Supplementary information

Role of the complement cascade on the biological fate of liposomes in rodents

Iara Maíra de Oliveira Viana,[a,b] Philippe Grenier, [b] Joan Defrene,[c] Frédéric Barabé,[c] Eliana Martins Lima *[a] and Nicolas Bertrand *[b]

[a] Laboratory of Pharmaceutical Technology (FarmaTec), Federal University of Goiás, Goiânia, 74605-220 Goiás, Brazil.

[b] Faculty of Pharmacy, Université Laval and CHU de Québec-Université Laval Research Center, 2705 Laurier Blvd, Québec, Canada G1V 4G2.

[c] Department of Medicine, Faculty of Medicine, Université Laval and CHU de Québec-Université Laval Research Center, 2705 Laurier Blvd, Québec, Canada G1V 4G2

*** Correspondence to:**

Eliana Martins Lima, emlima@ufg.br
FarmaTec, Federal University of Goiás, Goiânia, 74605-220 Goiás, Brazil.

Nicolas Bertrand, nicolas.bertrand@pha.ulaval.ca
2705 Laurier Blvd, Room T4-13, Quebec City, QC, Canada, G1V 4G2.

Supplementary Materials and Methods

Transmission Electron Microscopy (TEM)

Liposomes diluted in HEPES (1:3) were deposited on formvar-coated 300 mesh copper grids and stained by uranyl acetate solution 2% as described by Adair and Yearger [1]. Samples were visualized in a Jeol JEM-2100 microscope equipped with EDS (Thermo Scientific, Tokyo, Japan).

***In vitro* complement activation**

To assess terminal complement complex (C5b-9 or SC5b-9), 20 µL of liposomes (~30 mM of total phosphorus) were incubated in 40 µL of serum (mice or human) for 1 hour at 37 °C. Sterilized PBS solution (4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) and 5 mg mL^{-1} of Zymosan A were used as controls. Fourty µL of 25 mM EDTA solution were added to stop complement activation. All samples were frozen at -20 °C until further use. Terminal complement complex was assessed by ELISA (C5b-9 Mouse ELISA kit, LS-F22262, LSBio, USA and SC5b-9 Human ELISA kit, MicroVue™ SC5b-9 Plus kit, Quidel, USA).

Adjustment of rabbit red blood cells (RBC)

Rabbit RBC (Complement Technology, USA or *"Costa e Melo Produtos Agropecuários"*, Brazil) were separated from free hemoglobin at 1000 *g* for 3 min and resuspended in buffer (gelatin veronal buffered saline without calcium and magnesium, GVB^0 buffer, Complement Technology, USA; or HEPES buffer solution at 300 mOsm L^{-1}). Twenty µL of resuspended RBC were diluted in 980 µL of water; the optical density (O.D.) of this hemolyzed solution was read at 412 nm in microplate reader (Tecan infinite 200 pro, Switzerland; or Multiskan Spectrum, Thermo Scientific, Finland). Concentration of RBC suspension was adjusted, knowing that an O.D. of 0.6284 corresponded to 5 x 10^8 cells mL^{-1}.

Influence of complement cascade in release of hemoglobin

For complement activation in mouse serum, 25 µL of RBC at 5 x 108 cells mL^{-1} were added to 20 µL of mouse serum diluted in GVB with Ca^{2+} and Mg^{2+} in ratio 1:1 (v/v). In sequence, the RBC in mouse serum were diluted with 55 µL of GVB with Ca^{2+} and Mg^{2+} or with 55 µL of GVB buffer with 10 mM EDTA. The samples were incubated for 30 min at 37 °C, under 50 rpm in an orbital shaker. Five hundred µL of cold GVB containing 10 mM EDTA were used to stop complement cascade. Samples were centrifuged at 1000 *g* for 10 min at 4 °C, and the supernatant was read at 412 nm in microplate reader (*Sample absorbance*). The absorbance of the RBC in water was assumed as total lysis (*Absorbance of 100% Lysis*). The absorbance of serum diluted in GVB with Ca^{2+} and Mg^{2+} and water (ratio 1:1:8, v/v/v) was subtracted from all samples (*Serum background*). Release of hemoglobin was calculated by the following equation:

Equation S1:
$$Release\ of\ hemoglobin\ (\%) = \frac{(Sample\ absorbance - Serum\ background)}{Absorbance\ of\ 100\%\ Lysis}\ x\ 100$$

For complement activation in rat and human sera, 100 µL of RBC at ~5 x 10^8 cells mL^{-1} were incubated in rat or human sera, with or without EDTA, in 1:1 v/v ratio, for 1 hour at 37 °C. RBC were incubated in HEPES, with and without EDTA, and used as control of the procedure. Two hundred µL of cold EDTA solution (20 mM) were used to stop complement cascade. All samples were centrifuged at 20000 *g* for 3 min at 24 °C. Supernatant was read at 412 nm in microplate reader (*Sample absorbance*). The absorbance given by 100 µL of RBC in 300 µL of 10% p/v Triton-X was used as 100% lysis (*Absorbance of 100% Lysis*). The serum background was given by absorbance of serum diluted in HEPES and 20 mM EDTA solution 1:1:2 v/v/v in ratio. Release of hemoglobin was calculated as showed above.

Ex vivo complement activation

Mice and rats were treated previously with CVF or PBS intraperitoneally as described in *Pharmacokinetics and biodistribution in rodents (main manuscript)*. Blood samples were collected, and centrifuged at 2000 *g* for 10 min at 4 °C. The residual ability to activate complement cascade was assessed *in vitro*.

To check complement activity of mice serum, 5 mg mL^{-1} Zymosan A were incubated in serum, 1:2 v/v in ratio, for 30 min at 37 °C. Complement activation was stopped by adding 25 mM EDTA in ratio 1:1 v/v. All samples were frozen at -20 °C. C5a protein was assessed by ELISA (C5a Mouse ELISA kit, ab193718, Abcam, USA).

To assess the complement activity of rat serum, the concentration of rabbit RBC was adjusted to 5 x 10^8 cells mL^{-1}. Calcium chloride and magnesium chloride were added to GVB^0 buffer in order to get 0.15 mM and 0.5 mM, respectively. Rat serum was diluted in GVB with Ca^{2+} and Mg^{2+} in ratio 1:1 (v/v). Six µL of RBC at 5 x 10^8 cells mL^{-1}, and 30 µL of diluted rat serum, were added to 4 µL of GVB with Ca^{2+} and Mg^{2+}. Negative control was prepared by adding rat serum, and RBC, to GVB buffer containing 10 mM EDTA. 6 µL of RBC were added to 34 µL of water and used as control of total RBC lysis (*Absorbance of 100% Lysis*). All samples were incubated for 30 min at 37 °C, under 50 rpm in an orbital shaker. Complement activation was stopped by addition of 200 µL of cold GVB with 10 mM EDTA, and centrifuged at 1000 *g* for 10 min at 4 °C. Supernatant was read at 412 nm in a microplate reader (Tecan infinite 200 pro, Switzerland). Serum was diluted in water (in ratio 1:7, v/v), and used as control of serum background (*Serum background*). Hemolytic activity, due to complement activation, was calculated by the following equation:

Equation S2:
$$Hemolytic\ activity\ (\%) = \frac{(Sample\ absorbance - Serum\ background)}{Absorbance\ of\ 100\%\ Lysis}\ x\ 100$$

Extension of complement depletion by CVF

The complement cascade was inhibited with two intraperitoneal injections of CVF 12 and 24 hours before the experiment (dose of 15U per mice). Blood samples (50 µL) were taken from the saphenous vein of Balb/c mice after 1, 2, 5, 7, 9 and 11 days post CVF injection. Plasma samples were incubated with Zymosan (5 mg mL^{-1}), 1:1 v/v in ratio, to activate complement cascade. To confirm the inhibition of the complement cascade, C5a proteins was assessed by ELISA (C5a Mouse ELISA kit, ab193718, Abcam, USA) according to the manufacturer protocol.

