Electronic Supplementary Material (ESI) for Nanoscale.

Supporting Information

Micellar nanoparticles inhibit breast cancer and pulmonary metastasis by modulating the recruitment and depletion of myeloid-derived suppressor cells

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Supplementary methods

1. Quantitative determination of the main components of RLA NPs and RLA/DOX/αGC NPs

Firstly, toluidine blue spectrophotometry was used to quantitatively determine the content of LMWH in NPs. Toluidine blue can be combined with negatively charged LMWH by electrostatic interaction in aqueous solution. The UV absorption of free toluidine blue can be detected at 629 nm. In a certain concentration range, the absorbance of free toluidine blue was negatively correlated with the concentrations of LMWH in samples. 2.5 mg of toluidine blue and 100.0 mg of NaCl were dissolved in deionized water and the volume was fixed in a 50 mL volumetric flask to obtain the 0.005% (g/mL) toluidine blue solution. 2.0 mg of LMWH was dissolved in PBS and the volume was fixed in a 50 mL volumetric flask to obtain the 40 μg/mL LMWH solution. A series of LMWH standard solutions with concentrations of 1 μg/mL, 2 μg/mL, 4 μg/mL, 8 μg/mL, 12 μg/mL and 14 μg/mL were obtained by further dilution with PBS. 2 mL of LMWH standard solution was mixed with equal volume of 0.005% (g/mL) toluidine blue solution and shook for 30s. Then 5 mL of n-hexane was added to each sample and shook for 1 more min. After that, the sample was left to stand and the water layer was separated with a separating funnel. The UV absorption of the water layer was detected at the wavelength of 629 nm. The standard curve was obtained, and the linear range was determined at the same time. 5.0 mg of LMWH-ATRA powder was dissolved in PBS to obtain a 10 μg/mL solution, and the content of LMWH was determined according to the above method. The content of ATRA in the LMWH-ATRA conjugates can be determined by subtraction method. Since RGD-LMWH-ATRA was synthesized on the basis of LMWH-ATRA, the content of RGD in RGD-LMWH-ATRA can also be calculated.

Secondly, the content of DOX in NPs was determined quantitatively by fluorescence spectrophotometry. 2.0 mg of DOX·HCl was dissolved in deionized water and the volume was fixed in a 50 mL volumetric flask to obtain the DOX stock solution. A series of DOX standard solutions with concentrations of 0.1 μg/mL, 0.2 μg/mL, 0.5 μg/mL, 1.0 μg/mL, 2.0 μg/mL and 5.0 μg/mL were obtained by further dilution. The fluorescence value of each sample was measured under E_X = 500 nm, E_m = 595 nm to obtain the standard curve and determine the linear range. 5.0 mg of freeze-dried RLA/DOX NP powder or RLA/DOX/αGC NP powder was dissolved in 2 mL of deionized water, a small amount of DMSO was added to destroy the micelles and let the encapsulated DOX release. Then samples were diluted with deionized water to obtain solutions with DOX concentration of about 2.5 μg/mL. The accurate content of DOX was determined by the above method. The drug loading and encapsulating efficiency of DOX in RLA/DOX NPs or RLA/DOX/αGC NPs was further calculated.

2. Determination of the critical micelle concentration of RLA NPs

5.0 mg of pyrene was dissolved in acetone and the volume was fixed in a 10 mL brown volumetric flask to obtain pyrene stock solution (pyrene: 2.5×10^{-3} mol/L). Pyrene test solution (pyrene: 1.5×10^{-5} mol/L) was obtained by further dilution using acetone. 10.0 mg of RGD-LMWH-ATRA powder was dissolved in deionized water and the volume was fixed in a 10 mL volumetric flask to obtain RGD-LMWH-ATRA stock solution. A series of RGD-LMWH-ATRA sample solutions with concentrations of 0.1×10^{-3} mg/mL, 0.5×10^{-3} mg/mL, 0.1×10^{-2} mg/mL, 0.5×10^{-2} mg/mL, 0.1×10^{-1} mg/mL, 0.5×10^{-1} mg/mL, 0.1 mg/mL, 0.5 mg/mL and 1.0 mg/mL were obtained by further

dilution. 80 μL of pyrene test solution was added to each 2 mL brown volumetric flask and dried under nitrogen flow. Pyrene was laid on the bottom of each flask in a thin film. The above sample solutions were added to the scales respectively, and samples were shook in the shaking bed at 75 rpm, 37 ℃ for 24 h. Fluorescence spectrophotometer was used to detect the emission fluorescence intensity (I) of each sample at 373 nm and 384 nm under E_X = 336 nm. I_{373}/I_{384} and the logarithm of RGD-LMWH-ATRA concentration (log C) were used to plot the curves. The concentration corresponding to the mutation of I_{373}/I_{384} was the critical micelle concentration of RLA NPs.

3. Determination of the in vitro serum stability of NPs

RLA NPs, RLA/DOX NPs and RLA/DOX/αGC NPs solutions with a concentration of 2 mg/mL were prepared according to the above methods. An appropriate amount of fetal bovine serum (FBS) filtered by 0.22 μm microporous membrane was mixed with the above solutions to obtain samples with micelle concentration of 1 mg/mL with serum volume ratio of 10% and 50% respectively (n = 3). Samples were shook in the shaking bed at 75 rpm, 37 ℃. The particle size changes of each sample were detected at the time points of 0 h, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h and 48 h after the beginning of incubation.

4. Determination of the in vitro hemolysis of NPs

The blood was taken into the anticoagulant tube through the mice orbit, centrifuged at 200 g (RCF) for 5 min, and the supernatant was discarded. The precipitate was resuspended with an appropriate amount of PBS and centrifuged again. The red blood cells were obtained after washing repeatedly for 3 times. An appropriate amount of PBS was added to obtain 2% (V/V) red blood cell suspension.

RLA NPs solutions with concentrations of 0.30 mg/mL, 0.60 mg/mL and 1.20 mg/Ml were prepared according to the above methods. These solutions were mixed with red blood cell suspension in equal volume to obtain a series of sample solutions with RLA NP concentrations of 0.15 mg/mL, 0.30 mg/mL and 0.60 mg/mL (n = 3). Red blood cell suspension mixed with equal volume of PBS was used as hemolysis negative control group. Red blood cell suspension mixed with equal volume of 0.5% (g/mL) Triton-X 100 was used as hemolysis positive control group. The above samples were placed in a shaking bed and incubated at 37 ℃ and 75 rpm. At the time points of 1, 2, 4 and 8 h after the start of incubation, each sample was centrifuged under the condition of 200 g (RCF) for 5 min. Each sample took 100 μL of the supernatant as the test sample. After sampling, samples were resuspended and continue the incubation. After sampling at the 8^{th} hour, each group took an appropriate amount of samples to make smears, and the morphology of red blood cells was observed under the optical microscope. The obtained test samples were placed in a 96-well plate. The absorbance of each sample was measured by a multimode reader (Varioskan Flash, Thermo Scientific, USA) under the 540 nm wave length. The hemolysis rate was calculated according to the following formula:

$$\text{Hemolysis rate (\%)} = (A_{sample} - A_{PBS}) / (A_{Triton\ X\text{-}100} - A_{PBS}) \times 100\%$$

5. Evaluation of the in vitro drug release of RLA/DOX NPs

RLA/DOX NPs solution with DOX concentration of 100 μg/mL was prepared according to the above method, and free DOX of the same concentration was used as the control group. The solution was divided into several 1 mL samples. Each sample was sealed into a dialysis bag (MWCO 1000 Da), immersed in 50 mL of PBS (containing 1% (g/mL) tween 20) with pH values of

5.0, 6.8 and 7.4 and 37 ℃ and incubated in a shaking bed at 37℃ and 75 rpm for 48 h (n = 3). 200 μL of release medium was taken as test samples at the 0.5 h, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h and 48 h time point after the start of incubation, blank release medium with the same temperature, volume and pH was supplemented to continue the incubation. The DOX fluorescence value of each test sample was measured under E_X = 500 nm, E_m = 595 nm. The DOX release rate of the sample at each time point was calculated according to the above DOX fluorescence-concentration standard curve, and the release curve was plotted.

6. Determination of pharmacokinetic parameters

Healthy Balb/c mice (female, 18-22 g, 5-week-old, SPF) were randomly divided into two groups with 3 mice in each group. Each mouse was given free DOX or RLA/DOX NPs through the tail vein (0.2 mL each, micelle dosage: 90 mg/kg, DOX dosage: 3 mg/kg). The sampling time points were set at 0.2 h, 0.5 h, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h and 48 h after administration. At each time point, about 100 μL of whole blood of each mouse was collected from the orbit. The blood sample was centrifuged at 2300 g (RCF), 4℃ for 10 min, and 30 μL of upper plasma was carefully collected. 120 μL of acetonitrile was added to each plasma sample, whirled at high speed and ultrasonicated for 15 min to precipitate proteins. After that, the sample was centrifuged at 13400 g (RCF), 4℃ for 10 min and the supernatant was carefully collected. The obtained supernatant was filtered by a 0.22 μm microporous filter membrane to obtain the sample to be tested, which was analyzed by LC-MS/MS method (API-3000, AB SCIEX, USA).

7. Cell Lines and animals

Murine breast cancer cells (4T1 and EMT-6) and HUVECs were obtained from Shanghai Institutes for Biological Sciences, CAS (SIBS, Shanghai, China). 4T1-luciferase (4T1-Luc) cells were obtained by transfection with luciferase expressing-lentivirus titer according to the instructions.

