps were weighed and lysed in Cell Lysis Buffer 2 (R&D Systems) for 30 min at 37 °C. The supernatant was collected after centrifugation. Three different inflammatory cytokines IL-1 α, TNF- α and IL-6 in the supernatant were quantified using Magnetic Luminex Assay (R&D Systems).

*Myocardial IRI model and fluorescence molecular tomography*

Female CD-1 mice (4-5 weeks old) were anaesthetised with 4% isoflurane and then maintained at 2% in 100% $O_2$. Mice received 0.024 mg buprenorphine subcutaneously (1.1 mg $kg^{-1}$; Vetergesic, Alstoe Animal Health, UK), and were placed on a supine position, and intubated

763 and ventilated with a tidal volume of 250 μL and a respiratory rate of 150 breaths $min^{-1}$ (Hugo-
764 Sachs MiniVent type 845; Harvard Apparatus Ltd., Kent, UK). The chest was shaved and a skin
765 disinfectant was applied. A film dressing was placed over the chest to prevent furs entering the
766 wound. After a left thoracotomy in the fourth intercostal space, the pericardium was removed,
767 a 6-0 polyethylene suture was used to ensnare the left anterior descending (LAD) and tied
768 against a polythene tubing for 60 min (LAD ischaemia) after which the ligating suture was
769 loosened to allow reperfusion. The ligature was consistently positioned ~1mm below the atrio-
770 ventricular junction. At the end of surgery mice were given 0.5 mL saline by subcutaneous
771 injection to counter dehydration and allowed to recover in a heated chamber for 20 min, then
772 moved to a normal holding cage with supplemental heat for a few hours and mashed food at
773 floor level. Adequate post-operative care was provided. Electrocardiogram (ECG), heart rate
774 and body temperature were monitored throughout surgery. Consistency of the surgical IRI
775 model was ensured by application of pre-defined surgical exclusion criteria: clear ST segment
776 elevation on the ECG and distinct blanching of the myocardium after LAD ligation are primary
777 criteria used to confirm the occurrence of myocardial infarction.

778 To determine the biodistribution of injected neutrophils in mice with myocardial IRI, 24 h after
779 reperfusion, neutrophils were isolated from donor mice, labelled with VivoTrack 680 and
780 intravenously injected to mice with myocardial IRI ($3.36 \times 10^6$ neutrophils per mouse) and
781 healthy mice. 1 h and 2 h post *i.v.* injection, mice were killed and the hearts were perfused with
782 PBS and excised. Kidney, spleen, lung and liver were harvested as well for *ex vivo* imaging.
783 Five same organs from non-injected healthy mice were also harvested as the blank control. All
784 the organs were imaged using Fluorescence Molecular Tomography (FMT4000, PerkinElmer).

785 To investigate the biodistribution of liposome loaded neutrophils in mice with myocardial IRI,
786 24 h after reperfusion, formulated liposome/neutrophils (liposomes were labelled with DiD)
787 were intravenously injected to mice with myocardial IRI ($5.6 \times 10^6$ neutrophils per mouse) and

healthy mice. 1 h post *i.v.* injection mice were killed and the hearts were perfused with PBS and excised. Kidney, spleen, lung and liver were harvested and only a part of each organ was used for *ex vivo* imaging. Five same organs from non-injected healthy mice were also harvested as the control. All the organs were imaged using Fluorescence Molecular Tomography (FMT4000, PerkinElmer). The collected fluorescence images were reconstructed by FMT 4000 system software (TrueQuant v3.0, PerkinElmer) for the quantification of three-dimensional fluorescence signals in different organs.

*Statistics*

All the statistical analyses were conducted in GraphPad 8.0 (Prism). All the statistical tests were specified in the figure legends. Shapiro-Wilk test was used to assess normality and thus determine the statistical test. For non-normal distributions, the left healthy leg and right inflamed leg from the same mouse were preselected as a pair to analyse.

**Supplementary References**

[1] H. Zhang, in *Liposomes*, Springer, **2017**, pp. 17–22.

[2] U. Baxa, in *Charact. Nanoparticles Intend. Drug Deliv.*, Springer, **2018**, pp. 73–88.

[3] P. Kapusta, P. Gmbh, **2010**, 0.

[4] P. Müller, P. Schwille, T. Weidemann, *Bioinformatics* **2014**, *30*, 2532.

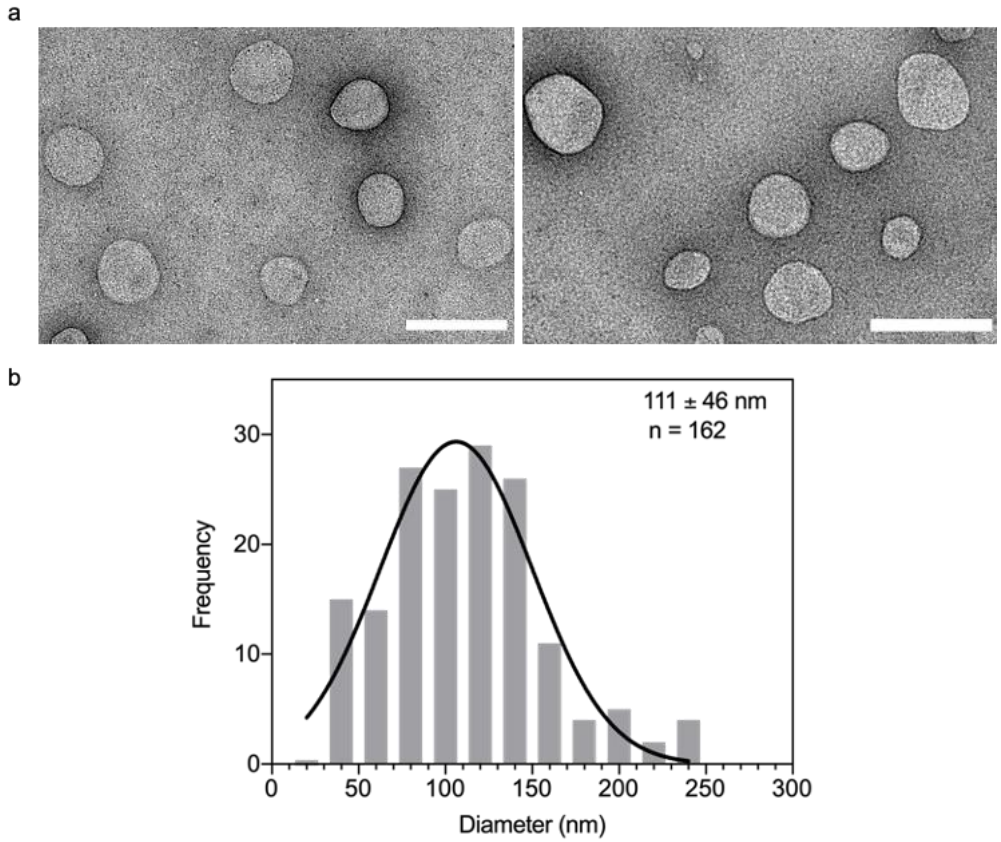


**Figure S1. TEM images and corresponding histogram for MTX-liposomes. a,** TEM images of MTX-liposomes. Scale bar: 200 nm. **b**, Size distribution of MTX-liposomes.