Equations for pharmacokinetic parameters

The blood concentration (%ID per g of blood) was calculated as follow:

Equation S3:
$$\%ID\ per\ g\ of\ blood\ (\%) = \frac{(Radioactivity\ of\ blood\ /\ mass\ of\ blood\ (g))}{Radioactivity\ of\ dose\ administered}\ x\ 100$$

Trapezoidal method to determine area under curve from 0 to 6 hours for mice ($AUC_{0\text{-}6h}$) and from 0 to 24 hours for rats ($AUC_{0\text{-}24h}$):

Equation S4:
$$AUC(0-6h) = t1\ x\ C1 + \ (((t2-t1)x(C2+C1) + (t3-t2)x(C3+C2) + (t4-t3)x(C4+C3) + (t5-t4)x(C5+C4) + (t6-t5)x(C$$

Equation S5:
$$AUC(0-24h) = t1\ x\ C1 + \ (((t2-t1)x(C2+C1) + (t3-t2)x(C3+C2) + (t4-t3)x(C4+C3) + (t5-t4)x(C5+C4) + (t6-t5)x(C + (t7-t6)x(C7+C6) + \ (t8\ -t7)x(C8+C7)))/2$$

where C1 is the %ID per g of blood at t1 (0.25 hours); C2 is the %ID per g of blood at t2 (0.5 hours); C3 is the %ID per g of blood at t3 (1 or 1.5 hours); C4 is the %ID per g of blood at t4 (2 or 2.5 hours); C5 is the %ID per g of blood at t5 (4 hours); C6 is the %ID per g of blood at t6 (6 hours); C7 is the %ID per g of blood at t7 (12 hours); C8 is the %ID per g of blood at t8 (24 hours).

Concentration at time 0 (C_0):

Equation S6:
$$C0 = e^{intercept\ of\ Ln(\%ID)x\ time(h)\ plot}$$

AUC from 6 hours (t6), or 24 hours (t8), to infinity, and Ke:

Equation S7:
$$AUC(t6\ to\ infinite) = C6\frac{C6}{|slope\ of\ Ln(\%ID)x\ time(h)\ plot|}$$

Equation S8:
$$AUC(t8\ to\ infinite) = C8\frac{C8}{|slope\ of\ Ln(\%ID)x\ time(h)\ plot|}$$

Equation S9: $$Ke = \frac{C0}{AUC(0\ to\ infinite)}$$

Supplementary Tables and Figures

Liposomes

Table S1: Phospholipid composition, Z-average and PdI of liposomes.

Liposomes (molar ratio)	Z-average	PdI	Experiment
HSPC:cholesterol:DSPE-PEG 2000 (47.5:50:2.5)	114 ± 3	0.07 ± 0.07	Zeta potential determination (Table S4).
HSPC:cholesterol (62:38)	110.5 ± 0.4	0.10± 0.01	
HSPC:cholesterol:DSPE-PEG 2000 (57:38:5)	110 ± 1	0.04 ± 0.02	*In vitro* complement activation in mice serum by ELISA C5a assay (Fig. 1A)
HSPC:cholesterol (62:38)	111 ± 1	0.10 ± 0.01	
Soy phosphatidycholine:cholesterol: DSPE-PEG 2000 (57:38:5)	98 ± 3	0.09 ± 0.01	*In vitro* complement activation in human serum by ELISA C5a assay (Fig. 1B)
Soy phosphatidycholine: cholesterol (62:38)	100 ± 2	0.05 ± 0.01	
HSPC:cholesterol:DSPE-PEG 2000 (57:38:5)	115.9 ± 1.4	0.12 ± 0.02	*In vitro* complement activation in mice serum by ELISA C5b-9 assay (Fig. S2A)
HSPC:cholesterol (62:38)	115.9 ± 0.9	0.05 ± 0.02	
HSPC:cholesterol:DSPE-PEG 2000 (57:38:5)	103 ± 1	0.06 ± 0.01	*In vitro* complement activation in human serum by ELISA SC5b-9 assay (Fig. S2B)
HSPC:cholesterol (62:38)	100 ± 4	0.14 ± 0.04	
HSPC:cholesterol:DSPE-PEG 2000 (57:38:5) with HPTS and DPX encapsulated	102.2 ± 0.9*	0.05 ± 0.01*	*In vitro* dye release in mice, rat and human sera (Fig. 2)
HSPC:cholesterol (62:38) with HPTS and DPX encapsulated	105 ± 14**	0.10 ± 0.04**	
HSPC:cholesterol:DSPE-PEG 2000 (57:38:5) labelled with ^{14}C-cholesteryl oleate	103 ± 6***	0.04 ± 0.01***	Pharmacokinetics experiments (Fig. 3 and 4, Fig. S5 – S11)
HSPC:cholesterol (62:38) labelled with ^{14}C-cholesteryl oleate	105 ± 6****	0.07 ± 0.03****	
HSPC:cholesterol (62:38) labelled with DiD'	124 ± 4	0.14 ±0.01	Fluorescence-activated cell sorting (FACS) (Fig. 5)

Values represent mean ± SD of three measurements by dynamic light scattering of one formulation (n = 3); ***** two formulations (n = 6); ** four formulations (n = 12); ******* three formulations (n = 9) or ******** eleven formulations (n = 33).

Table S2: Doses and time points used for mice and rats pharmacokinetics experiments.

Experiment	Pharmacokinetic profile	Dose (µg per animal)	Time points
Mouse high dose	6 hours	500	0.25, 0.5, 1, 2, 4 and 6 hours
Mouse low dose	6 hours	50	0.25, 0.5, 1, 2, 4 and 6 hours
Mouse low dose	24 hours	50	0.25, 0.75, 1.5, 3, 6, 12 and 24 hours
Rat high dose	24 hours	5000	0.25, 0.5, 1 (or 1.5), 2 (or 2.5), 4, 6, 12 and 24 hours
Rat low dose	24 hours	500	0.25, 0.5, 1 (or 1.5), 2 (or 2.5), 4, 6, 12 and 24 hours

Table S3: Monoclonal antibodies used for cells labelling.

Antigen	Clone	Supplier – catalogue #	Fluorophore (ex/em)
Live Dead (FVS510)	-	BD Biosciences – 564406	BV512 (408/512 nm)

CD45	30-F11	BD Biosciences – 564279	BV395 (408/602 nm)
CD11b	M1/70	BD Biosciences – 612977	BUV661 (348/661 nm)
B220	RA3-6B2	BD Biosciences – 563708	BV605 (408/602 nm)
CD3e	145-2C11	BD Biosciences – 562286	PE-CF594 (496/594 nm)

Table S4: Zeta potential of liposomes.

Liposomes (molar ratio)	Z-average (mV)	
	in 1% HEPES solution	in 10% HEPES solution
HSPC:cholesterol:DSPE-PEG 2000 (47.5:50:2.5)	-5 ± 6	-2 ± 10
	-4 ± 7	-3 ± 15
	-4 ± 8	-3 ± 7
HSPC:cholesterol (62:38)	-	-2 ± 10
	-	-3 ± 7
	-	-3 ± 3

Values represent zeta potential ± standard deviation of each measurement by electrophoretic light scattering.