Cells were maintained in Roswell Park Memorial Institute 1640 culture medium (RPMI 1640, Gibco, USA) containing 10% fetal bovine serum (FBS, Invitrogen), 100 U/mL streptomycin and 100 U/mL penicillin (Invitrogen) at 37 °C, 5% CO_2 in a humidified cell incubator (Froma 311, Thermo Scientific, USA). Balb/c mice (female, 18-22 g, 5-week-old, SPF) were purchased from Dashuo experimental animal company (Chengdu, China).

8. Cellular uptake and intracellular drug release of RLA/DOX NPs

Mouse 4T1 breast cancer cells were incubated in RPMI 1640 culture medium with 10% FBS, 100 U/mL of penicillin G sodium salt and 0.1 mg/mL of streptomycin sulfate. The harvested 4T1 cells were seeded in a 6-well plate (4×10^5 cell/well) and cultured in a cell incubator at 37°C, 5% CO_2 overnight. Then, 2 mL of serum-free RPMI 1640 culture medium containing PBS, free DOX, LA/DOX NPs, RLA/DOX NPs or RLA/DOX NPs + c(RGDfk) (DOX concentration: 10 μg/mL, c(RGDfk) concentration: 100 μg/mL, c(RGDfk) was pre-incubated with 4T1 cells 0.5 h earlier) was added to each well and incubated in a cell incubator at 37 °C for 1.5 hours (n = 3). After incubation, the culture medium was discarded, the cells were collected after washing with PBS, and the DOX fluorescence intensity of cells in each group was detected by flow cytometry (FACS Celesta, BD Biosciences, USA).

Cover glasses were pre-placed at the bottom of 6-well plates. The uptake experiment was carried out according to the above method. Half an hour before the end of the uptake experiment, 1 μL of LysoTracker Red DND-99 (E_x = 577 nm, E_m = 590 nm) was added to each well to stain

lysosomes. After the experiment, the culture medium was discarded. Cells were fixed with 4% (g/mL) paraformaldehyde. Cell nuclei were stained with DAPI. The cover glasses were washed with PBS, mounted and observed under the confocal laser scanning microscope (CLSM) (LSM800, ZEISS, Germany).

9. Tumor targeting ability of RLA NPs in vivo

The fluorescence probe DiD instead of DOX was encapsulated in RLA NPs to obtain clearer in vivo fluorescence images. In brief, 2 mL of DiD chloroform solution (DiD concentration: 100 μg/mL) was put into a round bottom flask and the chloroform was removed with a rotary evaporator. DiD was dissolved in 0.2 mL of dichloromethane and mixed with 12 mg of RGD-LMWH-ATRA conjugate powder. The mixture was stood at room temperature for 10 min. After that, 2 mL of PBS was added and ultrasonic emulsification (100 W, 5s/5s, 10 min) was performed. The emulsion was moved into a round bottom flask and the organic phase was removed in 40°C water bath by a rotary evaporator. The RLA/DiD NPs solution with 100 μg/mL of DiD was obtained after filtrating through 0.22 μm microporous membrane. LA/DiD NPs were prepared by the same method.

Mouse 4T1 tumor model was established by the above method. The experiment was carried out when the average tumor volume reached about 500 mm^3. The mice were randomly divided into three groups, and were given PBS, LA/DiD NPs or RLA/DiD NPs (20 μg DiD/mouse) through the tail vein, respectively. Then, in vivo imaging system (IVIS Lumina Series III, PerkinElmer, USA) was used to observe the distribution of DiD fluorescence at specific time point (1, 4, 8 and 24 h). Mice were sacrificed after 24 h. Main organs and tumors were collected and observed.

10. Cytotoxicity evaluation of RLA NPs and RLA/DOX/αGC NPs

To evaluate the cytotoxicity of RLA NPs, the harvested 4T1 cells were seeded in a 96-well plate (4×10^3 cell/well) and cultured in a cell incubator at 37°C, 5% CO_2 overnight. Then, 180 μL of serum-free RPMI 1640 culture medium containing PBS, LMWH, ATRA, LA NPs or RLA NPs (in a series concentration of 1.54 μg/mL, 4.63 μg/mL, 13.89 μg/mL, 41.67 μg/mL, 125 μg/mL, 250 μg/mL and 500 μg/mL) was added to each well and incubated in a cell incubator at 37°C for 24 h (n = 5).

To evaluate the cytotoxicity of RLA/DOX/αGC NPs, the harvested 4T1 cells were seeded in a 96-well plate (4×10^3 cell/well) and cultured at 37°C, 5% CO_2 overnight. Then, 180 μL of serum-free RPMI 1640 culture medium containing PBS, free DOX, RLA NPs, RLA/DOX NPs or RLA/DOX/αGC NPs (in a series DOX concentration of 0.0625 μg/mL, 0.125 μg/mL, 0.25 μg/mL, 0.5 μg/mL, 1 μg/mL, 2 μg/mL and 4 μg/mL) was added to each well and incubated in a cell incubator at 37°C for 24 h (n = 5).

After incubation, 20 μL of MTT solution (5 mg/mL) was added into each well. Plates were incubated for another 4 hours. Then, culture medium in each well was removed carefully and 150 μL of DMSO was added into each well to dissolve the generated formazan. A multifunctional microplate reader (Varioskan Flash, Thermo Scientific, USA) was used to measure the absorbance of each well at the wavelength of 570 nm. The drug-free group was used as the negative control and the cell-free group as the positive control. The cell viability (%) was calculated according to the following formula:

$$\text{Cell viability (\%)} = (A_{sample} - A_{positive}) / (A_{negative} - A_{positive}) \times 100\%$$

11. The preliminary safety evaluation in vivo

Healthy Balb/c mice were randomly divided into 7 groups with 3 mice in each group.

Preparations were administrated according to the scheme in section 2.16.

To analyze the blood cells and serum biochemical indexes, on the 25th day, about 150 μL of whole blood was taken from the eye orbit of each mouse and transferred into the tube containing anticoagulant. Then, blood samples were analyzed by an automatic blood cell analyzer (BC-2800Vet, Mindray, China). Besides, 40 μL of serum was taken from each mouse and analyzed by an automatic biochemical analyzer (Chemray800, Rayto, China).

To conduct the pathological observation, mice were sacrificed on the 25th day. Then, 6 mL of PBS and 6 mL of 4% (g/mL) paraformaldehyde were perfused through the heart to discharge the blood and conduct tissue fixation. The main organ tissues of mice were collected and dehydrated to prepare paraffin embedded sections followed by HE staining. The obtained sections were observed with optical microscope (DM2000 LED, Leica, Germany).

Supplementary tables

Table S1. Characterizations of RLA NPs and RLA/DOX/αGC NPs. (means ± SD, n = 3)

Micelle	LMWH: ATRA (w:w)	Average size (nm)	PDI	Zeta potential (mV)	LMWH (w/w) (%)	DOX drug loading capacity (%)
RLA NPs	1:1	precipitate				
	2:1	257.2 ± 13.3	0.422 ± 0.147	-31.1 ± 3.7		
	5:2 (best)	128.9 ± 4.6	0.212 ± 0.078	-31.8 ± 2.0	79.4 ± 3.1	
	3:1	206.2 ± 10.8	0.414 ± 0.113	-32.7 ± 2.7		
RLA/DOX NPs	5:2	177.3 ± 9.5	0.221 ± 0.096	-32.2 ± 0.9		2.9 ± 0.2 (best)
	5:2	248.8 ± 15.7	0.356 ± 0.171	-33.1 ± 1.2		3.8 ± 0.4
RLA/DOX/ αGC NPs	5:2	173.5 ± 9.8	0.251 ± 0.056	-32.1 ± 1.8		2.9 ± 0.3

Table S2. Pharmacokinetic parameters of free DOX and RLA/DOX NPs. (i.v., DOX: 3 mg/kg) (means ± SD, n = 3)

	$t_{1/2}$ (h)	V1 (L/kg)	CL (L/h/kg)	AUC(0-t) (mg/L*h)	AUC(0-∞) (mg/L*h)
Free DOX	0.344 ± 0.051	2.431 ± 0.069	4.607 ± 0.289	0.544 ± 0.042	0.653 ± 0.056
RLA/DOX NPs	4.892 ± 0.310	6.193 ± 0.152	1.005 ± 0.062	2.620 ± 0.118	2.979 ± 0.133

Table S3. Data of the survival test. The data were processed by GraphPad Prism 5. (n = 7)

Group	PBS	LMWH	ATRA	Free DOX	RLA NPs	RLA/DOX NPs	RLA/DOX/ αGC NPs
Earliest time of death (d)	36	42	42	44	46	54	64
Latest time of death (d)	46	58	56	60	64	68	----
Survival at the 80th day	0	0	0	0	0	0	3
Median survival (d)	42	54	48	50	54	66	70