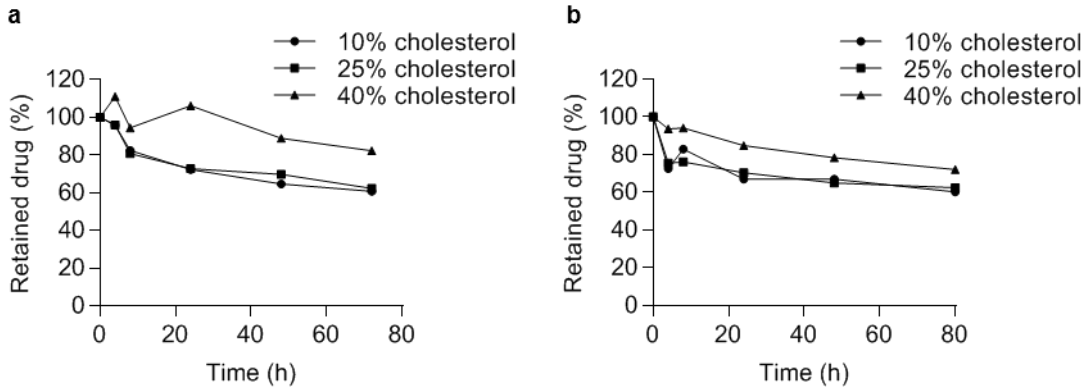


**Figure S2. The effect of cholesterol percentage of liposomes (liposome composition: 16:0-18:1 PC (POPC), 18:0 TAP and cholesterol) on drug retention behaviour.** Quantity of retained drug (MTX concentration used during liposome formation: **a**: 5 mg $mL^{-1}$; **b**: 10 mg $mL^{-1}$) inside liposomes with different cholesterol percentages over 3 days in PBS pH 7.4 at room temperature measured by LC-MS (n = 1).

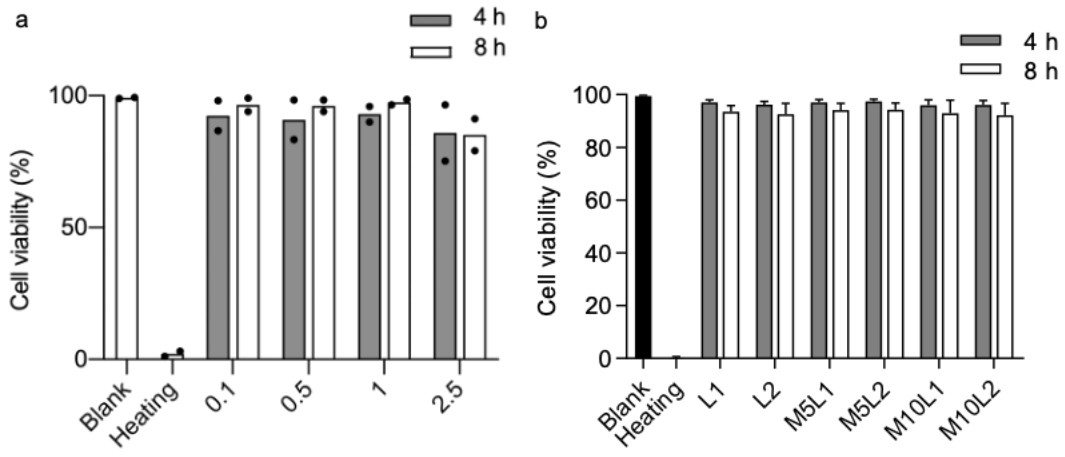


**Figure S3. Cytocompatibility of different liposome treatments to neutrophils after incubation for 4 h and 8 h**. **a**, Isolated neutrophils were incubated with blank liposomes at different lipid concentrations (Data shown as mean, n = 2). b, Isolated neutrophils were incubated with blank liposomes and MTX-liposomes at different lipid concentrations. Zombie Green was used to measure neutrophil viability by flow cytometry. Neutrophils heated at 70 °C for 10 min were used as the negative control (dead cells) (mean ± s.d., n = 3).

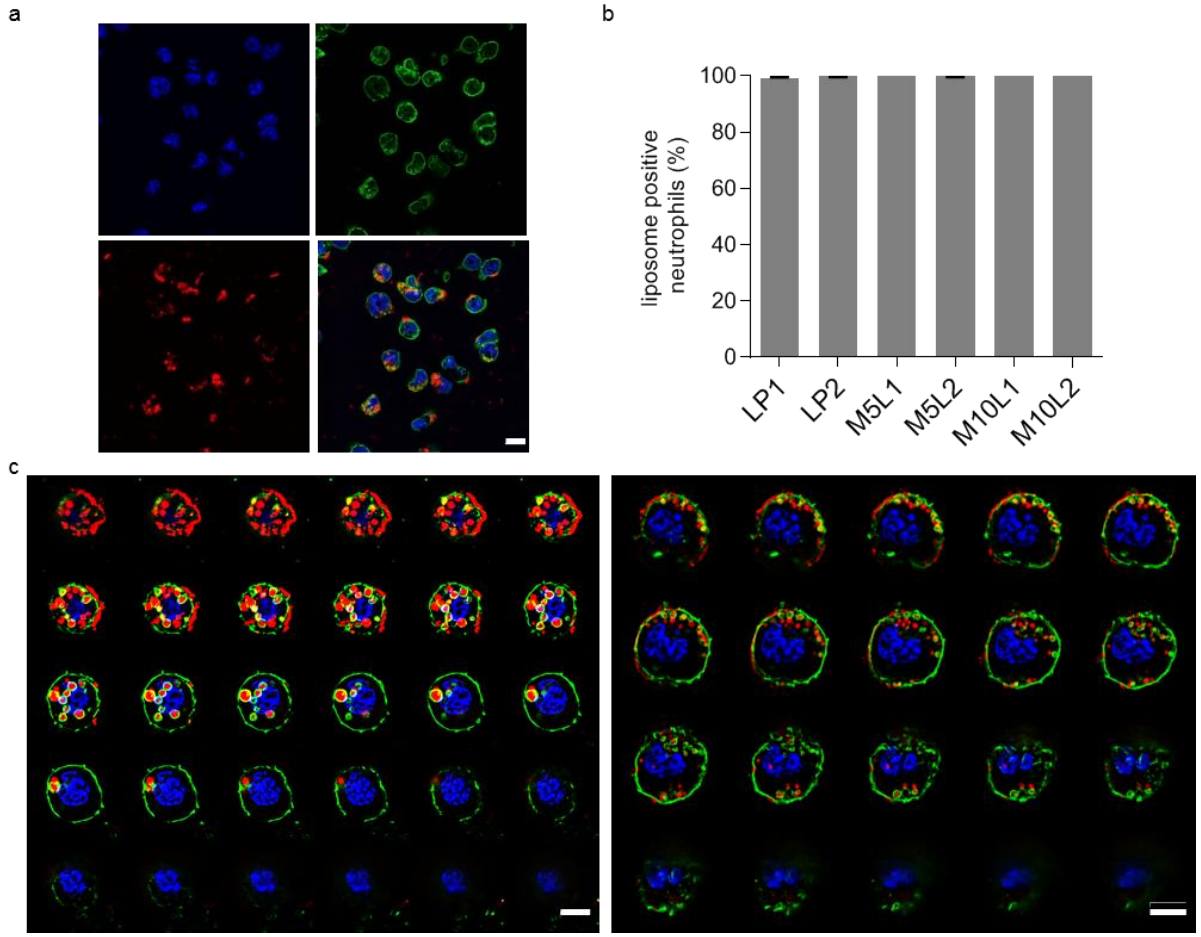


**Figure S4. Images of liposome loaded neutrophils. a**, CLSM images of MTX-liposome loaded neutrophils (DiD was used to label the liposome membrane (red), WGA was used to stain the cell membrane (green)). Scale bar: 10 μm. **b**, Flow cytometric analysis of liposome loaded neutrophils. Isolated neutrophils were incubated with blank liposomes and MTX-liposomes at different lipid concentrations for 1 h (mean ± s.d., n = 3). **c**, Super resolution images (SIM) showing the location of MTX-liposomes (red) on/in single neutrophil at different z position. Scale bars: 5 μm.

