Supplementary information

GRP78-targeted ferritin nanocaged ultra-high dose of doxorubicin for hepatocellular carcinoma therapy

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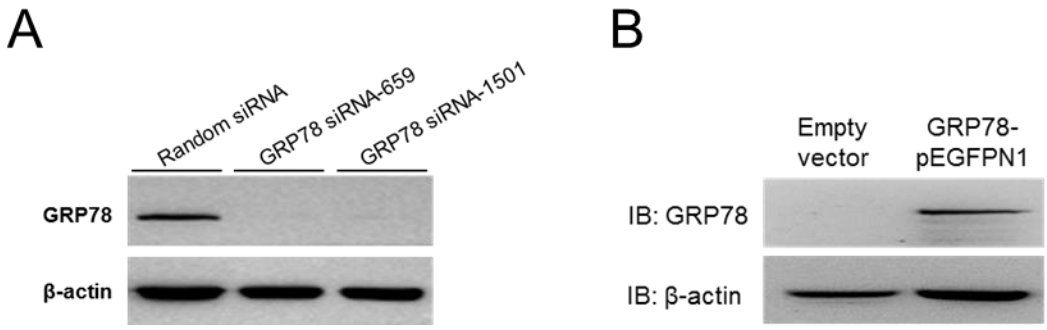

Figure S1 (A) Knockdown of GRP78 via the introduction of specific small interfering RNA (siRNA) (GRP78 siRNA-659, GRP78 siRNA-1501) into HepG2 cells through infection with viral vectors. The Random siRNA was used as a negative control. (B) Overexpression of GRP78 via the infection with GRP78-pEGFPN1 vector into 3T3 cells. Empty pEGFPN1 vector was used as a control.