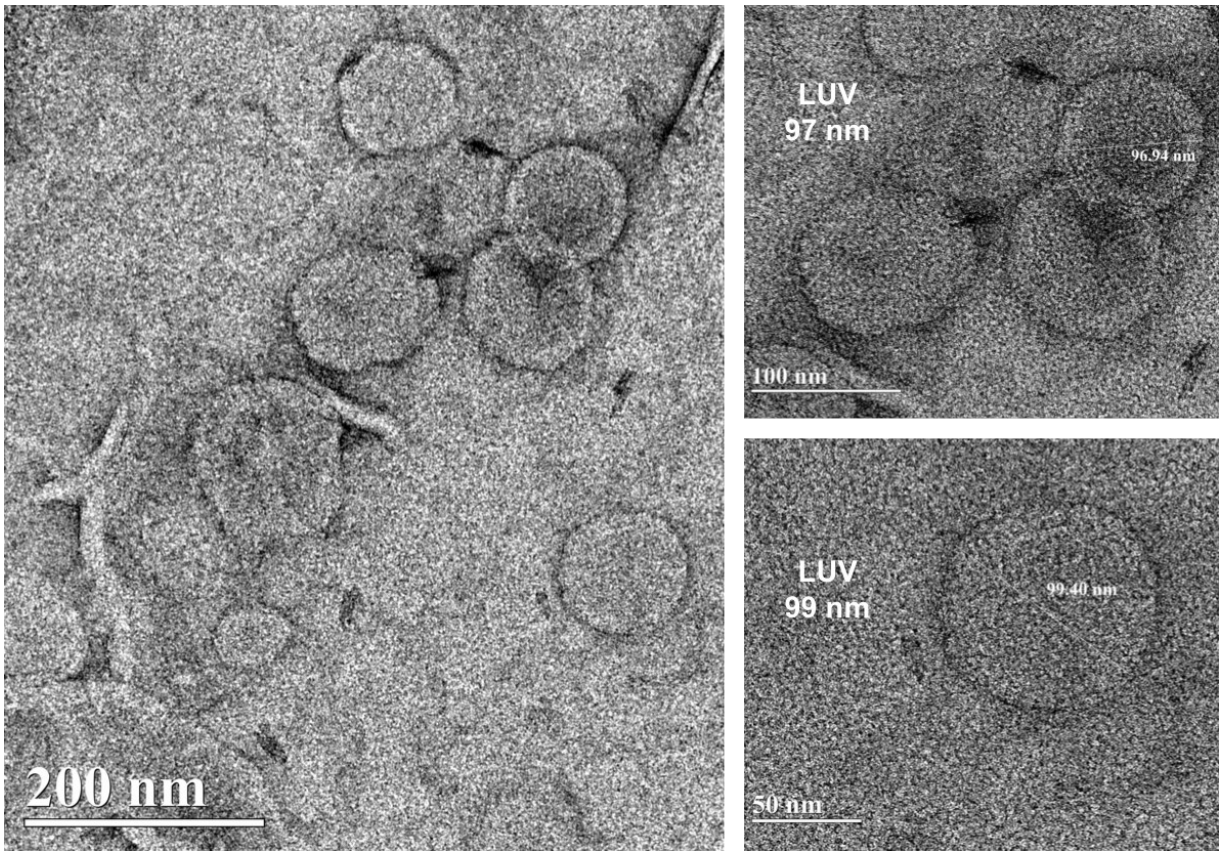


Fig. S1: TEM of liposomes. Non-PEGylated liposomes were diluted in HEPES (1:3), and stained by 2% uranyl acetate solution.

In vitro complement activation in different sera

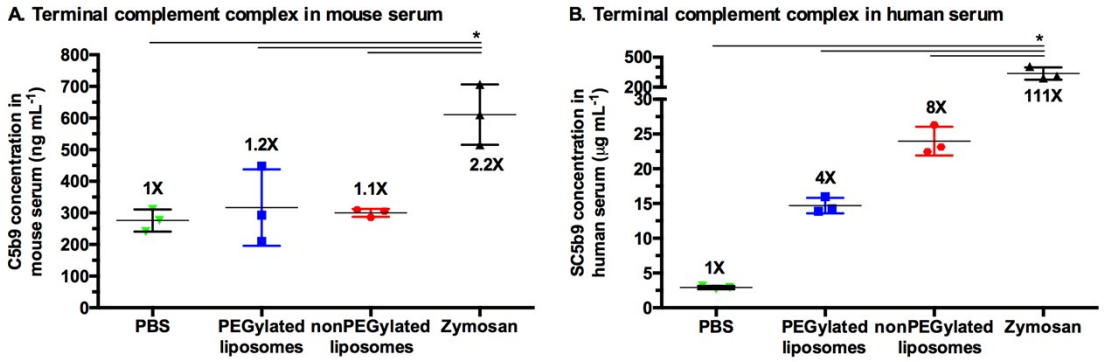


Fig. S2: Terminal complement complex levels in mouse and human sera after incubation with liposomes. Liposomes are not able to increase the levels of terminal complement complex in mouse serum (A) and led to minimal increase in human serum (B). Each replicate is shown (n = 3), * $p < 0.05$ for ANOVA between all samples.

Influence of complement cascade on the release of hydrophilic liposomal content

Table S5: Fluorescence of HPTS from liposomes in mice serum, with and without EDTA. Absolute values (release and 100% lysis) are expressed in scientific notation. Values represent mean ± SD of measurements (n = 3).

Liposomes	Mice serum with EDTA			Mice serum		
	Release (x10^6)	100% lysis (x10^6)	% release	Release (x10^6)	100% lysis (x10^6)	% release
non-PEGylated	12.1 ± 0.2	134 ± 2	9.0 ± 0.2	10.9 ± 0.8	116 ± 10	9.4 ± 0.3
PEGylated	5.2 ± 0.5	104 ± 10	5.1 ± 0.1	4.3 ± 0.2	96 ± 6	4.5 ± 0.1

Table S6: Fluorescence of HPTS from liposomes in rat serum, with and without EDTA. Values represent mean ± SD of measurements (n = 3-11).

Liposomes	Rat serum with EDTA			Rat serum		
	Release	100% lysis	% release	Release	100% lysis	% release
non-PEGylated	9 ± 4	164 ± 62	5.1 ± 0.5	16 ± 1	172 ± 63	11 ± 4
PEGylated	7.2 ± 0.6	230 ± 7	3.1 ± 0.4	5.6 ± 0.1	223 ± 12	2.5 ± 0.2

Table S7: Fluorescence of HPTS from liposomes in human serum, with and without EDTA. Values represent mean ± SD of measurements (n = 6-12).