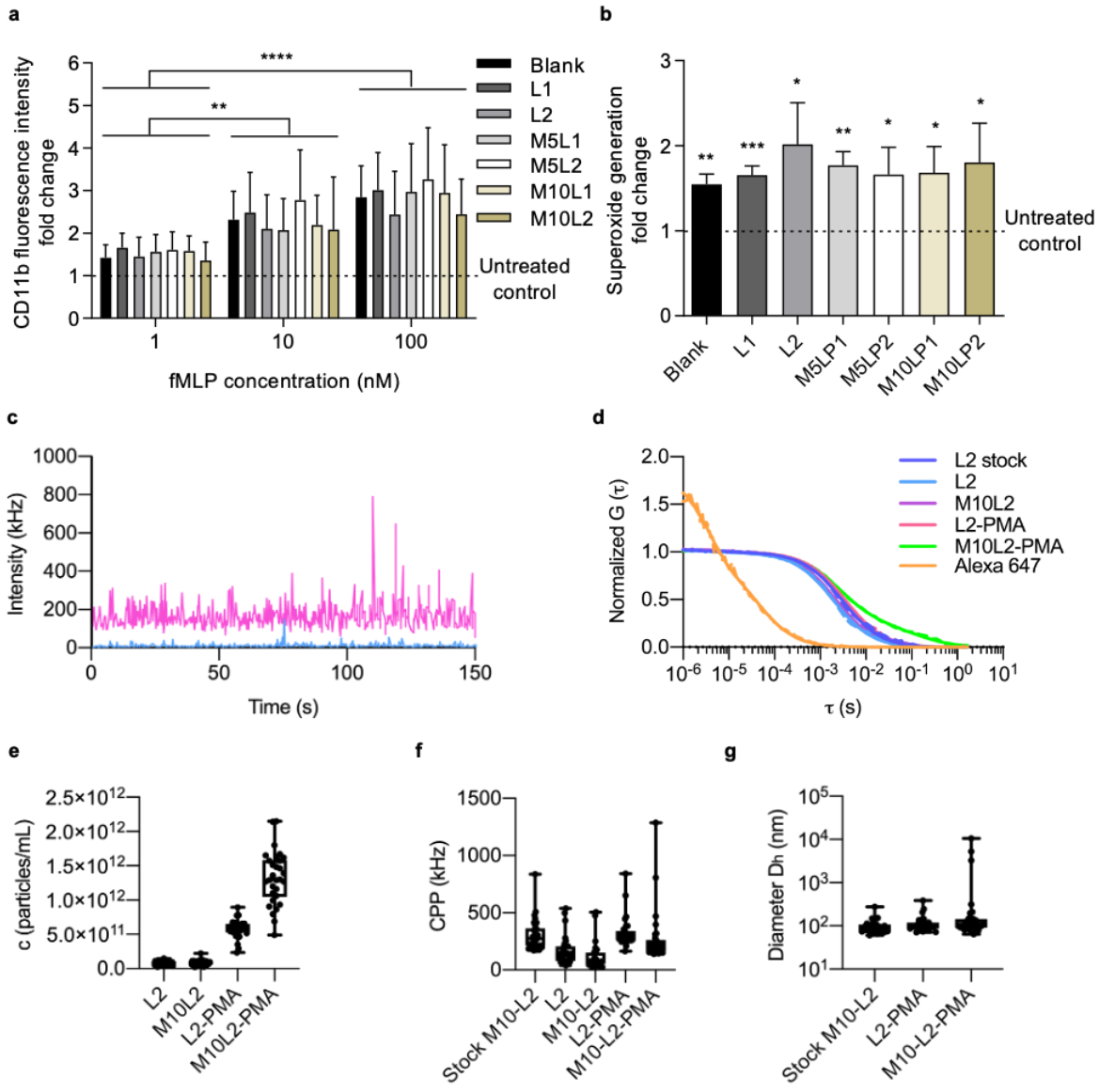


**Figure S5. Preservation of physiological functions of neutrophils after loading with different concentrations of MTX-liposomes and repeat of stimulated release of liposomes (DiD labelled) from neutrophils after treatment with PMA detected by FCS. a**, Change in the CD11b expression of liposome loaded neutrophils after treatment with fMLP at different concentrations. Neutrophils were loaded with blank liposomes and MTX encapsulated liposomes at different concentrations and then stained with PE anti-mouse CD11b antibody (mean ± s.d., n = 3 independent experiments). $^{**}P < 0.01$, $^{****}P < 0.0001$, two-way ANOVA, Bonferroni post hoc test. **b**, Change in the superoxide generation of liposome loaded neutrophils with and without fMLP treatment. The superoxide level of cells was detected using

dihydroethidium and compared to untreated control (mean ± s.d., n = 3 independent experiments). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, unpaired two-tailed Student's t test. **c**, Raw fluorescence intensity traces recorded for samples collected after incubation of liposomes/neutrophils with and without stimulated release (+/- PMA). **d**, Average autocorrelation curves from FCS measurements (n = 30 independent measurements, 5s each). **e**, Amount of released liposomes given in particles per mL, (**f**) signal (counts) per particle (CPP) and (**g**) hydrodynamic diameter ($D_h$) of liposomes was calculated from the fit parameters obtained in c. Centre line, the median; box limits, upper and lower quartiles; whiskers, minimum and maximum values (n = 30 measurements per sample).