Supplementary figures

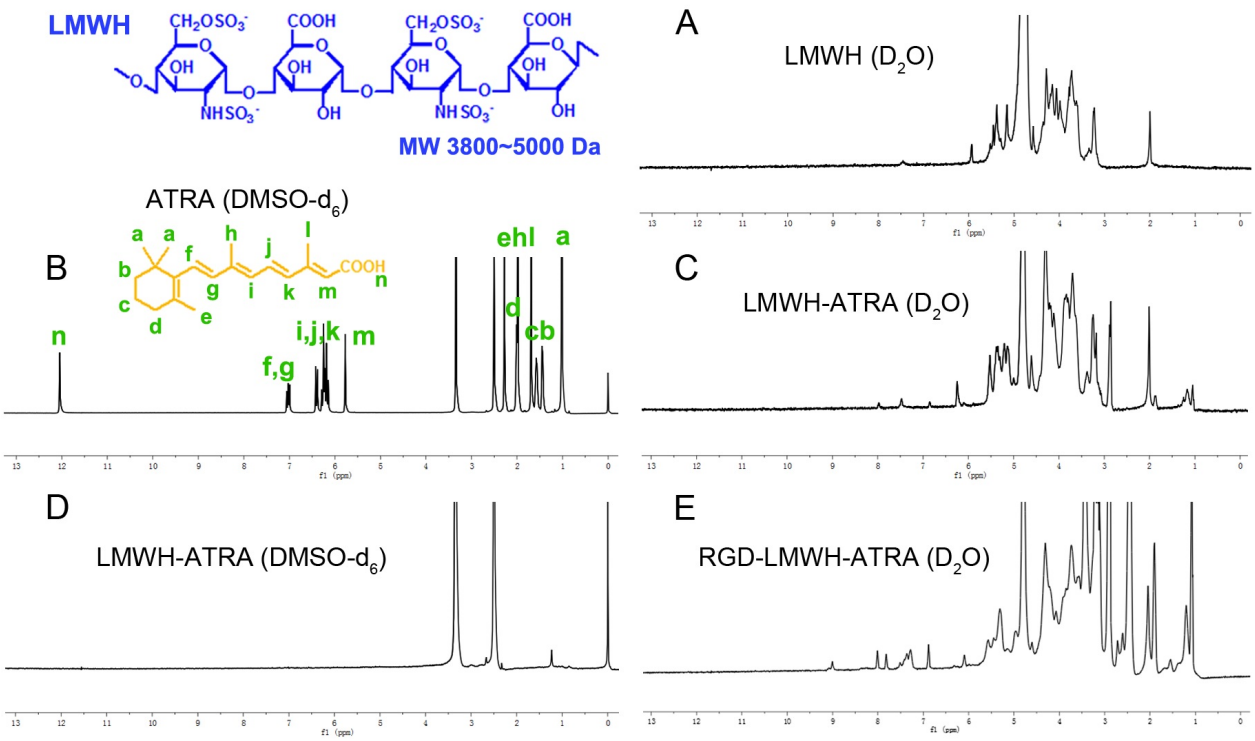

Fig.S1. ^{1}H-NMR spectra of (A) LMWH, (B) ATRA, (C)&(D) LMWH-ATRA and (E) RGD-LMWH-ATRA