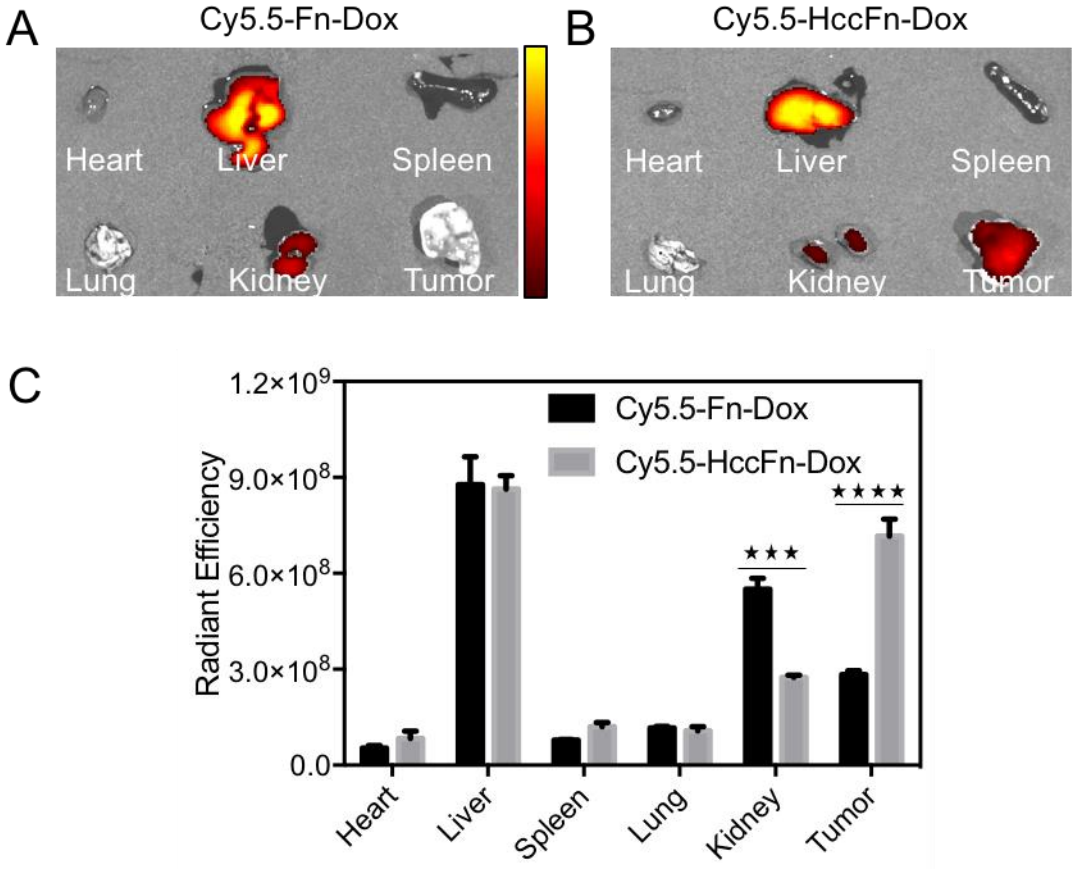

Figure S2 Tissue biodistribution of Cy5.5-HccFn-Dox nanocages in mice bearing subcutaneous HCC tumors. (A-B) Ex vivo imaging of the main organs and tumor of mice by measuring the Cy5.5 fluorescence signal after intravenous injection of Cy5.5-Fn-Dox (A) or Cy5.5-HccFn-Dox (B) at 4h. (C) Quantitative analysis of the Cy5.5 fluorescence signals of the main organs of mice after intravenous injection of Cy5.5-HccFn-Dox or Cy5.5-Fn-Dox at 4h.