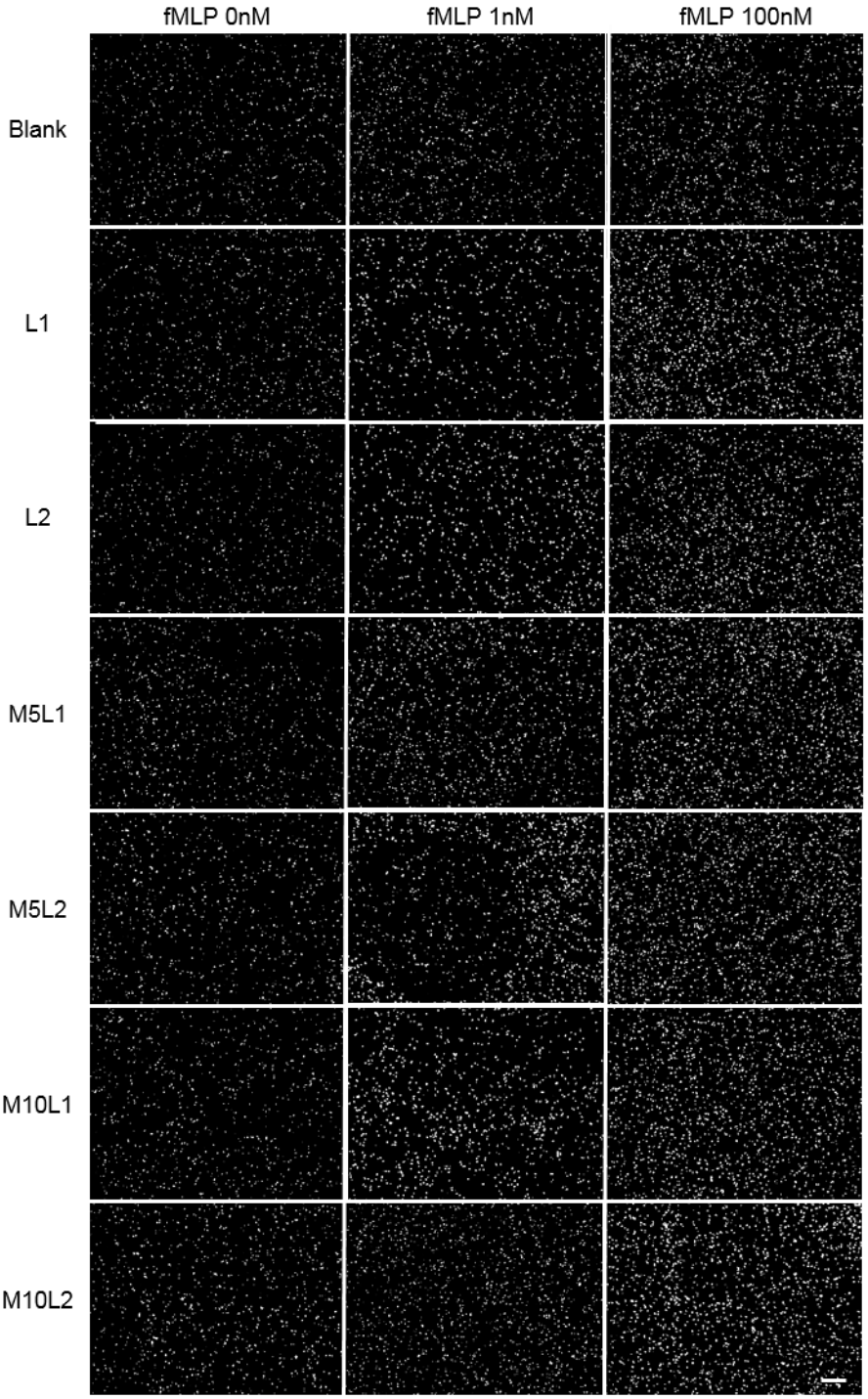


**Figure S6. Images of neutrophils at the bottom side of the transwell membrane after migration test**. Neutrophils were incubated with blank liposomes or MTX-liposomes at different lipid concentrations. Formulated MTX-liposome/neutrophils were put in the upper chamber of the transwell and activated by fMLP in the lower chamber. MTX-liposome/neutrophils migrated through the pores of the membrane. Membranes were taken out and the cells on the upper side were removed. The nuclei of neutrophils at the bottom side of

885 the membrane were stained with DAPI and imaged by fluorescence microscopy (DAPI channel).
886 Nuclei counts for the different conditions are shown in Fig 3b Scale bar: 100 µm.
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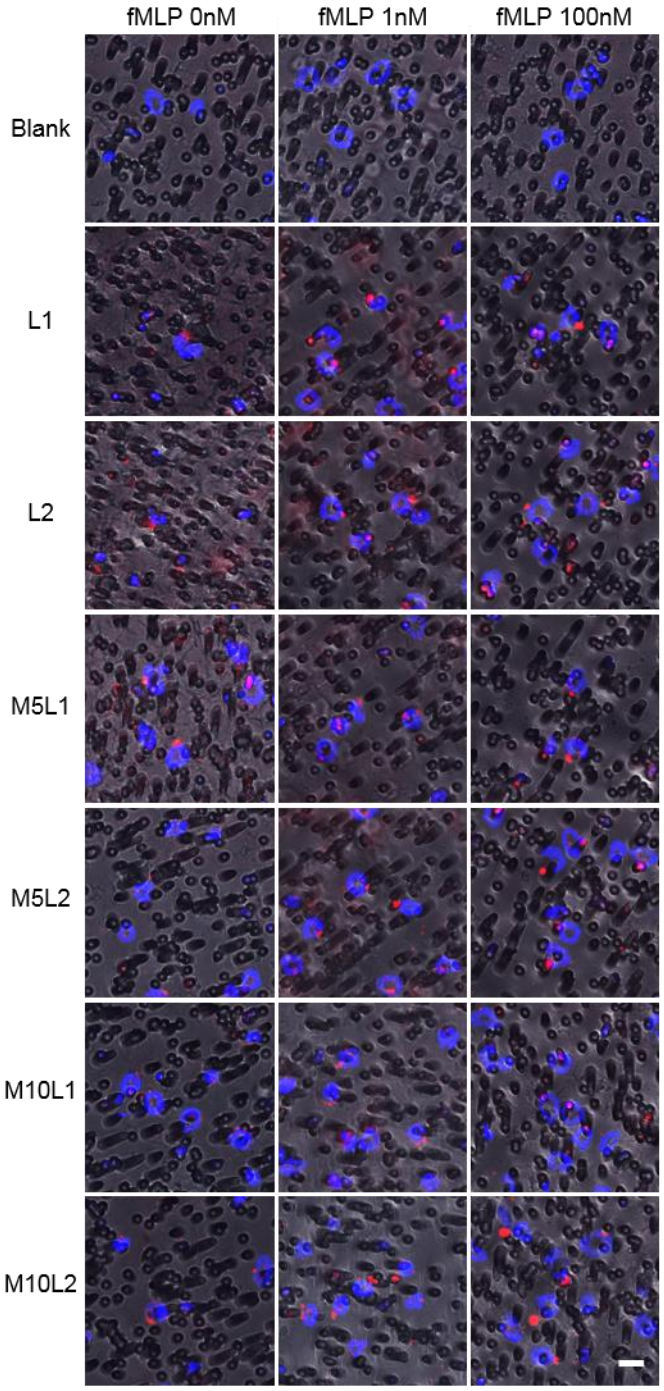


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894 **Figure S7. CLSM images of MTX-liposome/neutrophils on the transwell membrane.**
895 Formulated MTX-liposome/neutrophils (liposomes were labelled with DiD) were put in the
896 upper chamber of the transwell to measure the cell migration ability (details were described in
897 Figure S6). The membranes were cut out and put in an ibidi chamber slide and imaged using
898 confocal microscopy. Pores of the membrane (dark grey) were traversed by the liposome-
899 loaded neutrophils. Scale bars: 5 μm.