Liposomes	Human serum with EDTA			Human serum		
	Release	100% lysis	% release	Release	100% lysis	% release
non-PEGylated	10 ± 2	213 ± 51	5 ± 2	16 ± 6	222 ± 33	8 ± 4
PEGylated	5.9 ± 0.4	268 ± 22	2.2 ± 0.1	5.0 ± 0.5	235 ± 22	2.13 ± 0.08

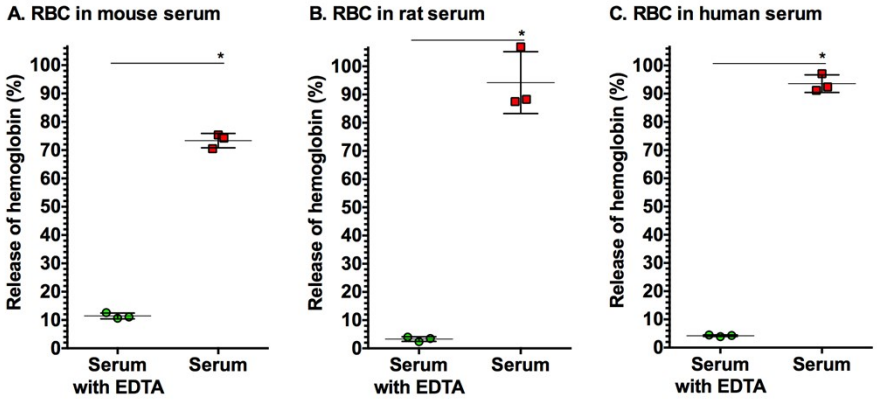
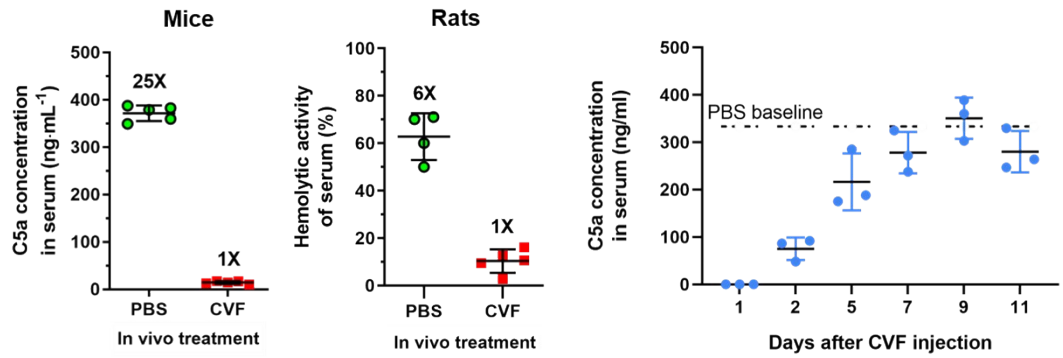


Fig. S3: Complement proteins can trigger release of content from RBC. RBC were separated from free hemoglobin at 1000 *g* for 3 min, and resuspended in isotonic buffer. RBC at approximately 5 x 10^8 cells mL^{-1} were incubated in mouse (A), rat (B), or in human (C) sera, with and without EDTA, at 37 °C. EDTA were added to stop complement activation, and all samples were centrifuged. Supernatant was read at 412 nm. The absorbance of RBC in water or 10% Triton-X was used as 100%. Data represent mean ± SD (n = 3 for RBC), * $p < 0.05$.



Influence of the complement cascade on the pharmacokinetics of liposomes in rodents

Fig. S4: Complement cascade was abrogated in animals treated in vivo with intraperitoneal injections of CVF. Control group was injected IP with PBS buffer. Blood samples from animals were collected 24 hours the first injection of CVF or PBS. Mice serum was incubated ex vivo with Zymosan A, and C5a protein was assessed by ELISA, while rat serum was incubated with RBC, and hemolytic activity was measured (A). CVF depletes the ability of animals to activate complement for 24-48h after the first injection (B). Values represent each animal (n = 4-5).

Table S8: Pharmacokinetics parameters of PEGylated liposomes administered at 20 mg kg^{-1} in rodents.

Time (h)	% ID per g of blood - Mice		% ID per g of blood - Rats	
	PBS group (n = 8)	CVF group (n = 7)	PBS group (n = 5)	CVF group (n = 5)
0.25	48 ± 4	49 ± 6	2.7 ± 0.4	2.4 ± 0.3
0.5	45 ± 3	47 ± 6	2.6 ± 0.4	2.4 ± 0.2
1	42 ± 3	45 ± 5	2.6 ± 0.4	2.4 ± 0.2
2	38 ± 3	42 ± 5	2.6 ± 0.4	2.3 ± 0.2
4	35 ± 2	37 ± 4	2.4 ± 0.4	2.2 ± 0.2
6	28 ± 2	29 ± 4	2.3 ± 0.3	2.1 ± 0.2
12	-	-	2.0 ± 0.3	1.8 ± 0.1
24	-	-	1.4 ± 0.2	1.2 ± 0.1
AUC (%ID h·g^{-1})	222 ± 14	236 ± 26	47 ± 7	43 ± 3
Ke (h^{-1})	0.088 ± 0.009	0.090 ± 0.003	0.028 ± 0.005	0.028 ± 0.001

Table S9: Pharmacokinetics parameters of non-PEGylated liposomes administered at 20 mg kg^{-1} in rodents.

Time (h)	% ID per g of blood - Mice		% ID per g of blood - Rats	
	PBS group (n = 8)	CVF group (n = 11)	PBS group (n = 10)	CVF group (n = 8)
0.25	43 ± 6	43 ± 5	3 ± 1	3 ± 1
0.5	39 ± 6	40 ± 6	3.0 ± 0.9	2.9 ± 0.9
1	34 ± 6	35 ± 5	2.9 ± 0.6	2.5 ± 0.9
1.5	-	-	2.8 ± 0.9	3.1 ± 0.7
2	29 ± 6	30 ± 5	2.7 ± 0.7	2.3 ± 0.8
2.5	-	-	2.6 ± 0.8	3.0 ± 0.7
4	24 ± 6	24 ± 4	2.5 ± 0.7	2.4 ± 0.7
6	17 ± 4	18 ± 3	2.2 ± 0.6	2.1 ± 0.6
12	-	-	1.6 ± 0.4	1.4 ± 0.4
24	-	-	0.9 ± 0.3	0.7 ± 0.3
AUC (%ID h·g^{-1})	165 ± 33	169 ± 27	42 ± 11	39 ± 12
Ke (h^{-1})	0.15 ± 0.02	0.14 ± 0.01	0.05 ± 0.01	0.061 ± 0.004

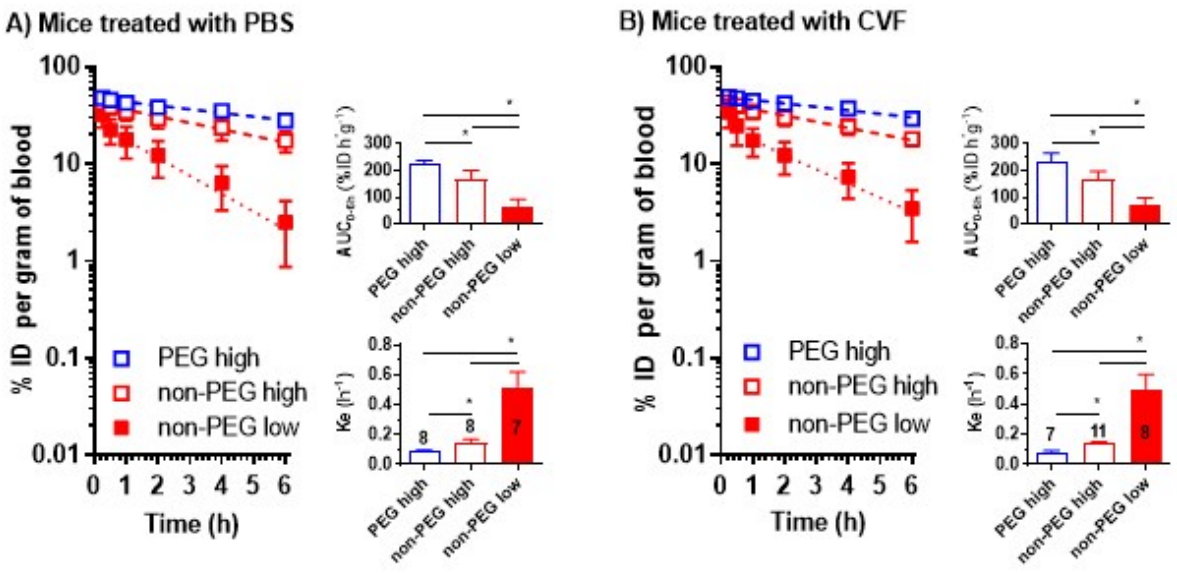


Fig. S5: Comparison of pharmacokinetics between PEGylated and non-PEGylated liposomes injected at various dose in mice. Liposomes were administered at 20 mg kg^{-1} (high dose) or 2 mg kg^{-1} (low dose) in mice treated with PBS (A) and CVF (B). Data represent mean ± SD (n = 7-11), * $p < 0.05$ for ANOVA between all samples.