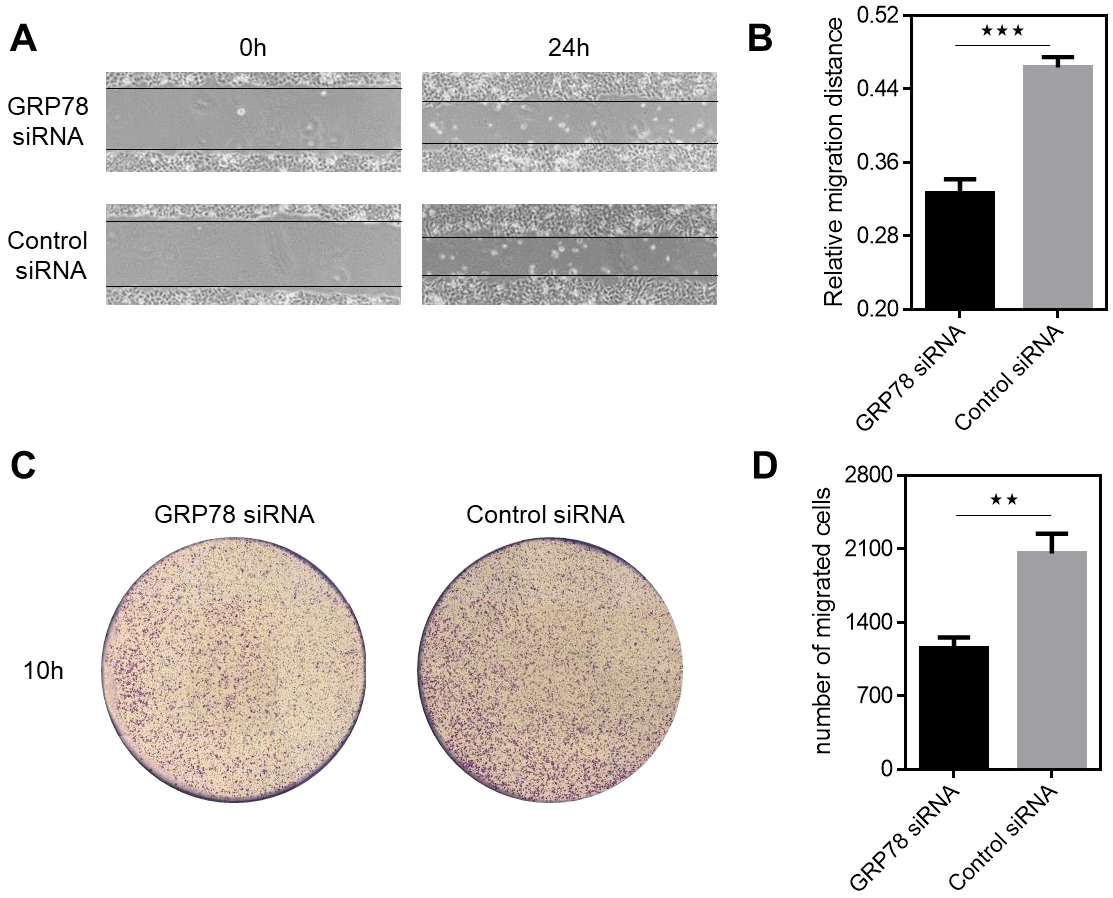

Figure S3 Knockdown of GRP78 suppresses the migration of HCC cells. GRP78 siRNA was transfected into HepG2 cells to knockdown GRP78, a random siRNA was used as control. (A) Wound healing analysis of the migration activities of HepG2 cell transfected with GRP78 siRNA or control siRNA in 24h. (B) Relative migration distance analysis. The migration distance was significantly decreased in GRP78 siRNA group compared to control siRNA group (Mean ± SD, n=3. ***p<0.001, unpaired t test,). (C) Panorama view of Transwell analysis of the migration activities of HepG2 cells transfected with GRP78 siRNA or control siRNA in 10h. (D) Quantitative statistics of transwell migrated cells. The number of migrated cells was significantly decreased in GRP78 siRNA group compared to control siRNA group (Mean ± SD, n=3. **p<0.01, unpaired t test,). All experiments were repeated three times and data represent the means ± SD of triplicates.

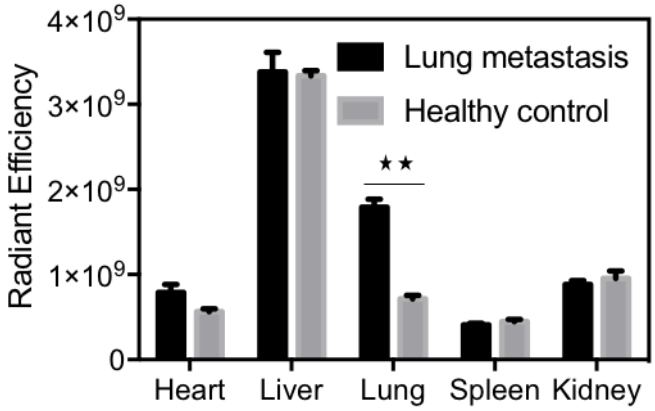

Figure S4 Tissue biodistribution of Cy5.5-HccFn-Dox nanocages in a HCC lung metastases mouse model. Quantitative analysis of the Cy5.5 fluorescence signals of the main organs of lung metastasis mice and healthy control mice after intravenous injection of Cy5.5-HccFn-Dox at 4h. The concentration of HccFn-Dox nanocages in HCC metastatic lung was significantly higher than that of healthy control lungs (Mean ± SD, n=3. **p<0.01, unpaired t test). All experiments were repeated for three times and data represent the means ± SD of triplicates.