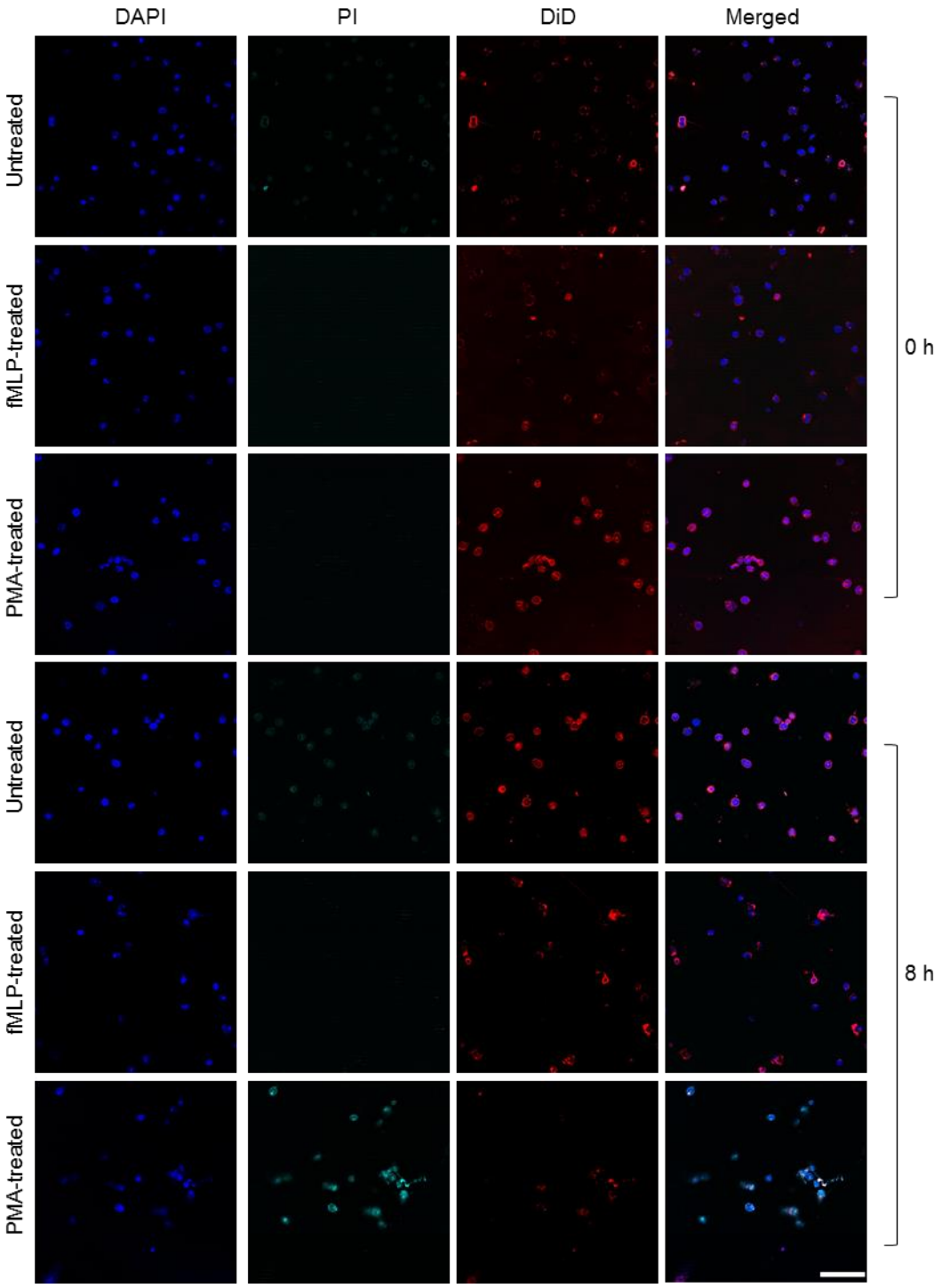
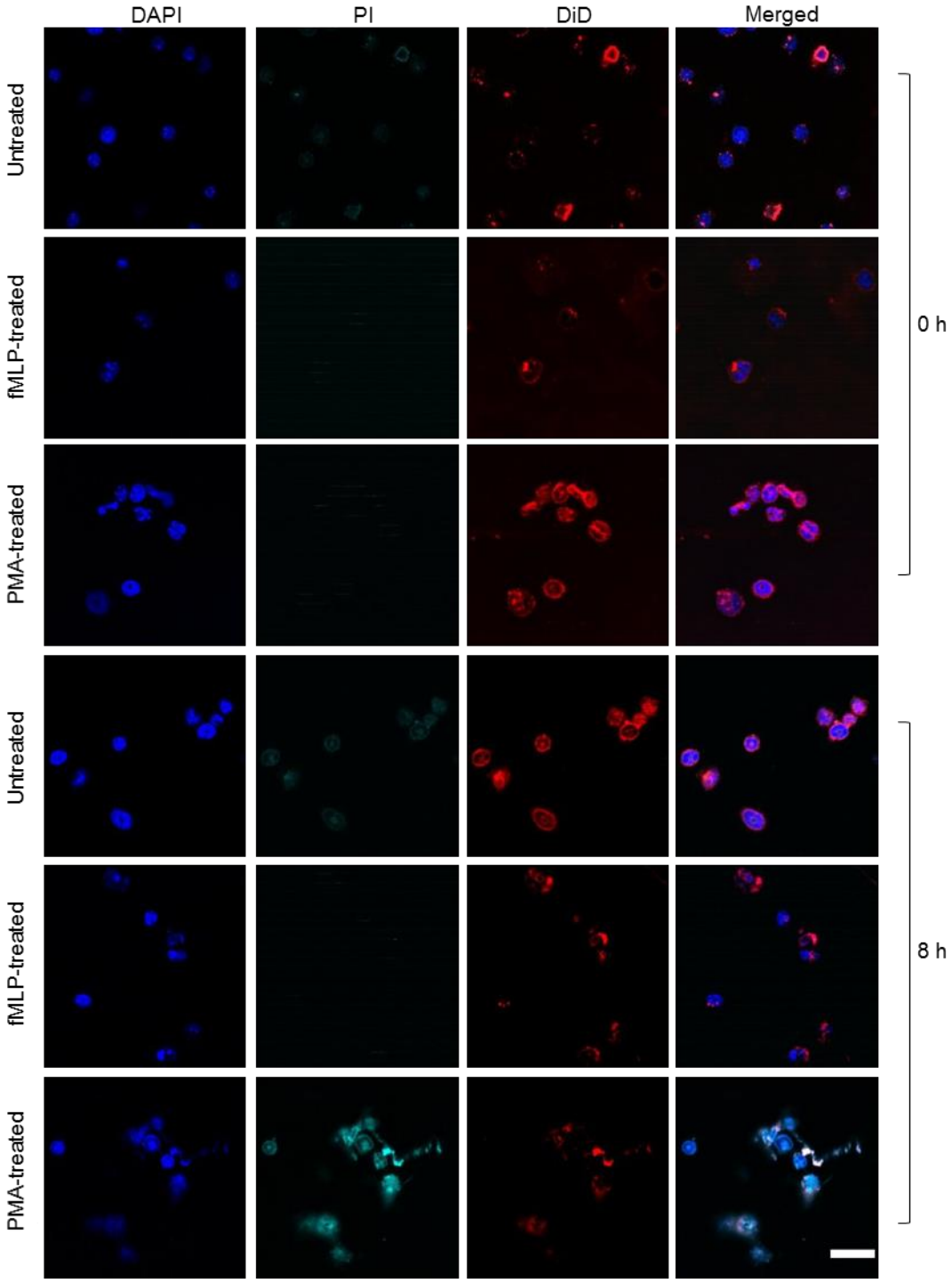



**Figure S8. CLSM images of DiD-liposome/neutrophils before and after treatment with fMLP or PMA.** Liposomes were labelled with DiD, followed by incubation with neutrophils to form liposome/neutrophils. Formulated liposome/neutrophils were incubated in presence of fMLP or PMA for 0 h and 8 h. The nuclei of neutrophils were stained with DAPI, the released

905 DNA fragments were stained with PI. The merged image is the overlay of the three individual
906 images. Scale bar: 50 µm.

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908 **Figure S9. Zoom of CLSM images from Figure S8**. Scale bar: 10 µm.

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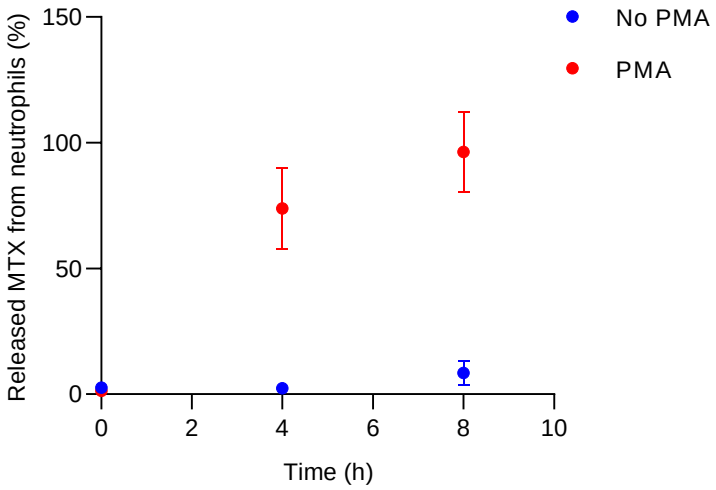


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913 **Figure S10. Percentage of released MTX from neutrophils.** Formulated MTX-
914 liposome/neutrophils were incubated with and without the inflammatory stimuli (+/- PMA).
915 The supernatants were collected at different time points and released MTX in the supernatants
916 was quantified by ELISA. Data shown as mean ± s.d., n = 3.