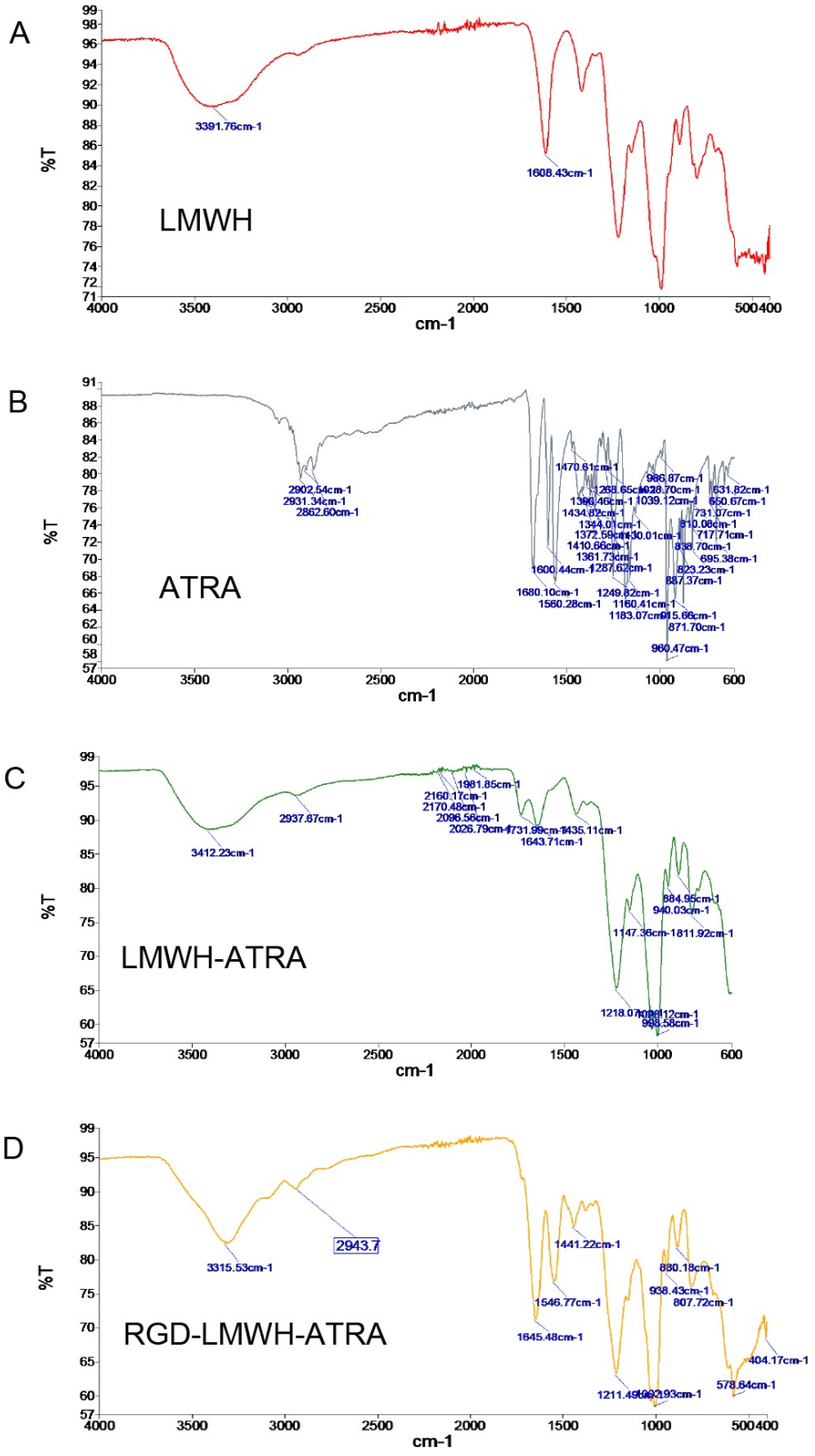

Fig.S2. IR spectra of (A) LMWH, (B) ATRA, (C) LMWH-ATRA and (D) RGD-LMWH-ATRA

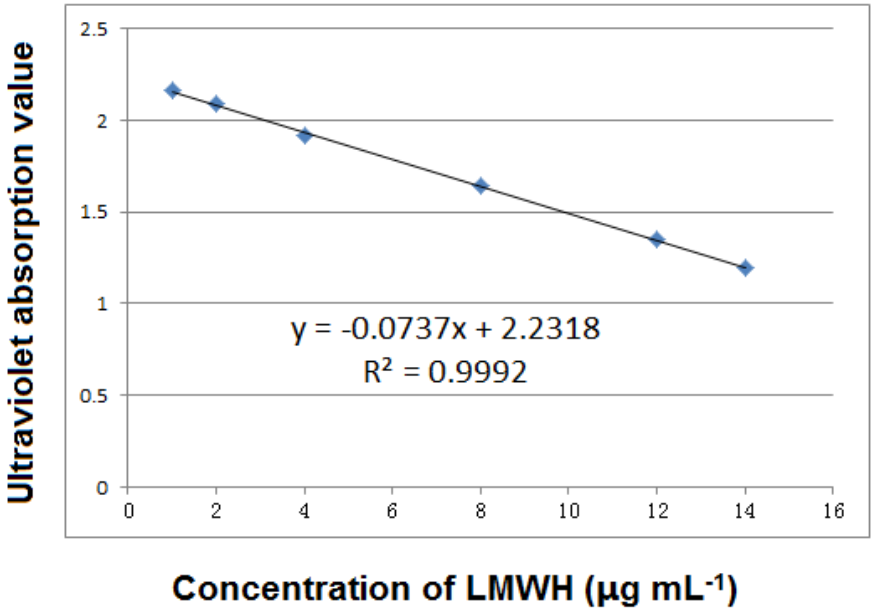

Fig.S3. The standard curve of toluidine blue spectrophotometry

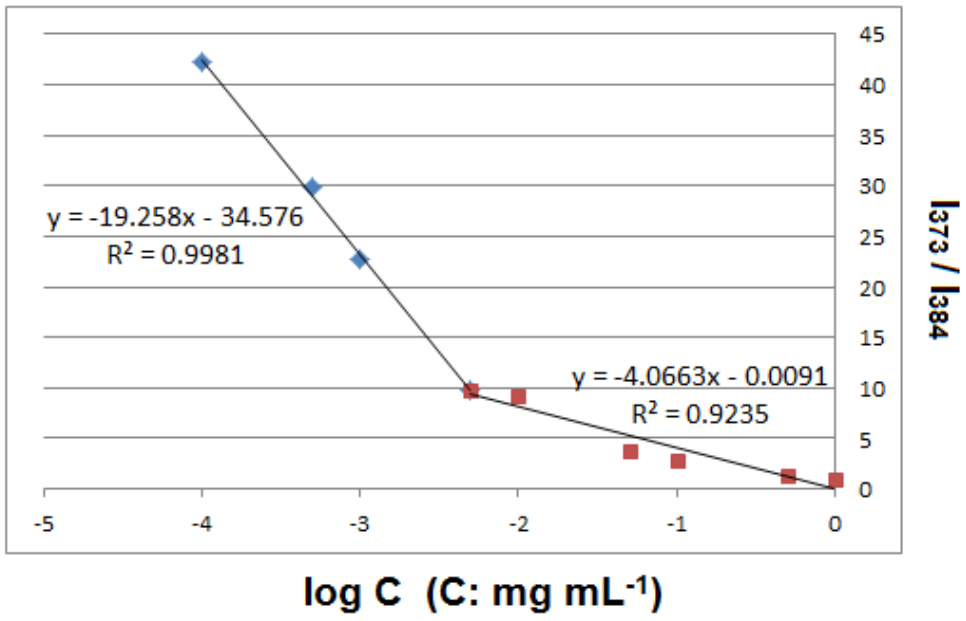

Fig.S4. Determination of the critical micelle concentration of RLA NPs

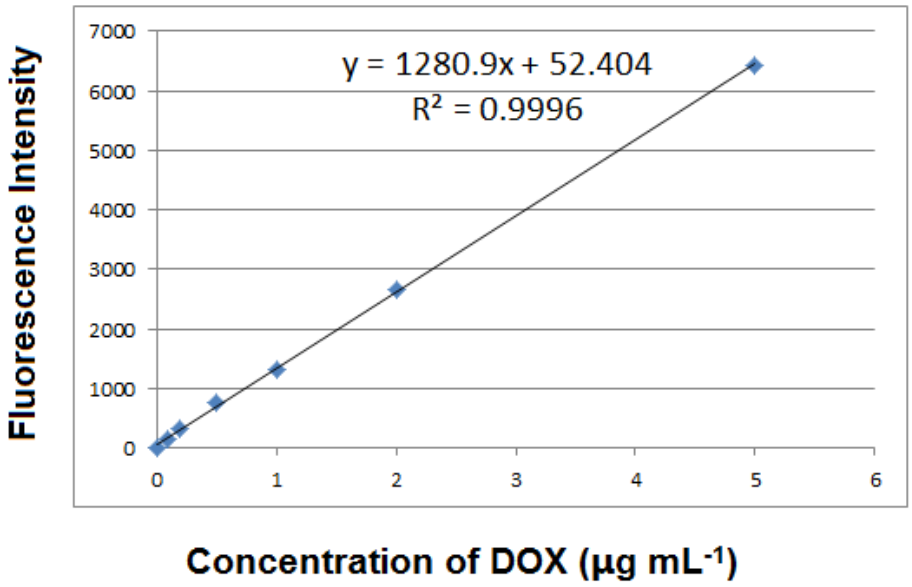

Fig.S5. The standard curve of DOX by fluorescence spectrophotometry

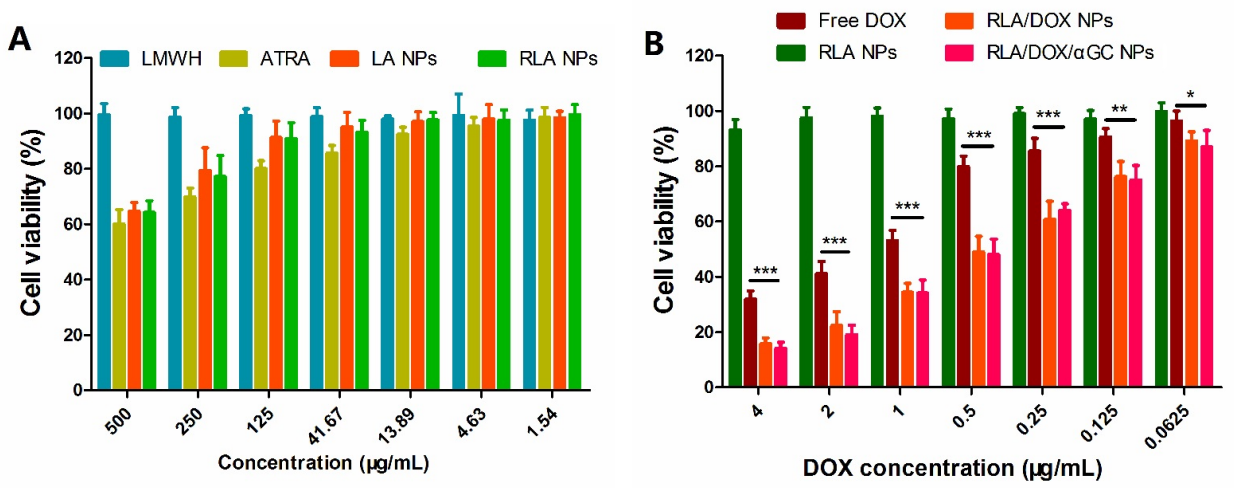

Fig.S6. (A) Cytotoxicity of RLA NPs and (B) RLA/DOX/αGC NPs to HUVECs. (n = 5, means ± SD, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$)

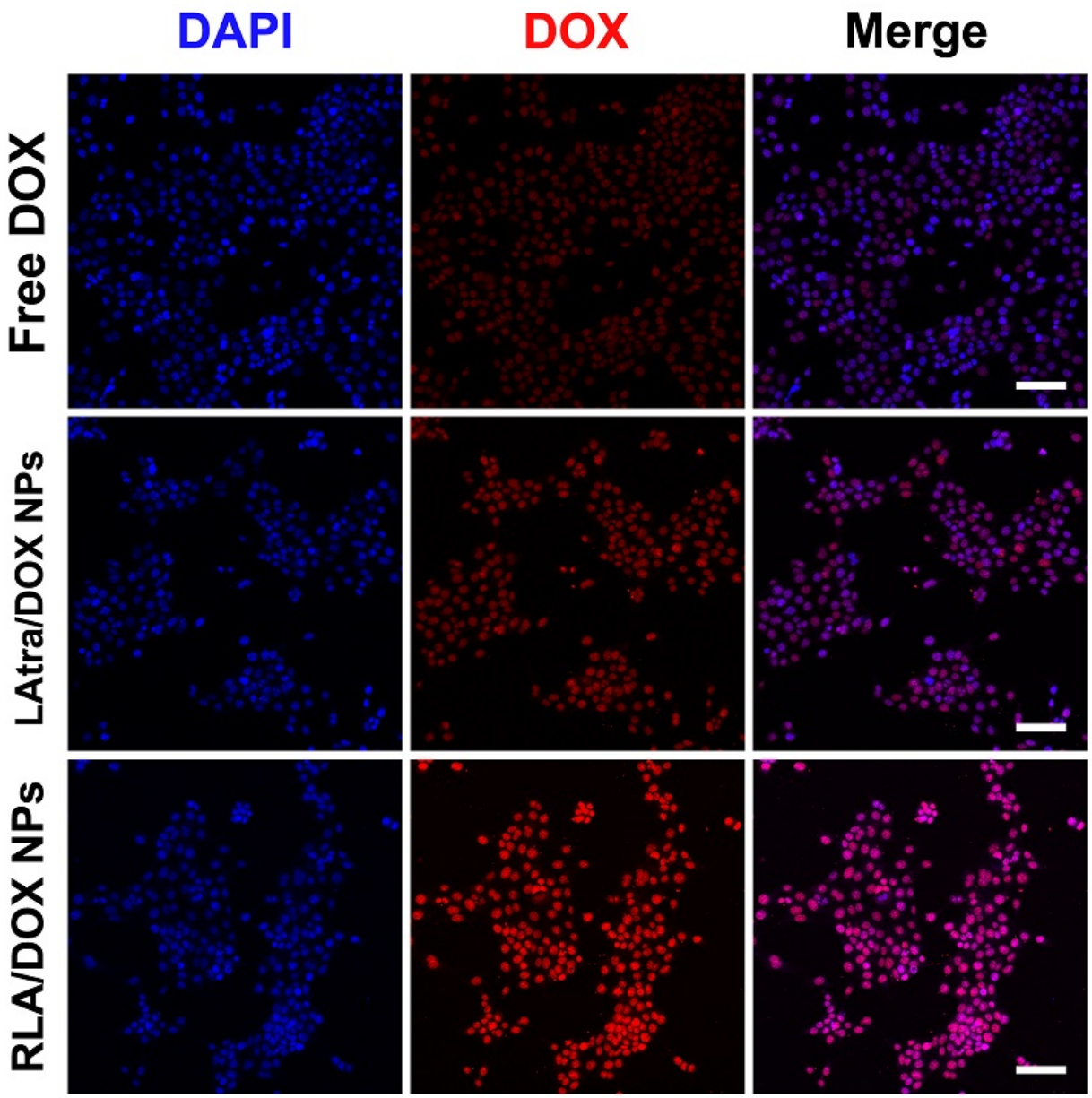

Fig.S7. Qualitative CLSM images of the cellular uptake assay (Showing DOX (red) and DAPI (blue). Scale bars represent 100 μm.)