Table 10: Pharmacokinetics parameters of non-PEGylated liposomes administered at 2 mg kg^{-1} in rodents.

Time (h)	% ID per g of blood - Mice		% ID per g of blood - Mice		% ID per g of blood - Rats	
	PBS group (n = 7)	CVF group (n = 8)	PBS group (n = 5)	CVF group (n = 4)	PBS group (n = 7)	CVF group (n = 6)
0.25	32 ± 8	34 ± 10	23 ± 5	28 ± 6	2.6 ± 0.8	3.3 ± 0.6
0.5	22 ± 6	25 ± 9	-	-	2.5 ± 0.7	3.2 ± 0.6
0.75	-	-	13 ± 4	17 ± 1	-	-
1	18 ± 6	17 ± 5	-	-	2.4 ± 0.4	2.9 ± 0.3
1.5	-	-	9 ± 3	11 ± 3	2.6 ± 1.1	3.0 ± 0.6
2	12 ± 5	12 ± 4	-	-	2.1 ± 0.3	2.7 ± 0.3
2.5	-	-	-	-	2.2 ± 0.9	2.8 ± 0.5
3	-	-	5.0 ± 0.9	8 ± 2	-	-
4	6 ± 3	7 ± 3	-	-	1.9 ± 0.5	2.5 ± 0.4
6	3 ± 2	3 ± 2	3.0 ± 0.2	4 ± 0.9	1.6 ± 0.5	2.3 ± 0.3
12	-	-	2.9 ± 0.5	3 ± 0.8	0.9 ± 0.5	1.5 ± 0.2
24	-	-	1.2 ± 0.4	2 ± 0.4	0.2 ± 0.1	0.6 ± 0.2
6 hours pharmacokinetic						
AUC (%ID h·g^{-1})	67 ± 24	72 ± 24	45 ± 10	61 ± 12	13 ± 4	16 ± 3
Ke (h^{-1})	0.5 ± 0.1	0.5 ± 0.1	0.52 ± 0.04	0.4 ± 0.1	0.08 ± 0.02	0.061 ± 0.007
24 hours pharmacokinetic						
AUC (%ID h·g^{-1})	-	-	87 ± 10	115 ± 17	26 ± 10	40 ± 7
Ke (h^{-1})	-	-	0.28 ± 0.06	0.23 ± 0.09	0.10 ± 0.02	0.07 ± 0.01

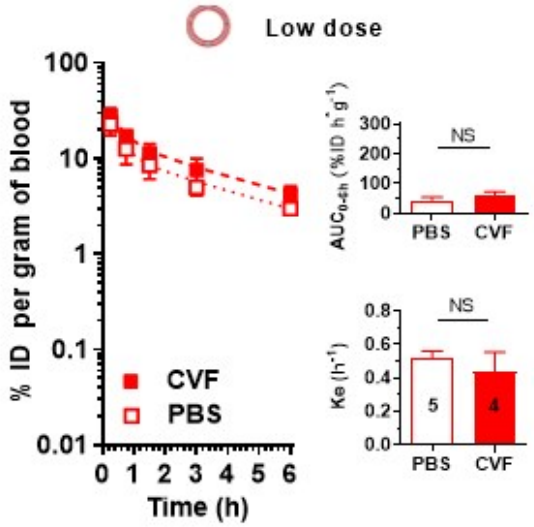


Fig. S6 Pharmacokinetic study of non-PEGylated liposomes at 2 mg kg^{-1} in mice treated previous with PBS and CVF (data up to 6 hours of same pharmacokinetic experiment shown in Fig. 3D). Data represent mean ± SD (n = 4-5), NS not statistically significant.

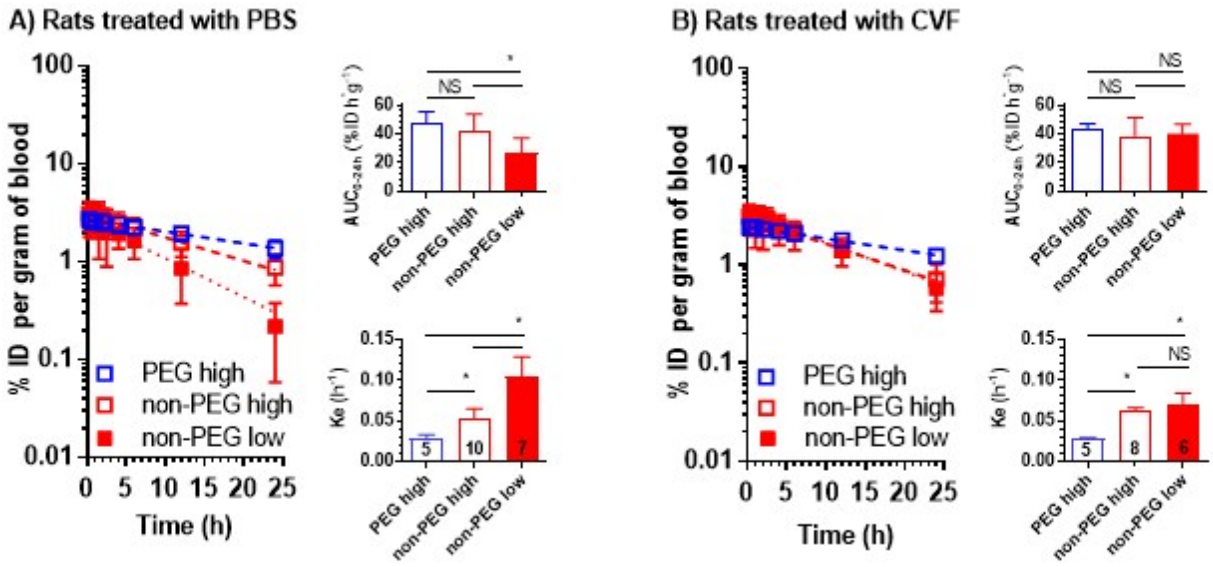


Fig. S7: Comparison of pharmacokinetics between PEGylated and non-PEGylated liposomes injected at various dose in rats. Liposomes were administered at 20 mg kg^{-1} (high dose) or 2 mg kg^{-1} (low dose) in rats treated with PBS (A) and CVF (B). Data represent mean ± SD (n = 5-10), * $p < 0.05$ for ANOVA between all samples, NS not statistically significant.