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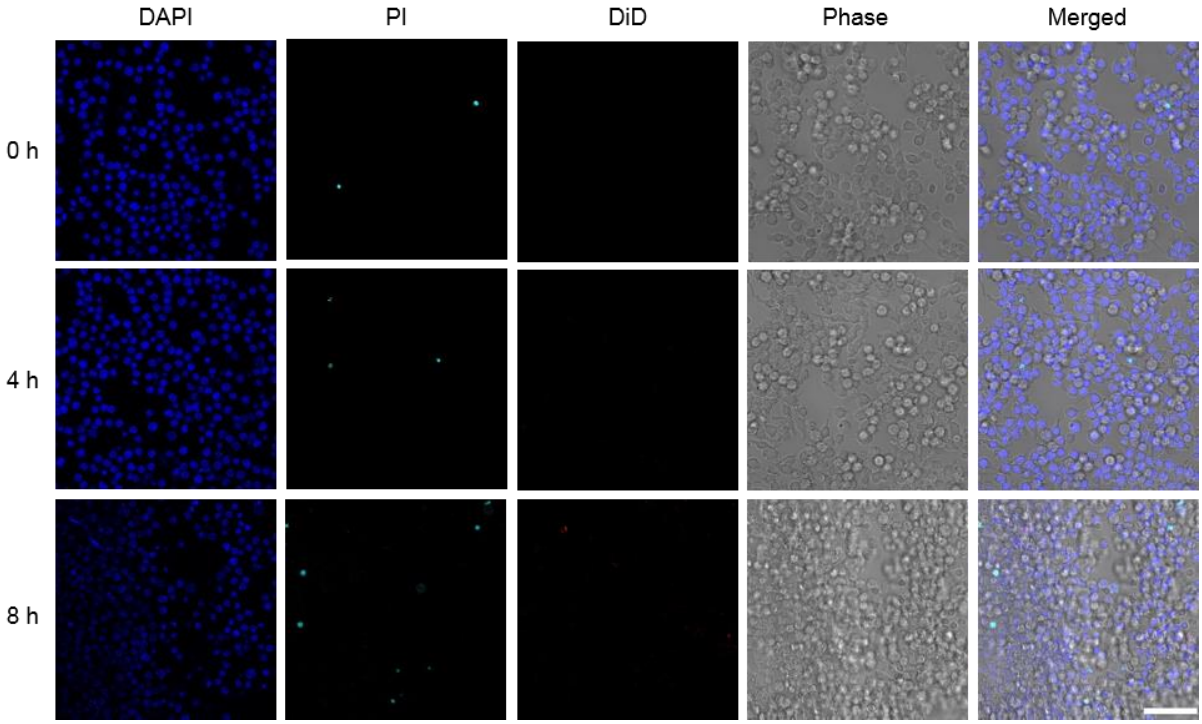


**Figure S11**. **CLSM images of RAW 264.7 cells after incubation with liposome/neutrophils for 0 h, 4 h and 8 h in the absence of PMA.** The nuclei of RAW 264.7 cells were stained with DAPI, the released DNA fragments were stained with PI and the liposomes were labelled with DiD. The merged image is the overlay of the four individual images. Scale bar: 50 µm.

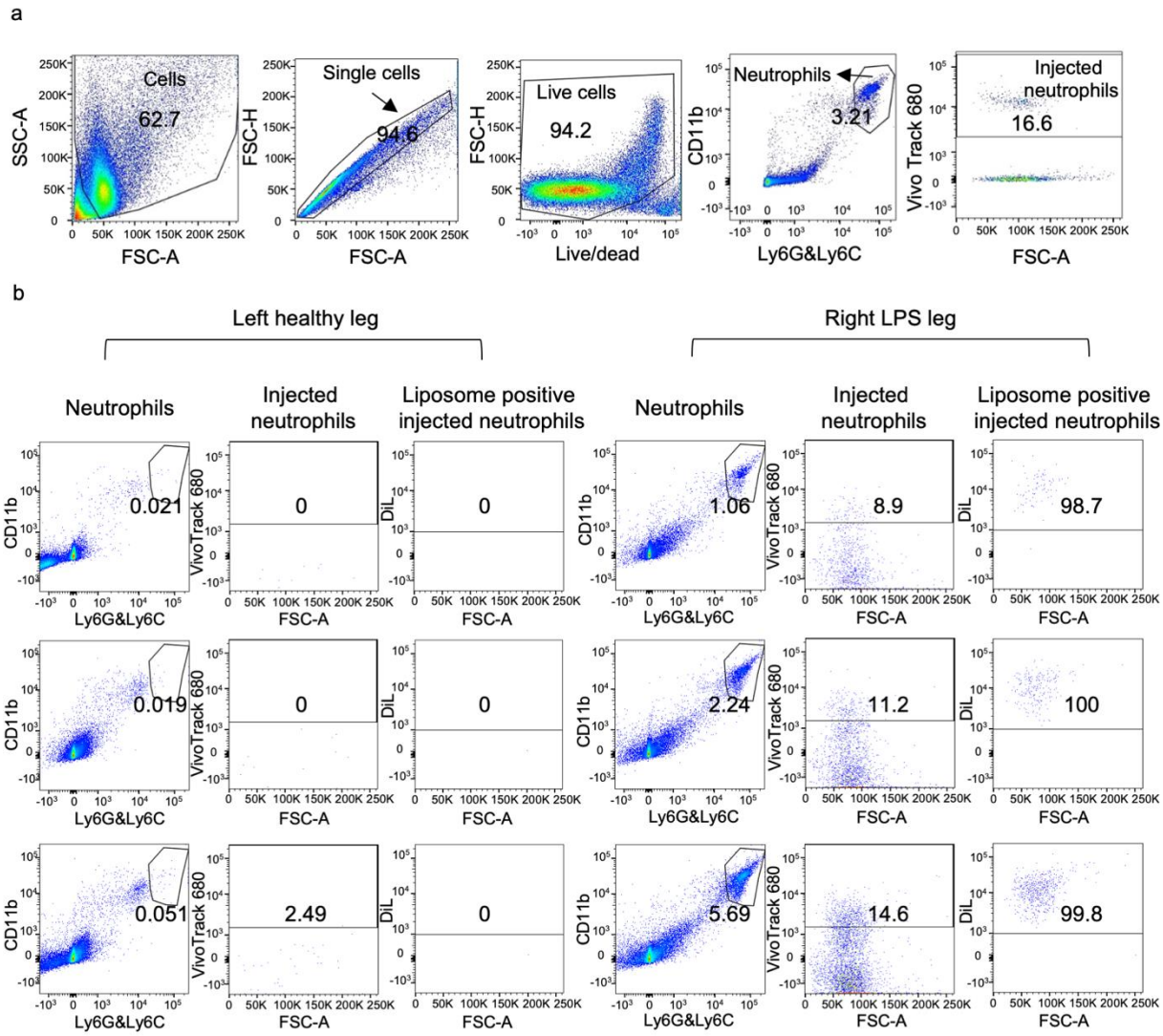


**Figure S12. The targeting effect of liposome/neutrophils to the inflamed quadriceps**. **a**, An example showing the gating strategy (data have been shown in Figure 4b). **b**, Flow cytometric analysis of the healthy quadriceps and the inflamed quadriceps after injection of liposome loaded neutrophils (liposomes were labelled with DiL; neutrophils were labelled with VivoTrack 680). First column: gate shows the double positive population of neutrophils within overall cell population. Second column: gate shows the VivoTrack 680 positive population of injection neutrophils compared to the total neutrophil population. Third column: gate shows DiL positive population of liposome in neutrophils compared to the total injected neutrophil population.

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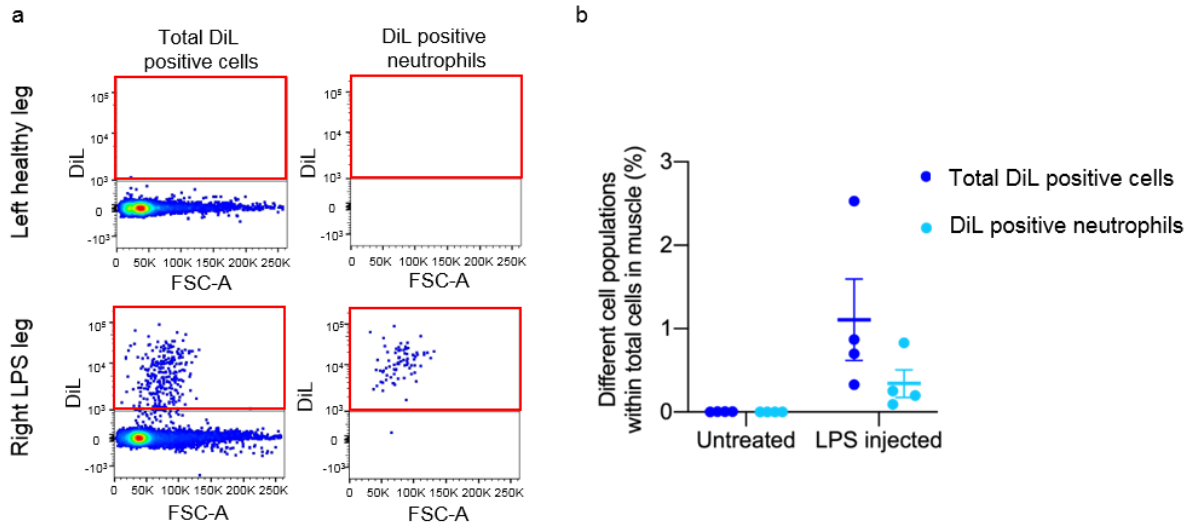