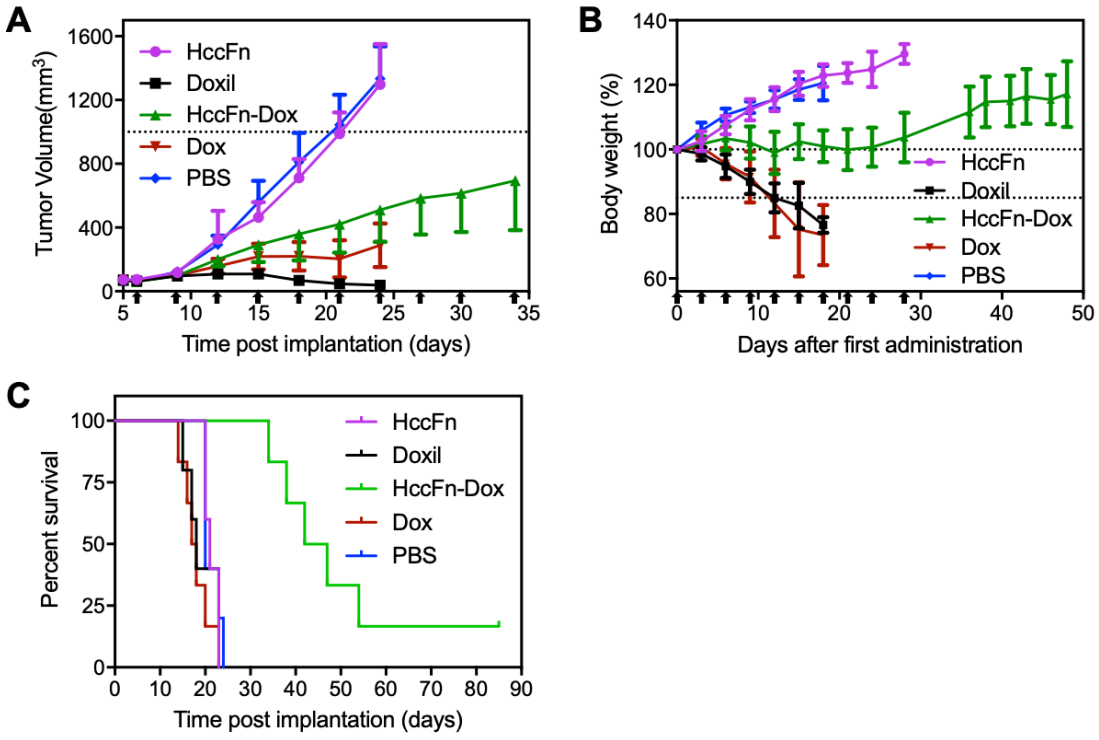

Figure S5 Multiple dose toxicity test of HccFn-Dox. HepG2 tumor cells were implanted subcutaneously into mice on day 0. Mice were intravenously administrated with HccFn-Dox (5 mg/kg Dox equivalents, n=6), and control substances such as Doxil (5 mg/kg Dox equivalents, n=5), Dox (5 mg/kg Dox equivalents, n=6), HccFn (12 mg/kg, n=5), or PBS (n=5) on day 6, 9, 12, 15, 18, 21, 24, 27, 30, 34. Tumor volume (A), Body weight (B) and survival time (C) were recorded. Black arrows indicated the administration time.

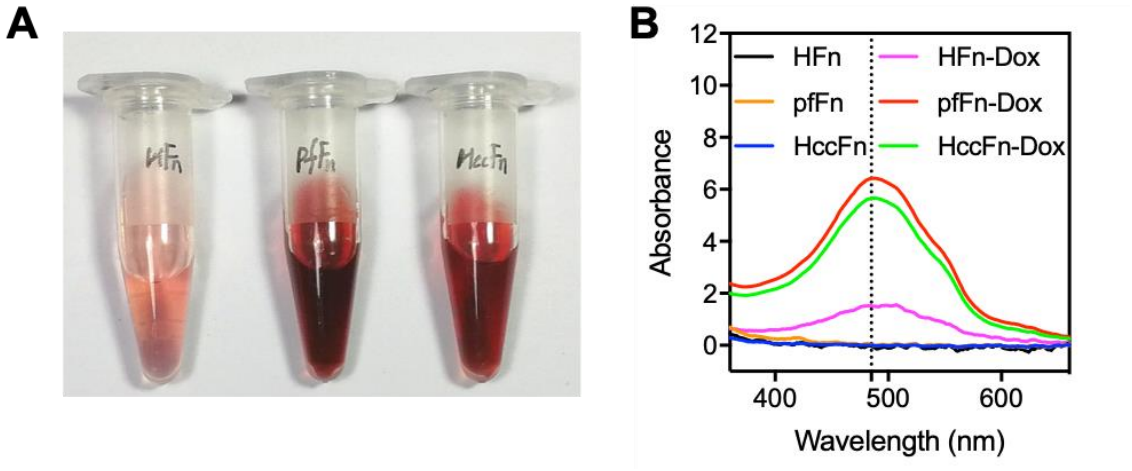

Figure S6 (A) Images of HFn-Dox (human heavy-chain ferritin), pfFn-Dox (*Pyrococcus furiosus* ferritin) and HccFn-Dox in PBS buffer, the protein concentration is 0.75 mg/ml. (B) UV-spectra of HFn, HFn-Dox, pfFn, pfFn-Dox, HccFn, HccFn-Dox in PBS. The maximum absorption wavelength of Dox is 485nm in PBS. The protein concentration of pfFn, pfFn-Dox, HccFn and HccFn-Dox are 0.75 mg/ml. The protein concentration of HFn and HFn-Dox are 2.25 mg/ml.