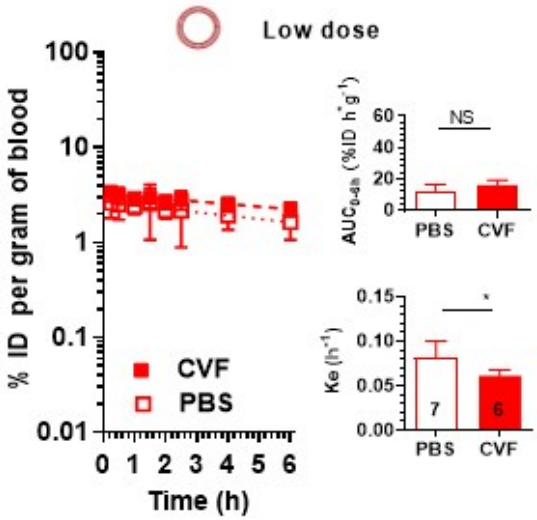


Fig. S8: Pharmacokinetic study of non-PEGylated liposomes at 2 mg kg^{-1} in rats treated previous with PBS and CVF (data up to 6 hours of same pharmacokinetic experiment shown in Fig. 4C). Data represent mean ± SD (n = 6-7), * $p < 0.05$, NS not statistically significant.

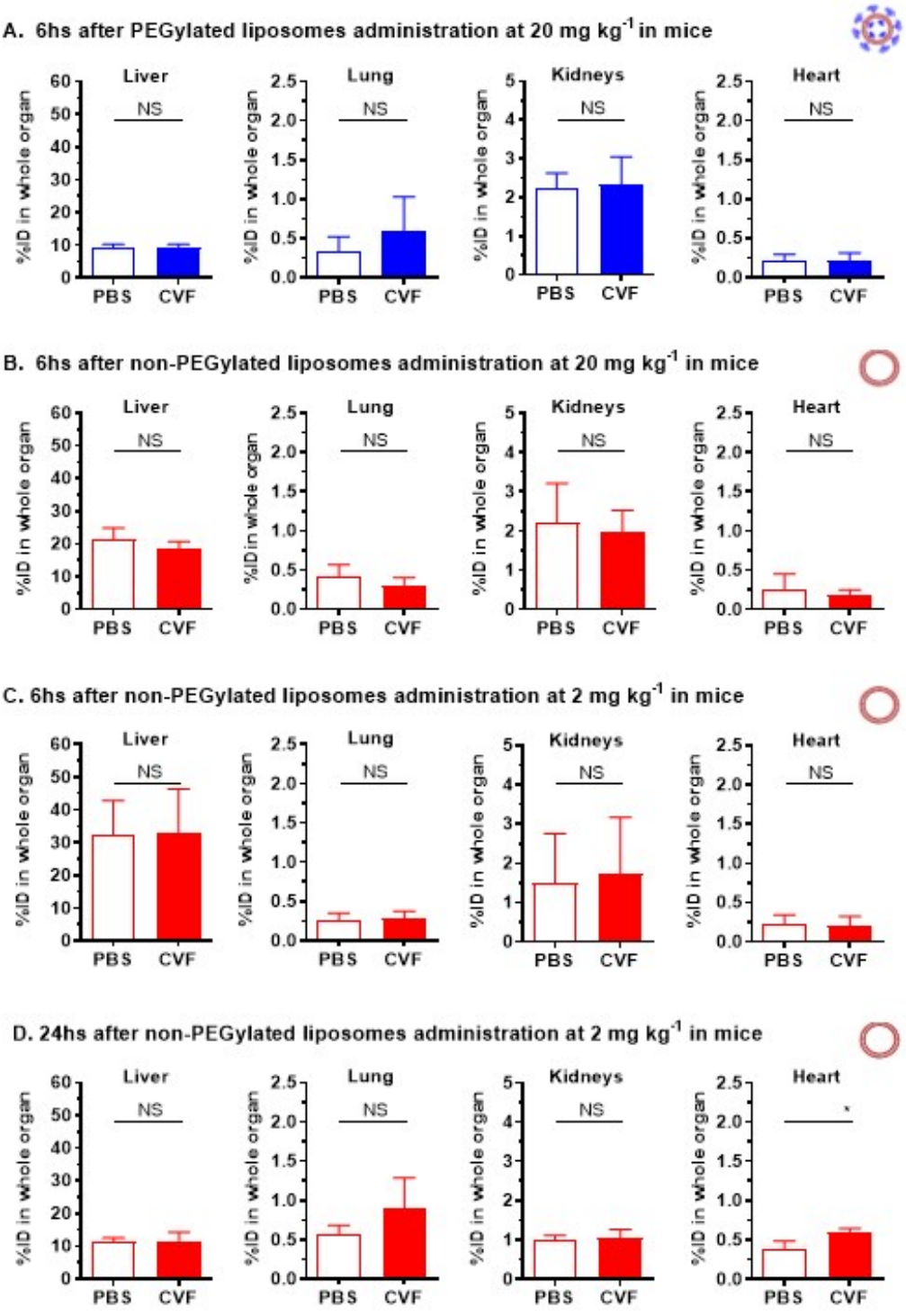


Fig. S9: Biodistribution of liposomes administered intravenously in mice. PEGylated liposomes were administered at 20 mg kg^{-1} and organs collected after 6 hours (A). Non-PEGylated liposomes were administered at 20 mg kg^{-1} and organs collected after 6 hours (B); or at 2 mg kg^{-1} and organs collected after 6 hours (C); or at 2 mg kg^{-1} and organs collected after for 24 hours (D). Data represent mean ± SD (n = 4-11), * $p < 0.05$, NS not significant.