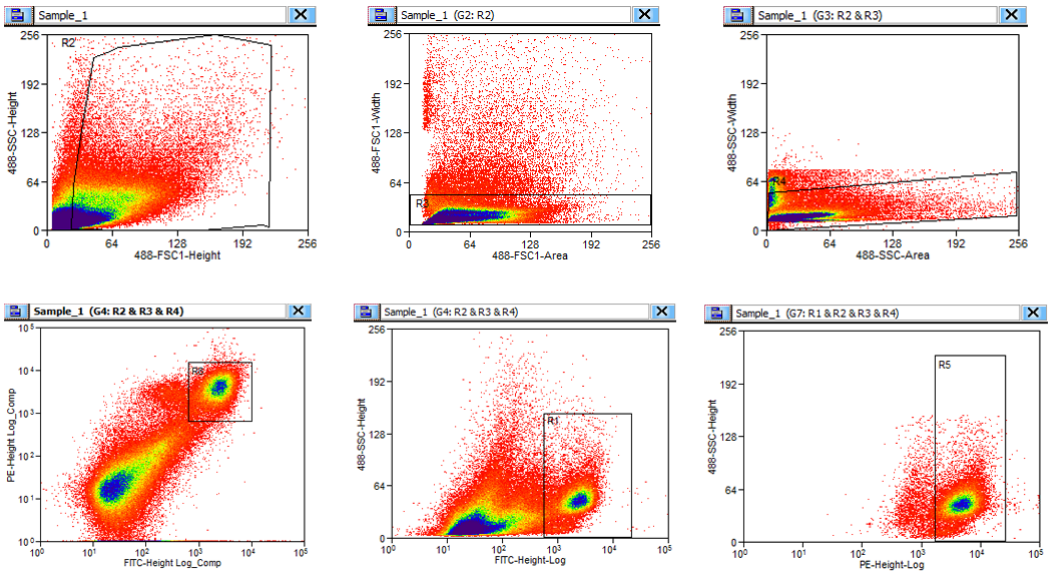

Fig.S8. The flow cytometry raw data of MDSCs isolation

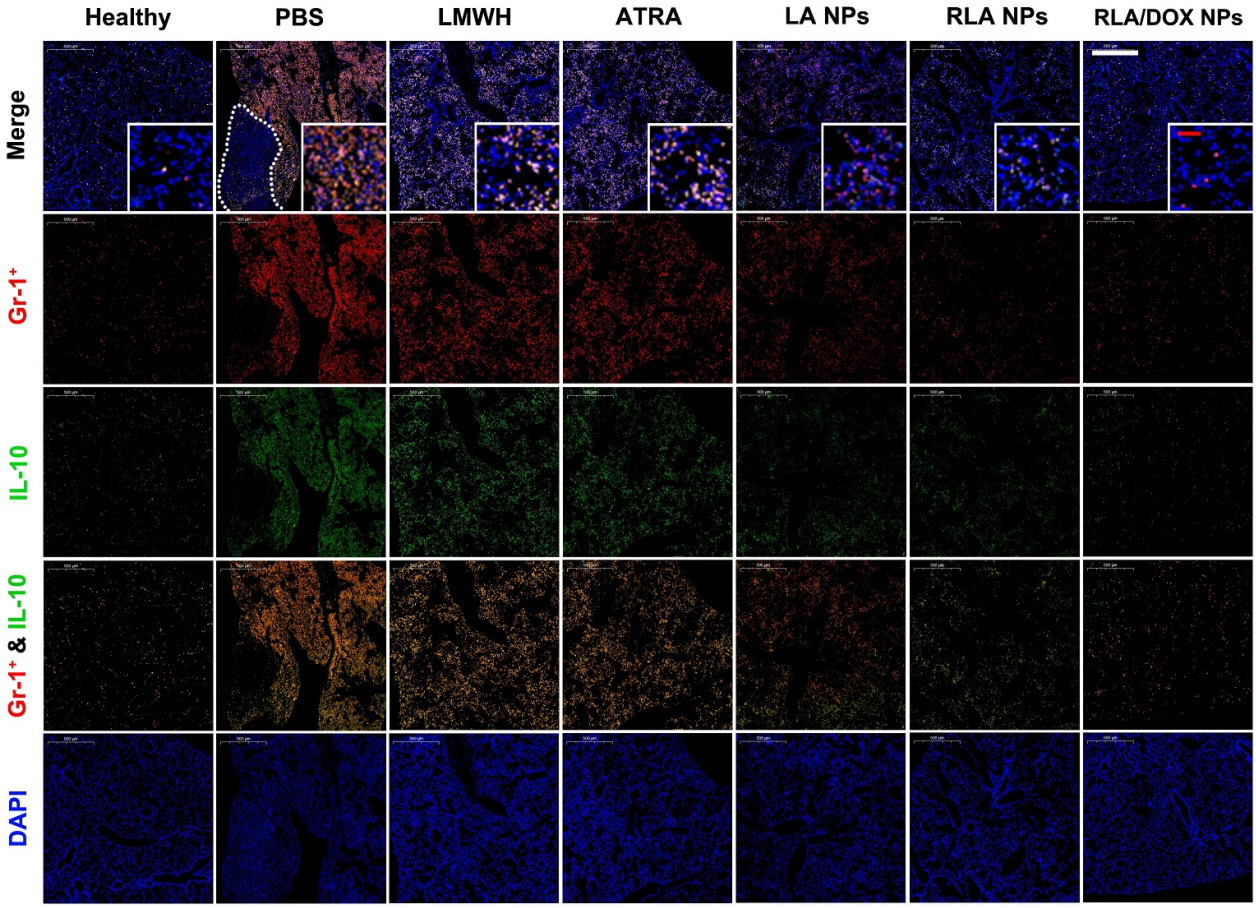

Fig.S9. CLSM images of the distribution of MDSCs and IL-10 in 4T1 tumor-bearing mice (Showing Gr-1 (red), IL-10 (green) and DAPI (blue), white scale bar represents 500 μm, red scale bar represents 50 μm. The circled part was the metastatic nodule.)

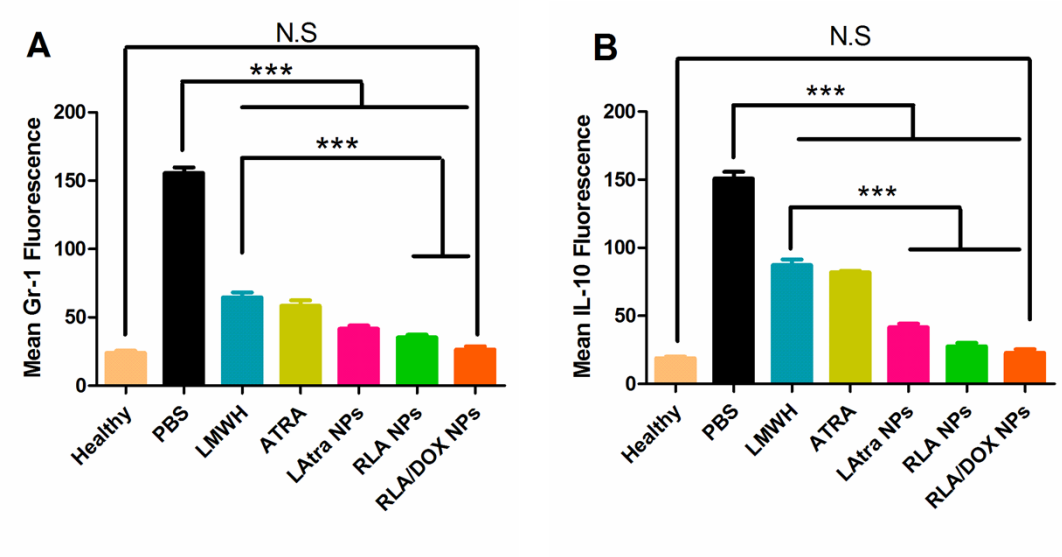

Fig.S10. Semi-quantitative analysis results of (A) Gr-1 and (B) IL-10 fluorescence (n = 3, means ± SD, *** $p < 0.001$)

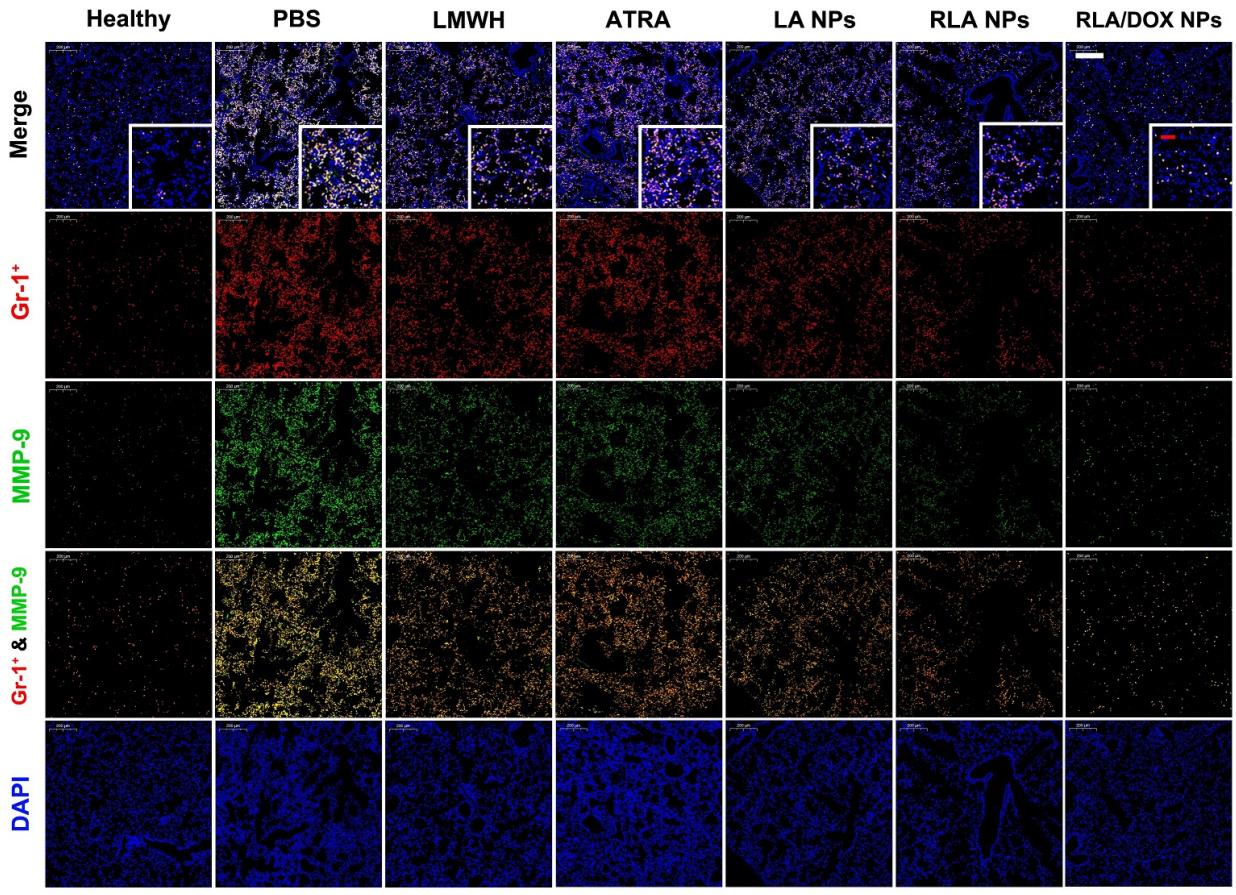

Fig.S11. CLSM images of the distribution of MDSCs and MMP-9 in 4T1 tumor-bearing mice (Showing Gr-1 (red), MMP-9 (green) and DAPI (blue), white scale bar represents 200 μm, red scale bar represents 50 μm)