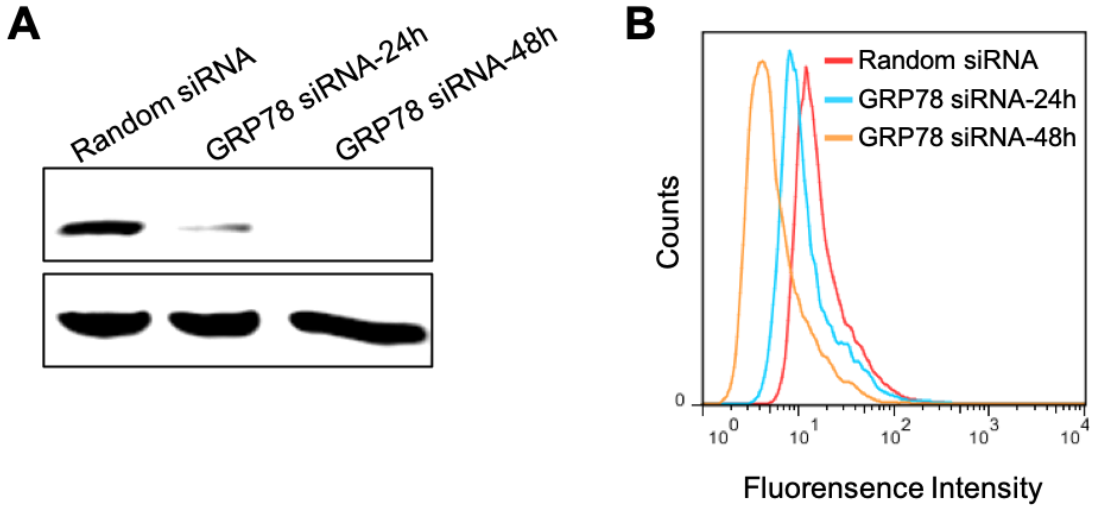

Figure S7 GRP78 gene knockdown reduces the uptake of HccFn nanocarriers. (A) Western blot analysis of the knock-down efficiency of GRP78-specific siRNA in HepG2 cells after transection for 24h and 48h. (B) Flow cytometry analysis of cellular uptake of FITC-HccFn nanocarriers in GRP78 gene knockdown HepG2 cells.

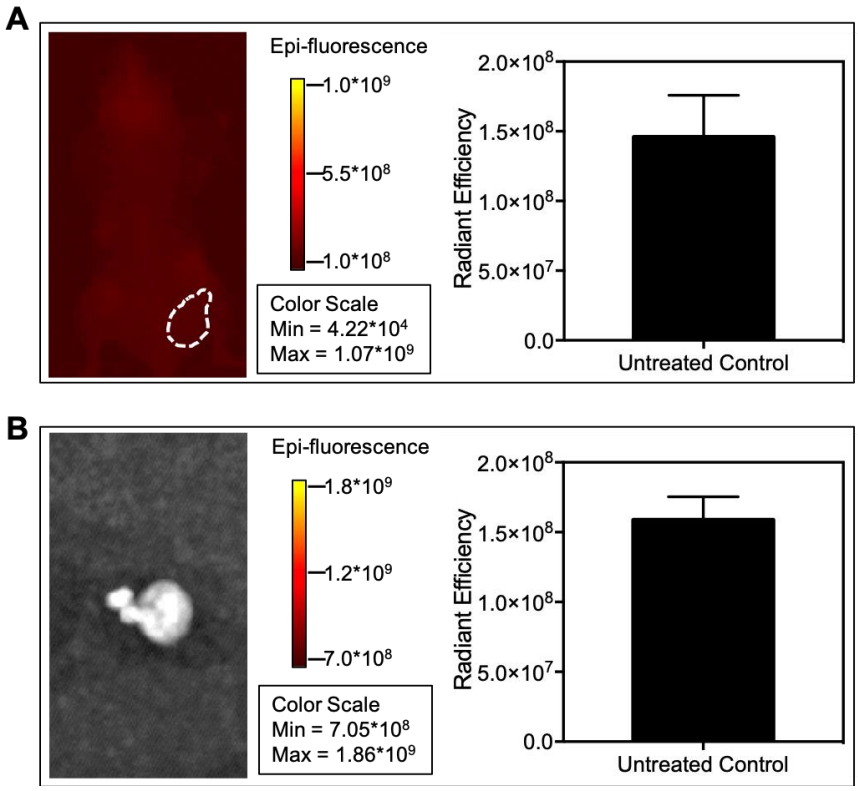

Figure S8 In vivo (A) and ex vivo (B) NIRF image of untreated control mouse and tumor. The white circle indicates the position of tumor in mice. Cy5.5 signals in the tumor area were quantitatively analyzed (Right panel, Mean ± SD, n=3).