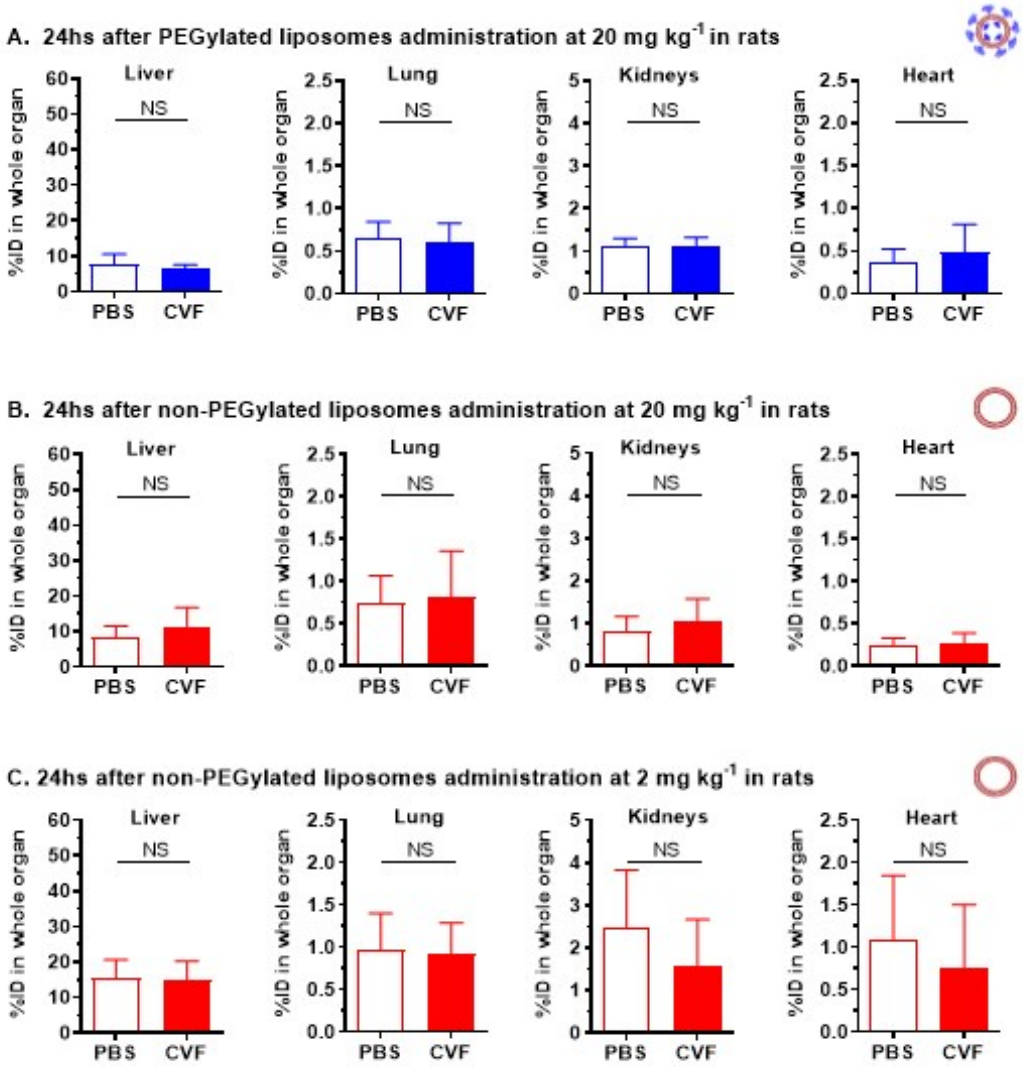


Fig. S10: Biodistribution study of liposomes administered intravenously in rats. PEGylated liposomes were administered at 20 mg kg^{-1} (A). Non-PEGylated liposomes were administered at 20 mg kg^{-1} (B); or at 2 mg kg^{-1} (C). The organs were collected after 24 hours of liposomes administration. Data represent mean ± SD (n = 5-10), * $p < 0.05$, NS not significant.

Table S11: Biodistribution of PEGylated liposomes administered at 20 mg kg^{-1} in rodents, PBS and CVF groups.

Organ	%ID in whole organ – Mice 6 hours		%ID in whole organ – Rats 24 hours	
	PBS group (n = 8)	CVF group (n = 7)	PBS group (n = 5)	CVF group (n = 5)
Liver	9 ± 1	9 ± 1	8 ± 3	6 ± 1
Spleen	1.4 ± 0.2	1.5 ± 0.1	1.8 ± 0.4	2.0 ± 0.3
Lung	0.3 ± 0.2	0.6 ± 0.4	0.6 ± 0.2	0.6 ± 0.2
Kidneys	2.2 ± 0.4	2 ± 1	1.1 ± 0.2	1.1 ± 0.2
Heart	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.5 ± 0.3

Table S12: Biodistribution of non-PEGylated liposomes administered at 20 mg kg^{-1} in rodents, PBS and CVF groups.

Organ	%ID in whole organ – Mice 6 hours		%ID in whole organ – Rats 24 hours	
	PBS group (n = 8)	CVF group (n = 11)	PBS group (n = 10)	CVF group (n = 8)
Liver	21 ± 3	18 ± 2	8 ± 3	11 ± 5
Spleen	3 ± 1	2 ± 1	3 ± 1	2 ± 1
Lung	0.4 ± 0.1	0.3 ± 0.1	0.7 ± 0.3	1 ± 1
Kidneys	2 ± 1	2 ± 1	0.8 ± 0.3	1.1 ± 0.5
Heart	0.3 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1

Table S13: Biodistribution of non-PEGylated liposomes administered at 2 mg kg^{-1} in rodents, PBS and CVF groups.

Organ	%ID in whole organ – Mice 6 hours		%ID in whole organ – Rats 24 hours	
	PBS group (n = 7)	CVF group (n = 8)	PBS group (n = 7)	CVF group (n = 6)
Liver	32 ± 10	33 ± 12	15 ± 5	15 ± 5
Spleen	2 ± 1	0.7 ± 0.2	4 ± 1	2 ± 1
Lung	0.3 ± 0.1	0.3 ± 0.1	1.0 ± 0.4	0.9 ± 0.3
Kidneys	2 ± 1	2 ± 1	2 ± 1	2 ± 1
Heart	0.2 ± 0.1	0.2 ± 0.1	1 ± 1	1 ± 1

Table S14: 24 hours biodistribution study of non-PEGylated liposomes administered at 2 mg kg^{-1} in mice, PBS and CVF groups.

Organ	%ID in whole organ – Mice 24 hours	
	PBS group (n = 5)	CVF group (n = 4)
Liver	11 ± 1	11 ± 3
Spleen	0.48 ± 0.02	0.49 ± 0.08
Lung	0.6 ± 0.1	0.9 ± 0.4
Kidneys	1.0 ± 0.1	1.0 ± 0.2
Heart	0.4 ± 0.1	0.61 ± 0.04

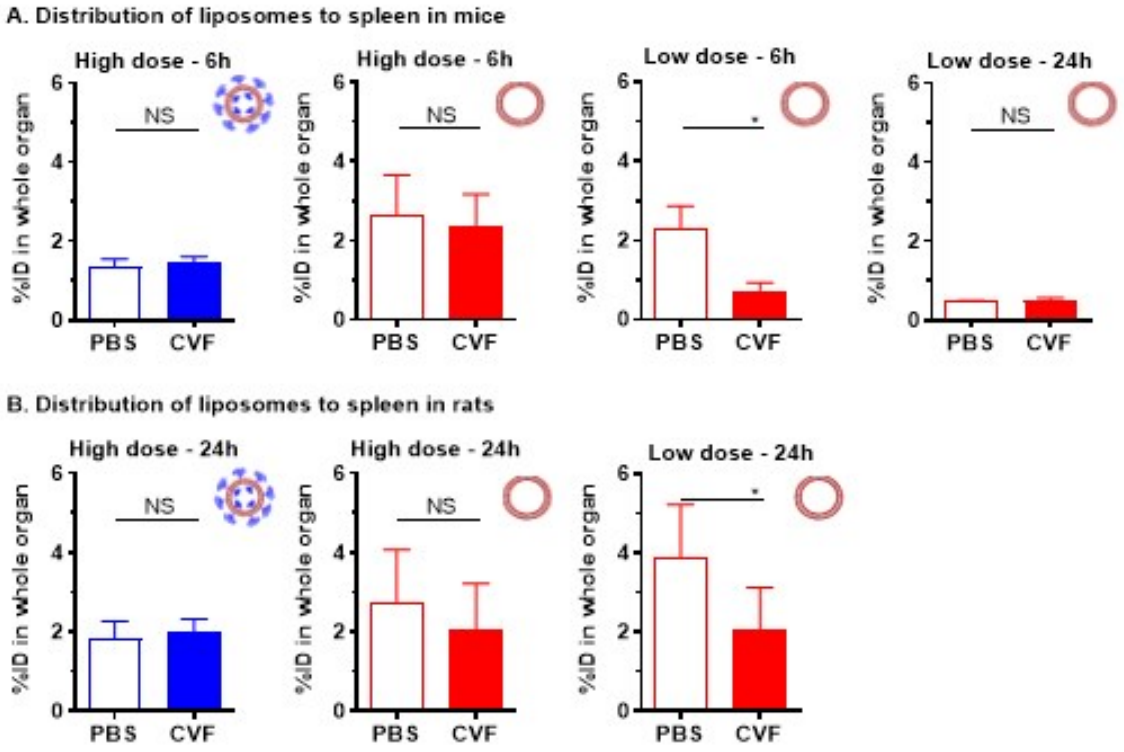
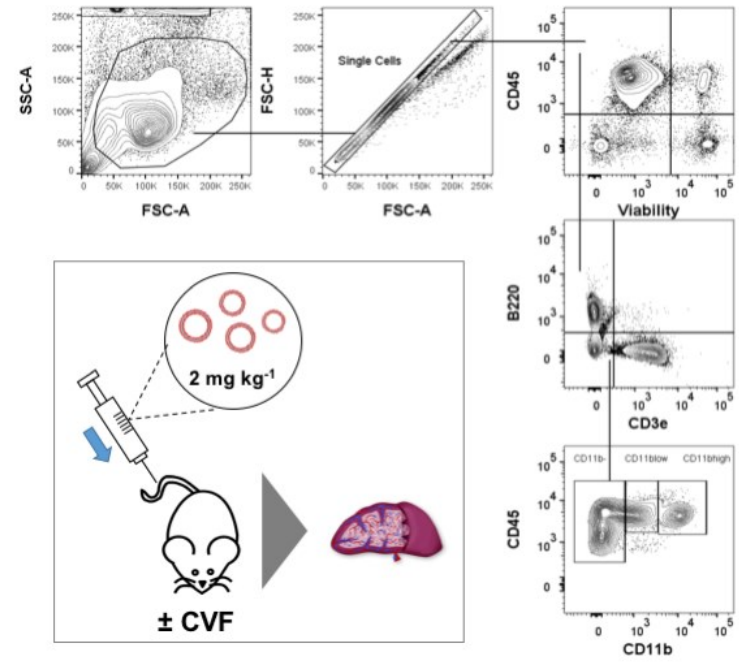