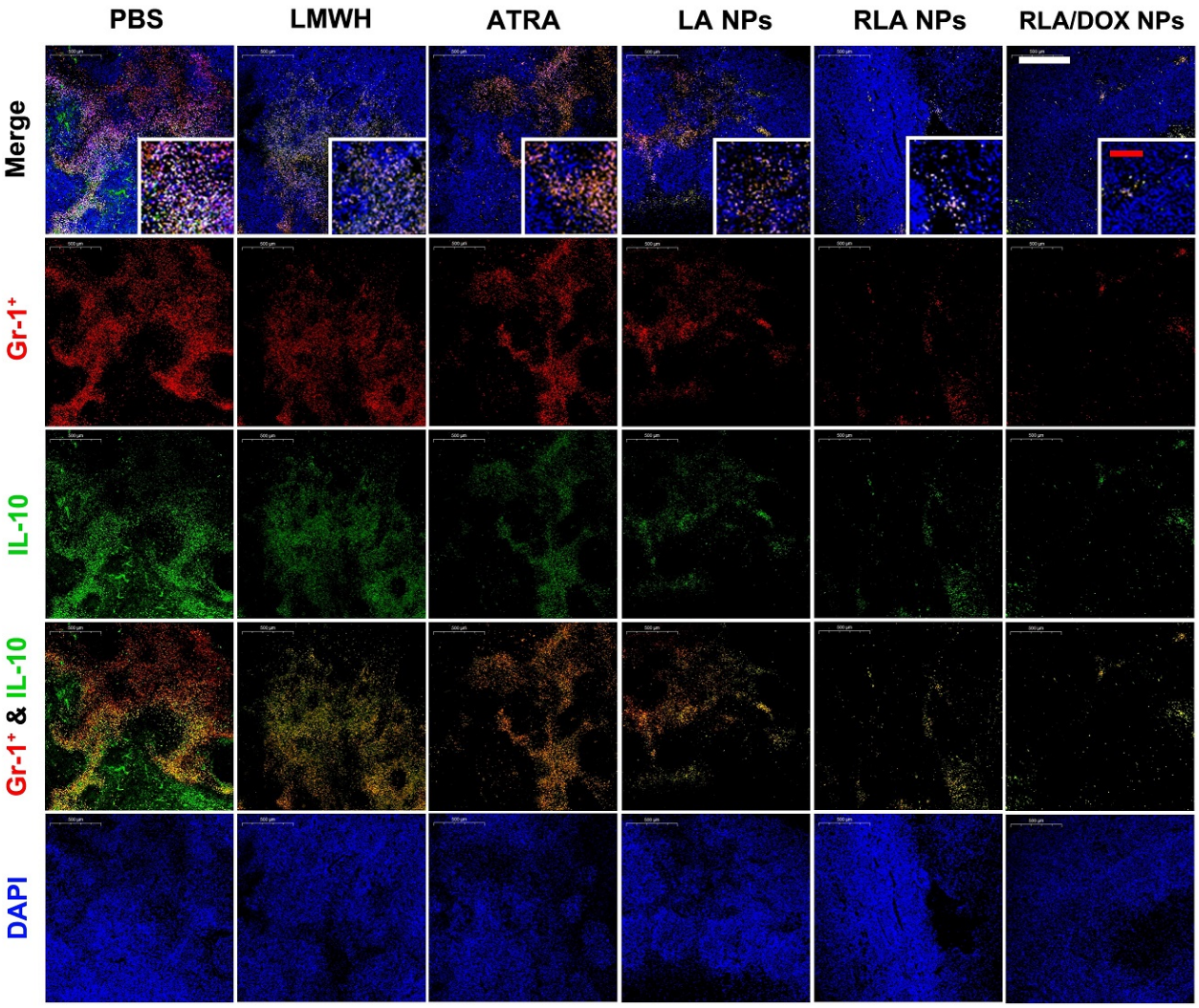

Fig.S12. CLSM images of the distribution of MDSCs and IL-10 in the tumors of 4T1 tumor-bearing mice (Showing Gr-1 (red), IL-10 (green) and DAPI (blue), white scale bar represents 500 μm, red scale bar represents 100 μm)

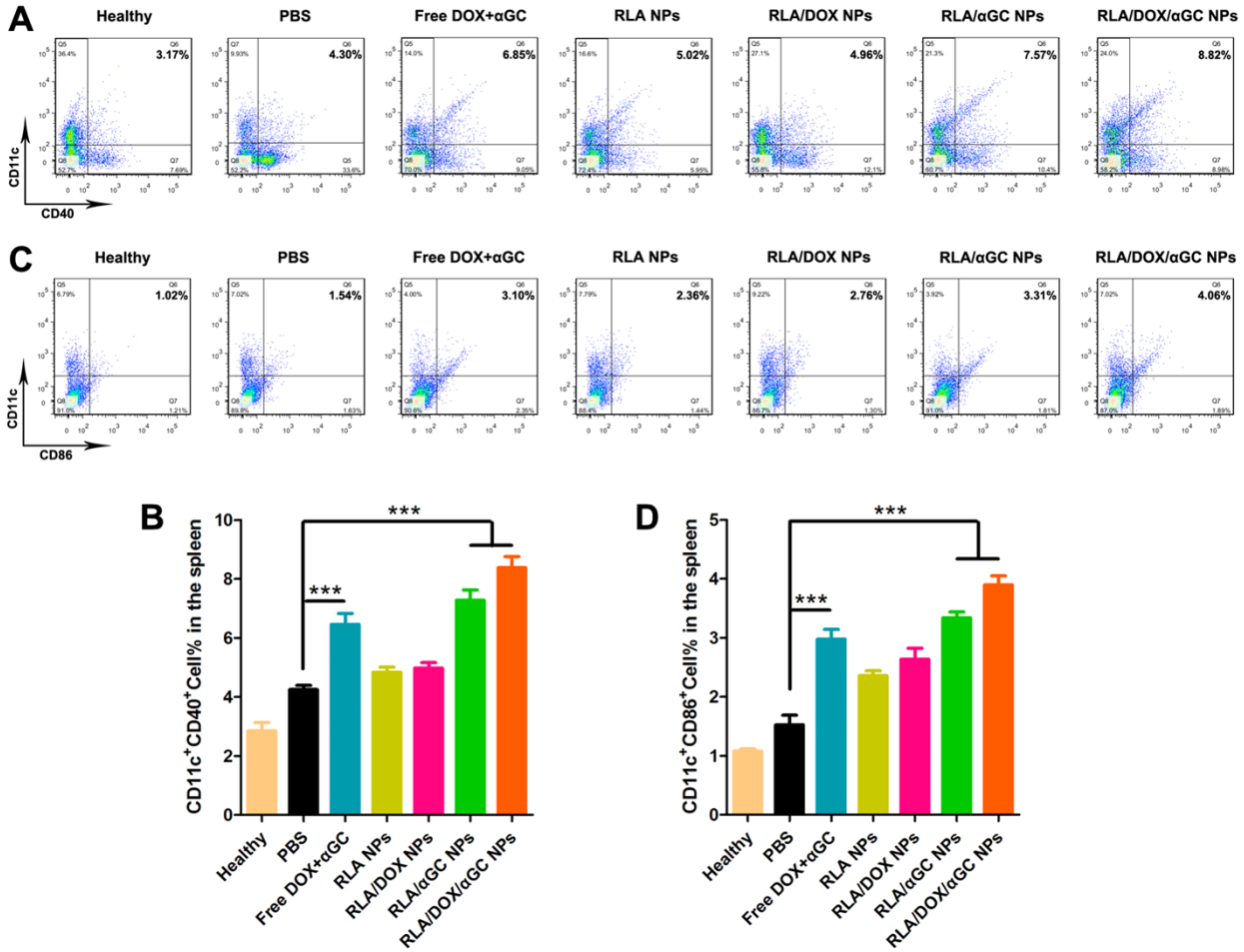

Fig.S13. Flow cytometry results of (A)&(B) CD40⁺ DC and (C)&(D) CD86⁺ DC in spleens of 4T1 tumor-bearing mice (n = 3, means ± SD, *** $p < 0.001$)