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943 **Figure S13. Delivery of cargo (DiL) to non-neutrophil cells *via* the liposome**
944 **(DiL)/neutrophil system. a**, Flow cytometric analysis of DiL positive cell populations in the
945 healthy quadriceps and the inflamed quadriceps after injection of liposome loaded neutrophils
946 (liposomes were loaded with DiL). Gates (red) showing populations of total DiL positive cells
947 (include DiL positive neutrophils) and DiL positive injected neutrophils (CD11b+Ly6G/6C+)
948 in the healthy quadriceps and LPS-injected quadriceps. **b**, The percentage of total DiL positive
949 cells and DiL positive injected neutrophils among total cells digested from the quadriceps.

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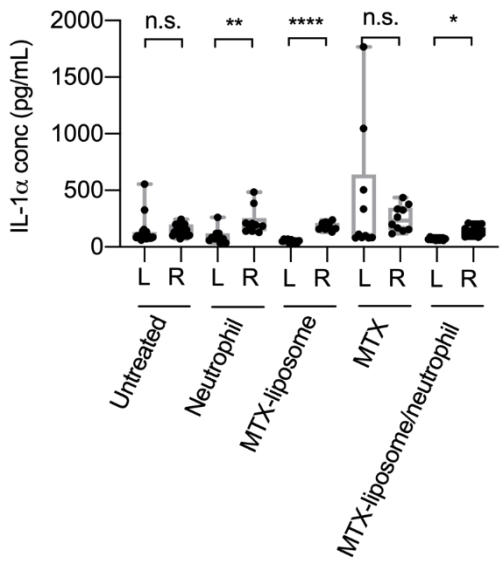



**Figure S14. IL-1α level in the healthy quadriceps and LPS-injected quadriceps after different treatments.** Centre line, the median; box limits, upper and lower quartiles; whiskers, minimum and maximum values. **P <0.01, ****P < 0.0001, n.s. not significant, Kruskal-Wallis, Corrected Dunn's post hoc test, n = 20 in the untreated group, n = 10 in other treated groups.

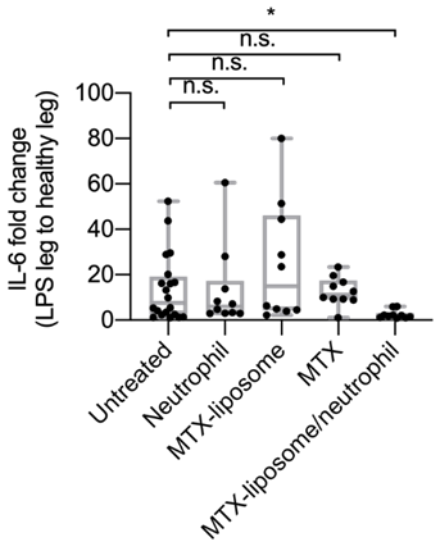



**Figure S15. IL-6 levels in inflamed versus healthy quadriceps after different treatments.** Fold-change of IL-6 expression level in LPS injected versus non-injected quadriceps. Centre line, the median; box limits, upper and lower quartiles; whiskers, minimum and maximum values, n = 20 in the untreated group, n = 10 in other treated groups. *$P < 0.05$, **$P < 0.01$, Kruskal-Wallis, Corrected Dunn's post hoc test.

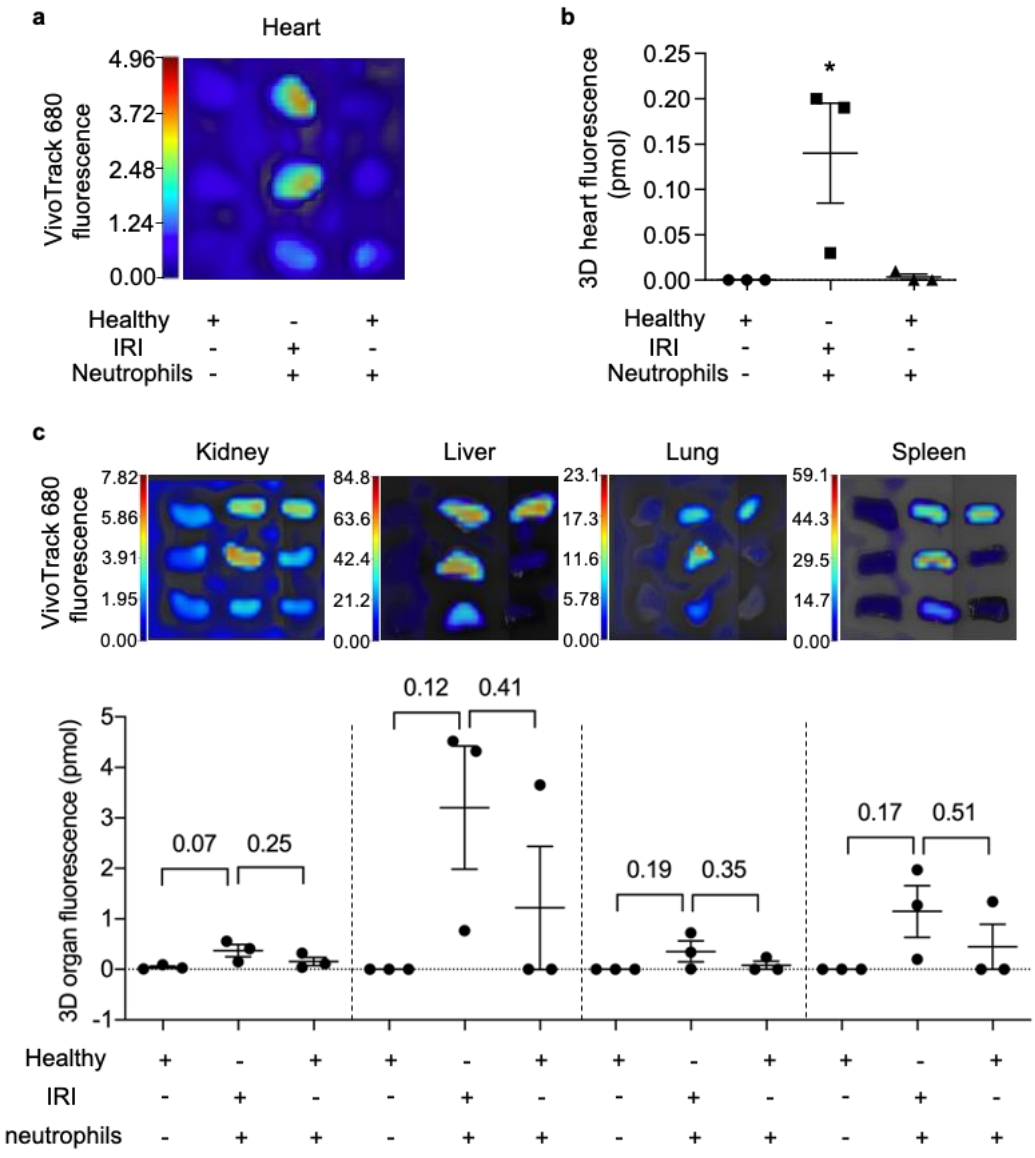



**Figure S16.** ***In vivo*** **accumulation of injected neutrophils (labelled with VivoTrack 680) in the heart after myocardial IRI surgery and biodistribution in other organs at 1 h post neutrophil injection. a**, Fluorescence image of hearts from mice after different treatments. **b**, Total amount of VivoTrack 680 dye in hearts (pmol, mean ± s.e.m.). *P < 0.05, one way ANOVA, Bonferroni post hoc test, n = 3. **c**, *In vivo* biodistribution of injected neutrophils in kidney, liver, lung and spleen (from left to right). Top row: fluorescence images of different organs from corresponding mice. Bottom row: total amount of VivoTrack 680 dye in different organs (pmol, mean ± s.e.m.). One-way ANOVA, Bonferroni post hoc test, n = 3.