Fig. S11: Distribution of liposomes to spleen in mice (A) and rats (B). Liposomes were administered intravenously at high dose (20 mg kg^{-1}) or low dose (2 mg kg^{-1}) in animals treated previously with PBS (empty bars) and CVF (full bars). The spleens were collected after 6 or 24 hours from injection of liposomes, PEGylated (in blue) or non-PEGylated (in red). Data represent mean ± SD (n = 4-11), * $p < 0.05$, NS not significant.



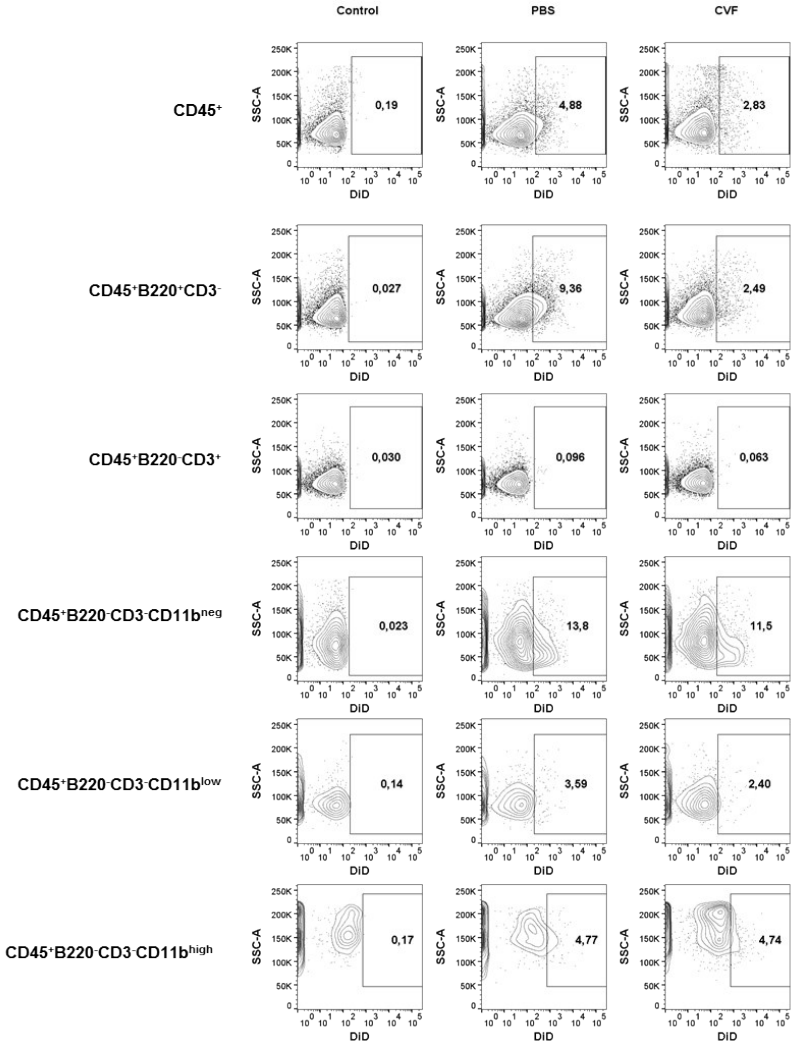


Fig. S12: Gating strategy.

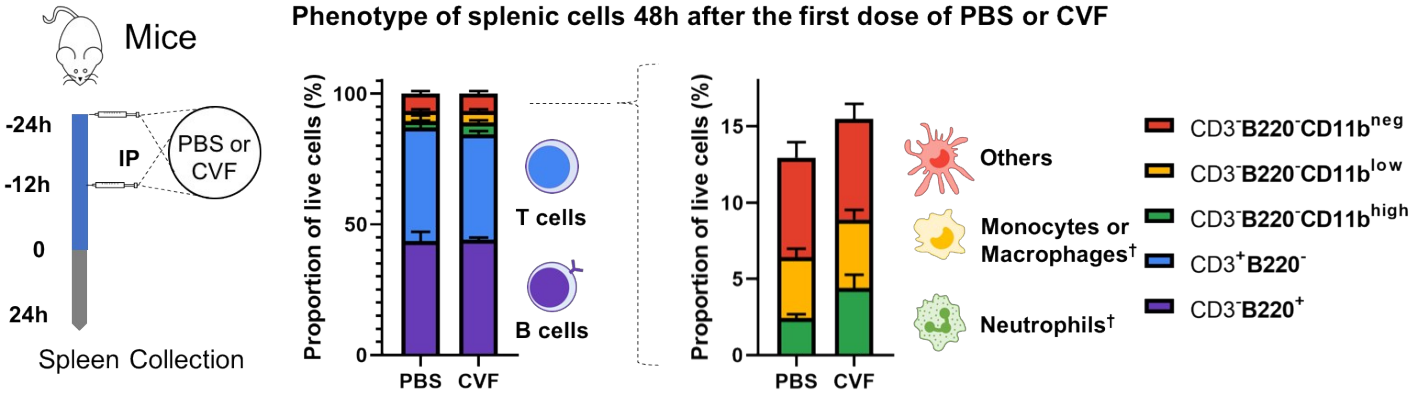


Fig. S13: Proportions of different leukocytes in the spleen of mice treated with PBS or CVF. Mice received two doses of PBS or CVF, 48 and 36 hours before euthanasia. The spleens were collected and analyzed by FACS. Viable cells $CD45^+$ cells, accounting for all leukocytes were counted (the total number of leukocytes correspond to 100%). CD45+ cells were further sorted according to their phenotype: T cells ($CD3^+B220^-$), B cells ($CD3^-B220^+$), non-lymphocytes cells ($CD3^-B220^-CD11b^{neg}$, $CD3^-B220^-CD11b^{low}$, $CD3^-B220^-CD11b^{high}$). Cells expressing CD11b receptors may correspond to monocytes, macrophages and neutrophils. The phenotype $CD3^-B220^-CD11b^{high}$ showed high granularity, suggesting they have a neutrophil phenotype. The percentage of each phenotype is represented as mean ± SD (n = 5). † Further analysis would be required to confirm absolute phenotype.

References:

1. B. D. Adair and M. Yeager, *Integrins*, 2007, **426**, 337-373.