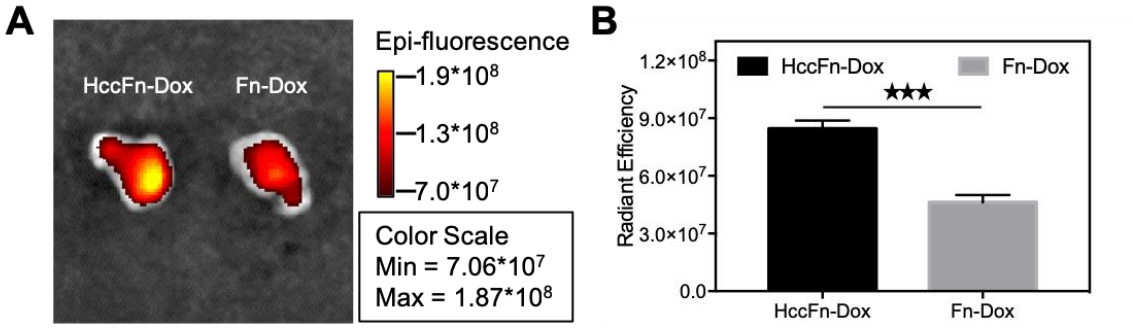

Figure S9 (A) Ex vivo NIRF imaging of the HepG2 tumors pre-treated i.v. with HccFn-Dox and Fn-Dox. (B) Dox signals in the tumor area were quantitatively analyzed, Mean ± SD, n=3 , ****p<0.0001, unpaired Student's t-test.

A

Cinical chemistry parameters	1 week after treatment			3 week after treatment		
	PBS	HccFn	HccFn-Dox	PBS	HccFn	HccFn-Dox
ALT(U/L)	42.97±4.78	61.90±29.06	46.56±6.01	43.20±1.91	59.95±20.41	64.67±21.16
AST(U/L)	101.30±36.76	124.50±51.79	136.40±34.30	163.07±37.97	225.75±55.06	156.20±21.21
CREA-J(μmol/L)	49.77±0.78	49.40±2.18	52.64±1.76★	49.53±3.48	48.95±6.24	52.44±6.08
UREA(mmol/L)	3.25±0.25	5.21±1.40	5.90±0.57★	7.74±1.32	8.66±1.75	9.45±1.79

B

Blood routine parameters	1 week after treatment			3 week after treatment		
	PBS	HccFn	HccFn-Dox	PBS	HccFn	HccFn-Dox
WBC	8.13±1.10	7.18±1,80	5.68±0.87★	6.57±2.74	6.83±1.39	7.40±1.75
RBC	9.09±0.32	8.00±1.83	8.51±0.74	9.15±0.48	9.57±0.45	9.14±0.89
HGB	166.67±8.62	144.75±33.98	157.00±14.35	168.00±7.94	173.50±3.79	163.60±11.59
HCT	49.23±1.99	43.88±10.67	46.32±3.64	48.43±2.51	51.15±1.35	48.56±4.57
MCV	54.10±0.44	54.68±1.61	54.46±0.55	52.93±0.32	53.40±2.01	53.10±0.84
MCH	18.30±0.44	18.05±0.25	18.46±0.58	18.40±0.61	18.13±0.67	17.92±0.73
MCHC	338.67±8.50	330.50±5.97	338.80±12.68	347.00-±9.64	339.50±9.29	337.80±14.65
PLT	483.00±12.12	450.50±47.28	542.40±25.60★	346.00±67.73	320.75±44.22	344.00±42.64
PCT	0.19±0.02	0.18±0.01	0.20±0.01	0.14±0.03	0.12±0.02	0.13±0.02
MPV	3.93±0.21	3.98±0.21	3.74±0.05	4.07±0.15	3.88±0.22	3.86±0.15
PDW	15.67±0.71	15.23±0.46	15.08±0.29	16.00±0.61	15.83±0.70	15.64±0.38
LYM	4.93±0.80	4.50±1.31	3.58±0.34★	4.03±1.47	4.08±0.93	4.38±1.31
MID	1.43±0.06	0.88±0.21★	0.74±0.18★	1.60±0.95	1.48±0.22	1.60±0.45
LYM%	60.47±5.75	62.18±7.23	63.58±3.95	61.90±3.30	59.45±3.85	58.98±5.23
MID%	17.83±3.04	12.50±1.80★	12.48±1.67★	23.23±4.42	22.13±3.43	21.30±2.93

Table S1 Clinical chemistry parameters (A) and blood routine parameters (B) of healthy mice treated with HccFn-Dox, HccFn, or PBS. Plasma samples were obtained 1 week and 3 weeks after treatment. ALT, alanine aminotransferase; AST, aspartate aminotransferase; CREA-J, creatinine; UREA, urea; WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, Platelets; PCT, thrombocytocrit; MPV, mean platelet volume; PDW, platelet distribution width; LYM, lymphocyte; MID, intermediate cell.