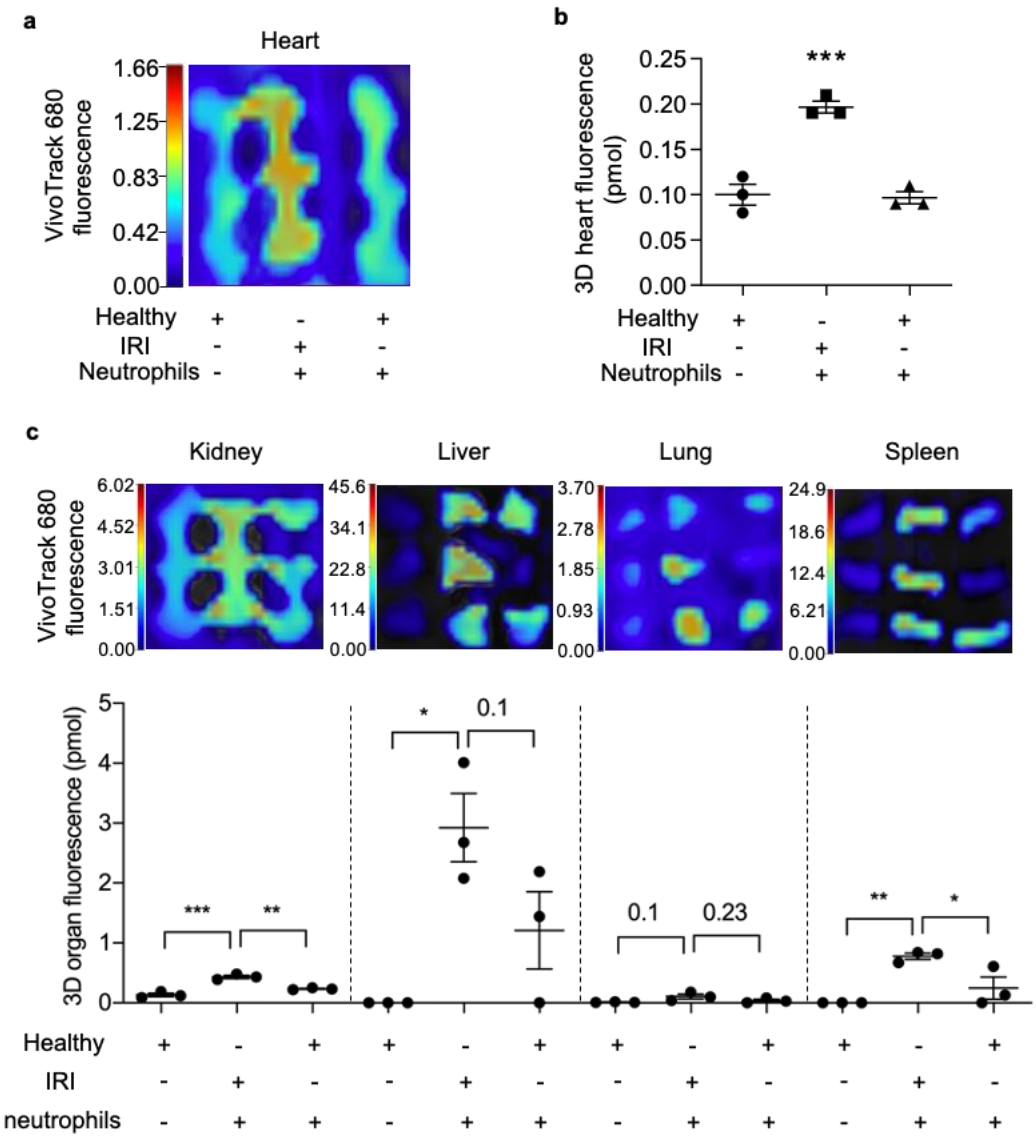
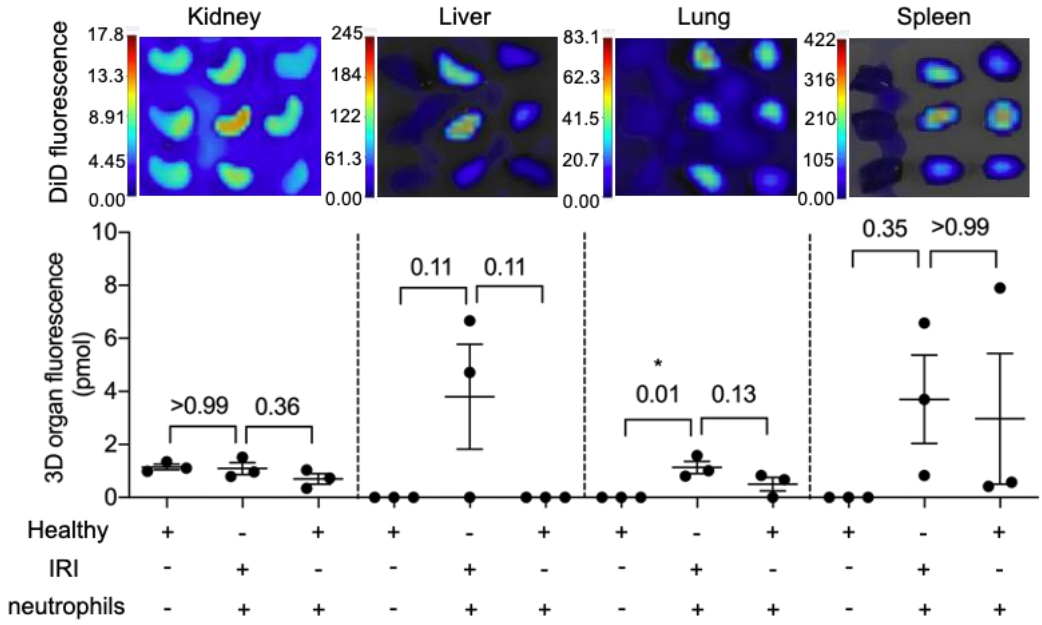



**Figure S17.** ***In vivo*** **accumulation of injected neutrophils (labelled with VivoTrack 680) in the heart after myocardial IRI surgery and biodistribution in other organs at 2 h post neutrophil injection. a**, Fluorescence image of hearts from mice after different treatments. **b**, Total amount of VivoTrack 680 dye in hearts (pmol, mean ± s.e.m.). ***P < 0.001, one way ANOVA, Bonferroni post hoc test, n = 3. **c**, *In vivo* biodistribution of injected neutrophils in kidney, liver, lung and spleen (from left to right). Top row: fluorescence images of different organs from corresponding mice. Bottom row: total amount of VivoTrack 680 dye in different organs (pmol, mean ± s.e.m.). *P < 0.05, **P < 0.01, ***P < 0.001, one-way ANOVA, Bonferroni post hoc test, n = 3.

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998 **Figure S18. *In vivo* biodistribution of liposomes in other organs in mice with myocardial**
999 **IRI at 1 h post liposome/neutrophil injection.** *In vivo* biodistribution of DiD labelled
1000 liposomes in the kidney, liver, lung and spleen (from left to right). Top row: fluorescence
1001 images of different organs from mice. Bottom row: total amount of DiD dye in the different
1002 organs (pmol, mean ± s.e.m.). *P < 0.05, one-way ANOVA, Bonferroni post hoc test, n = 3.

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