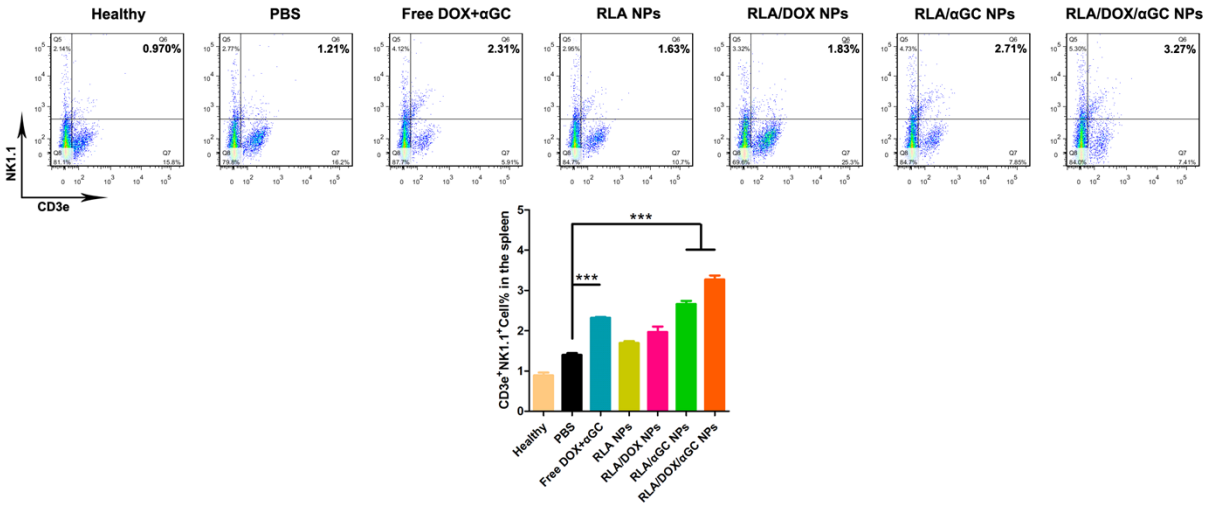

Fig.S14. Flow cytometry results of iNKT cells in spleens of 4T1 tumor-bearing mice (n = 3, means ± SD, *** $p < 0.001$)

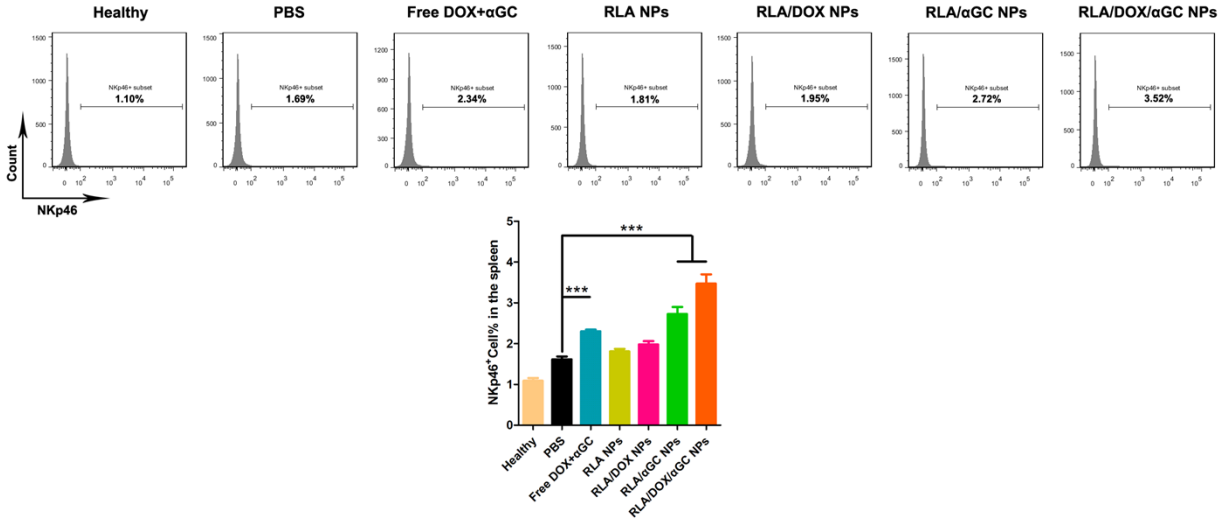

Fig.S15. Flow cytometry results of NK cells in spleens of 4T1 tumor-bearing mice (n = 3, means ± SD, *** $p < 0.001$)

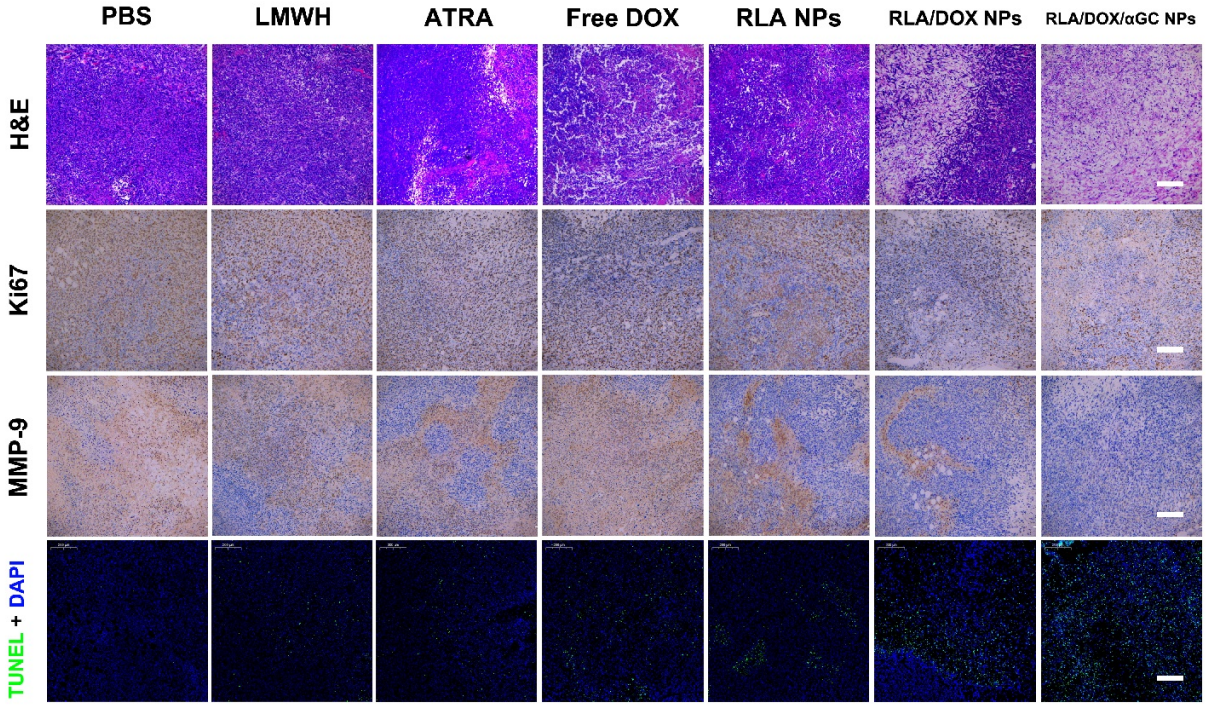

Fig.S16. Immunohistochemical staining results of tumor tissue sections of 4T1-Luc tumor-bearing mice (HE staining: hematoxylin stained nuclei (blue) and eosin stained cytoplasm and extracellular matrix (pink); Ki67 and MMP-9 staining: hematoxylin stained nuclei (blue), Ki67 positive cells (brown) and MMP-9 (brown); TUNEL staining: DAPI stained nuclei (blue) and TUNEL positive cells (green); scale bars represent 200 μm)

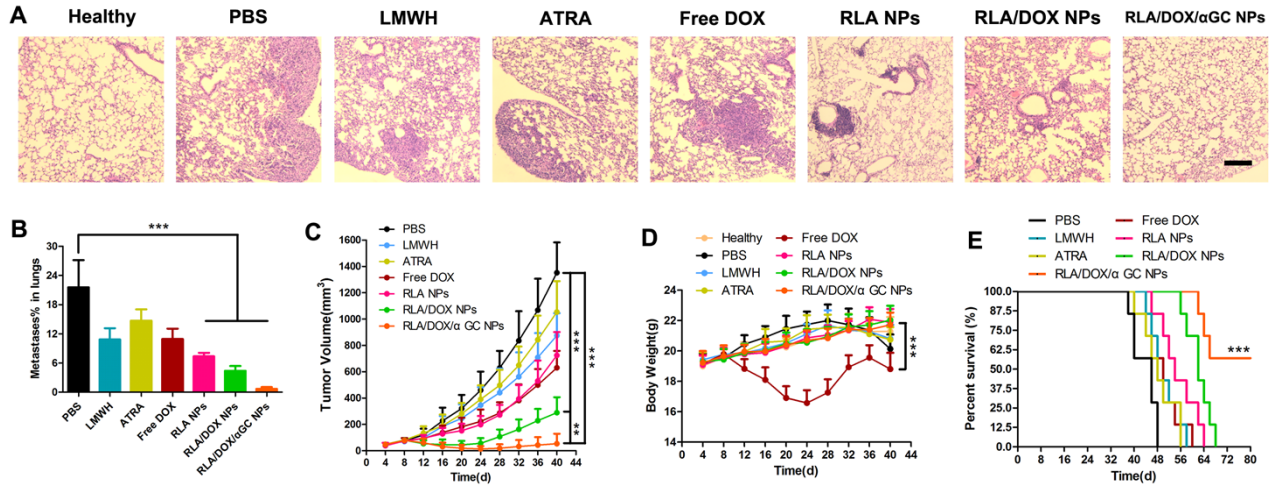

Fig.S17. RLA/DOX/αGC NPs inhibited the progression of EMT-6 breast cancer and pulmonary metastasis. (A) The distribution of metastatic nodules in the lungs of EMT-6 tumor-bearing mice. The dark purple areas in the images were the metastases. Scale bar represents 200 μm. (B) The semi-quantitative results of the metastatic area ratio. (means ± SD, n = 3, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$) (C) Tumor volume curves of mice in the therapeutic experiment. (means ± SD, n = 7, ** $p < 0.01$ and *** $p < 0.001$) (D) Body weight curves of mice in the therapeutic experiment. (means ± SD, n = 7, *** $p < 0.001$) (E) Overall survival curves of mice in the therapeutic experiment. (Log-rank Test, n = 7, *** $p < 0.001$)

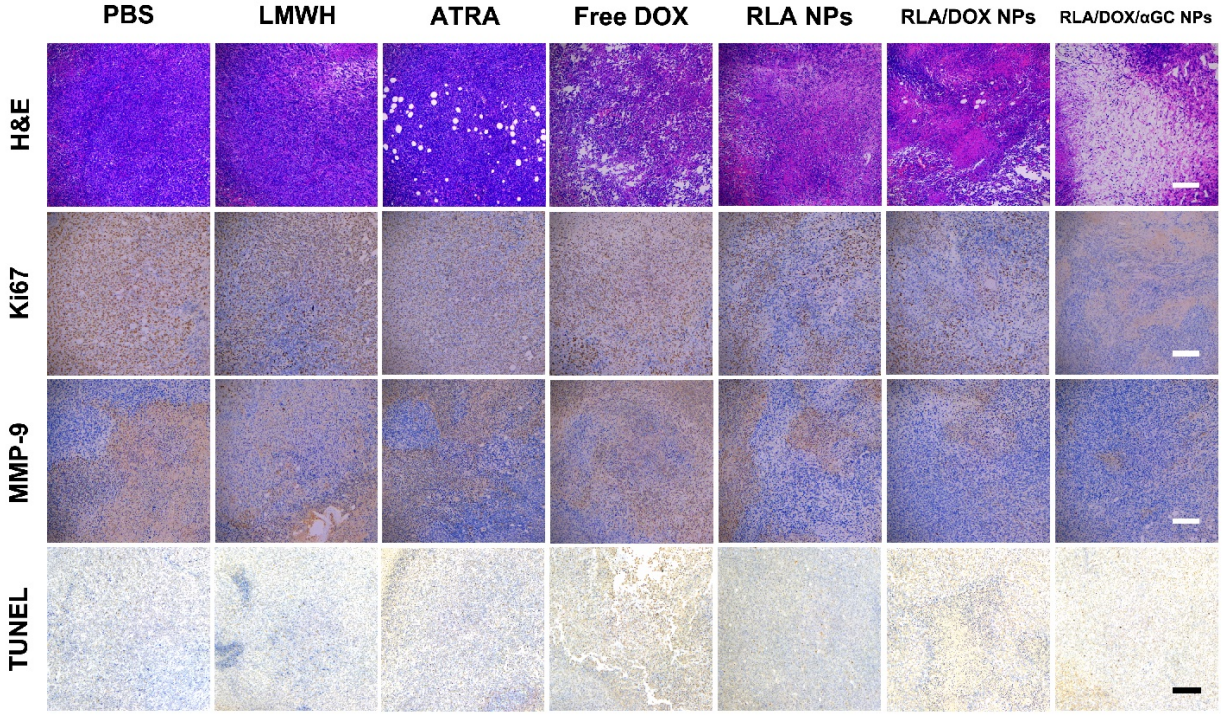

Fig.S18. Immunohistochemical staining results of tumor tissue sections of EMT-6 tumor-bearing mice (HE staining: hematoxylin stained nuclei (blue) and eosin stained cytoplasm and extracellular matrix (pink); Ki67, MMP-9 and TUNEL staining: hematoxylin stained nuclei (blue), Ki67 positive cells (brown), MMP-9 (brown) and TUNEL positive cells (brown); scale bars represent 200 μm)

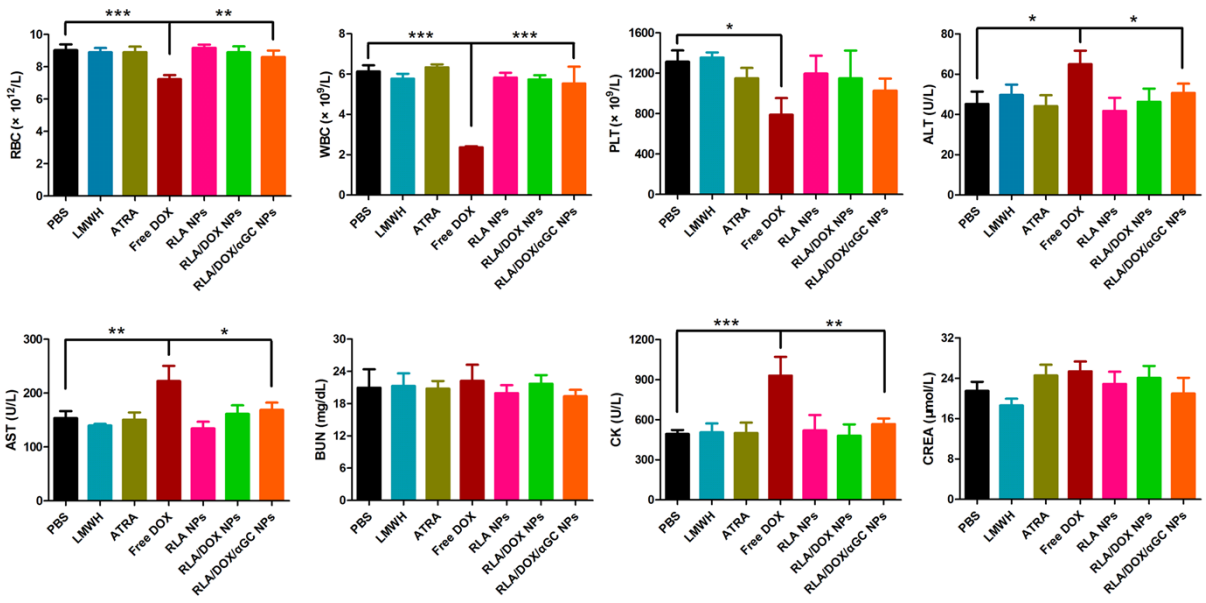

Fig.S19. Blood cells and main serum biochemical indexes in healthy mice after drug administration (RBC: red blood cells; WBC: white blood cells; PLT: platelets; ALT: alanine aminotransferase; AST: glutamic oxaloacetic transaminase; CK: creatine kinase; BUN: urea nitrogen; CREA: creatinine; n = 3, means ± SD, *p < 0.05, **p < 0.01 and *** p < 0.001)

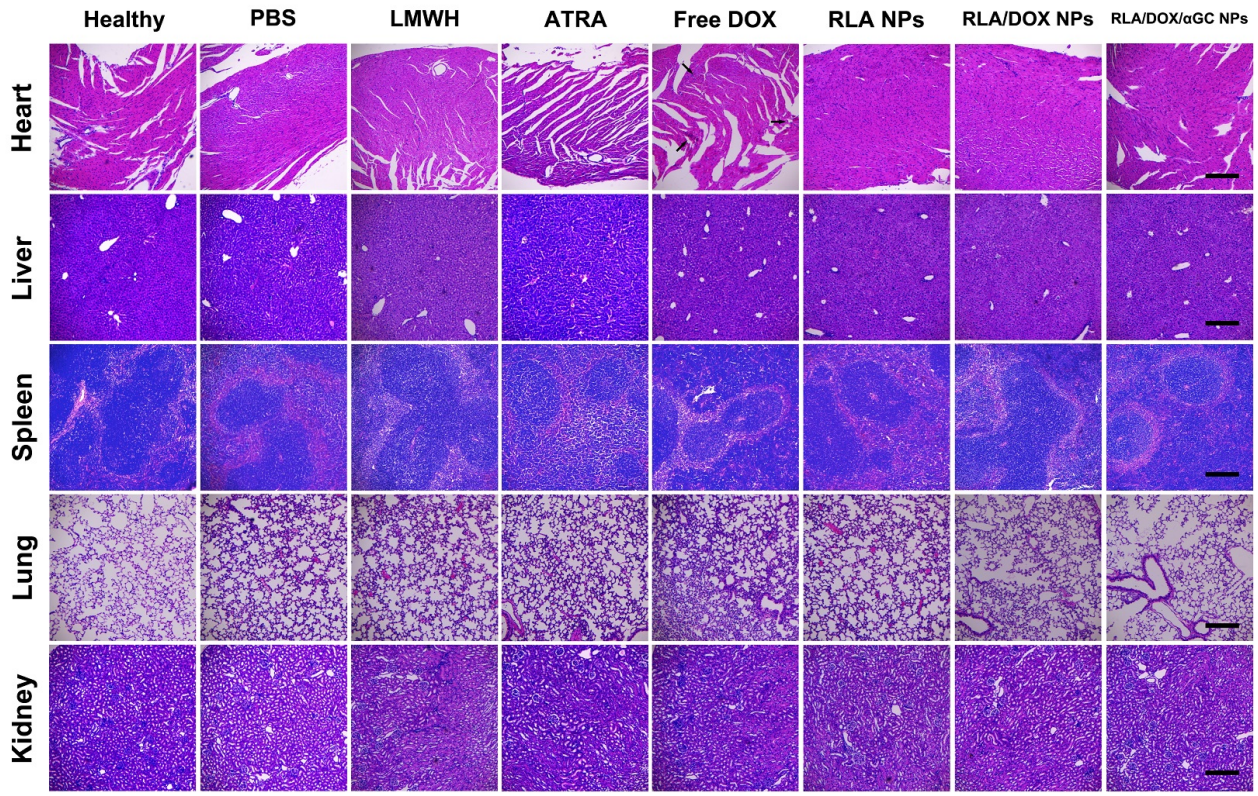

Fig.S20. HE staining results of main organs of healthy mice after drug administration (Black arrows indicate the sites of myocardial damages, scale bars represent 200 μm)