SUPPORTING INFORMATION

Immunocompatibility of Rad-PC-Rad liposomes *in vitro*, based on human complement activation and cytokine release

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1. Materials used for the experiments

The purchased chemical compounds were used without further purification.

Table S1. List of materials used in the experiments.

Name	Company	City, country
DSPE-PEG_{2000}	Lipoid AG	Steinhausen, Switzerland
Doxil®	Janssen Cilag Ltd.	Beerse, Belgium
AmBisome®	Gilead Sciences Ltd.	Foster City, CA, USA
Nitroglycerin Bioren 0.1% solution	Sintetica SA	Mendrisio, Switzerland
0.9 % saline solution	Bichsel AG	Interlaken, Switzerland
ELISA MicroVue kits (SC5b-9 Plus, C4d, Bb, C3a, C5a)	Quidel Corp.	San Diego, CA, USA
FITC Annexin V Apoptosis Detection Kit	BioLegend Ltd.	Budapest, Hungary
Cytometric Bead Array (Human inflammatory cytokine kit)	BD Biosciences	Budapest, Hungary
Dulbecco's phosphate buffered saline (DPBS) with and without $CaCl_2$, $MgCl_2$	Sigma-Aldrich Co.	Budapest, Hungary
R5 cell medium consisting of Roswell Park Memorial Institute (RPMI) medium with Glutamine		
10 % Fetal Bovine Serum (FBS)		
0.1 mM non-essential amino acids (NEAAs)		
1 % penicillin-streptomycin solution		
50 μM β-mercaptoethanol		
1 mM pyruvate		
Ethylenediaminetetraacetic acid (EDTA)		
Zymosan		
Millex-GV 0.22 μm syringe filter	Merck Millipore Ltd.	Cork, Ireland
Whatman Nuclepore Track-Etched Membranes	Sigma-Aldrich	Buchs, Switzerland
PD-10 desalting columns	GE Healthcare Bio-Sciences AB	Uppsala, Sweden
Hirudin-treated tubes	Roche Kft.	Budapest, Hungary
50 mM 5(6)-carboxyfluorescein, powder	Sigma-Aldrich	Buchs, Switzerland
10 mM HEPES buffer, powder		

2. Calculation of NTG encapsulation efficiency

Table S2. NTG encapsulation efficiency.

Formula	R3	R4
Averaged [Mw] molecular weight (g/mol)	760.00	862.2748
[C] measured conc. (g/L)	13.6	21.8
[M] molarity (mol/L) = [C]/[Mw]	0.00132	0.00116
Number of lipid molecules [No $_{lipid\ mol}$] = [M] * Avogadro No	7.92368×10^{20}	6.98385×10^{20}
[D] liposome diameter (nm)	114.5	97
[R1] liposome outer radius (nm) *	57.25	48.5
Liposome outer area (nm^2) [OA] = 4*3.14*R1^2	41187.06438	29559.24477
[R2] liposome inner radius (nm) **	53.25	44.5
Liposome inner area (nm^2) [IA] = 4*3.14*R2^2	35632.72867	24884.55498
Total area per liposome (nm^2) [TA] = [OA] + [IA]	76819.79305	54443.79976
[A] Area per lipid (nm^2)***	0.474	0.474
Number of lipids per liposome [No $_{lipids}$] = [TA]/[A]	162067.074	114860.337
Number of vesicles per 1L [N] = [No $_{lipid\ mol}$] / [No $_{lipids}$]	4.88914×10^{15}	6.0803×10^{15}
Volume of liposome (nm^3) [V] = (4/3)*3.14*R2^3	632480.9338	369120.8989
Entrapped volume per 1L (nm^3) [EV] = [N]*[V]	3.09229×10^{21}	2.24437×10^{21}
[EV] conversion nm^3 into L per 1L; mL/mL	0.003092287	0.002244365
Area under curve [AUC] of NTG 100% signal in 1 mL	501428.6667	501428.6667
Theoretical value of 100% signal in liposomes [EA]*[AUC]	1550.561388	1125.389191
Measured value of % signal in liposomes	590.157	132.237
Encapsulation efficiency of NTG (%) [EE] = [measured]/[theoretical]*100	38.06	11.75

* mean diameter from DLS measurement

** R2 = R1 - 4 nm (thickness of bilayer)

*** X-ray data, converted from $Å^2$ to nm^2

3. Differential scanning calorimetry (DSC)

DSC were directly measured from the prepared Rad-PC-Rad and Rad-PC-Rad/DSPE-PEG vesicle suspensions. Liposomal suspensions were degassed for 30 minutes using a TA degassing station. The alternative heating-cooling scans were recorded on a TA Nano DSC (TA Instruments, USA) from 5 °C to 90 °C with a scanning speed of 0.5 K/min. The experiment was performed twice, starting with new suspensions, in order to ensure reproducibility. The scans of the second heating-cooling scans are reported in Figure S1. Raw data was baseline corrected and converted to molar heat capacity (MHC) using the NanoAnalyze software (TA Instruments, USA).

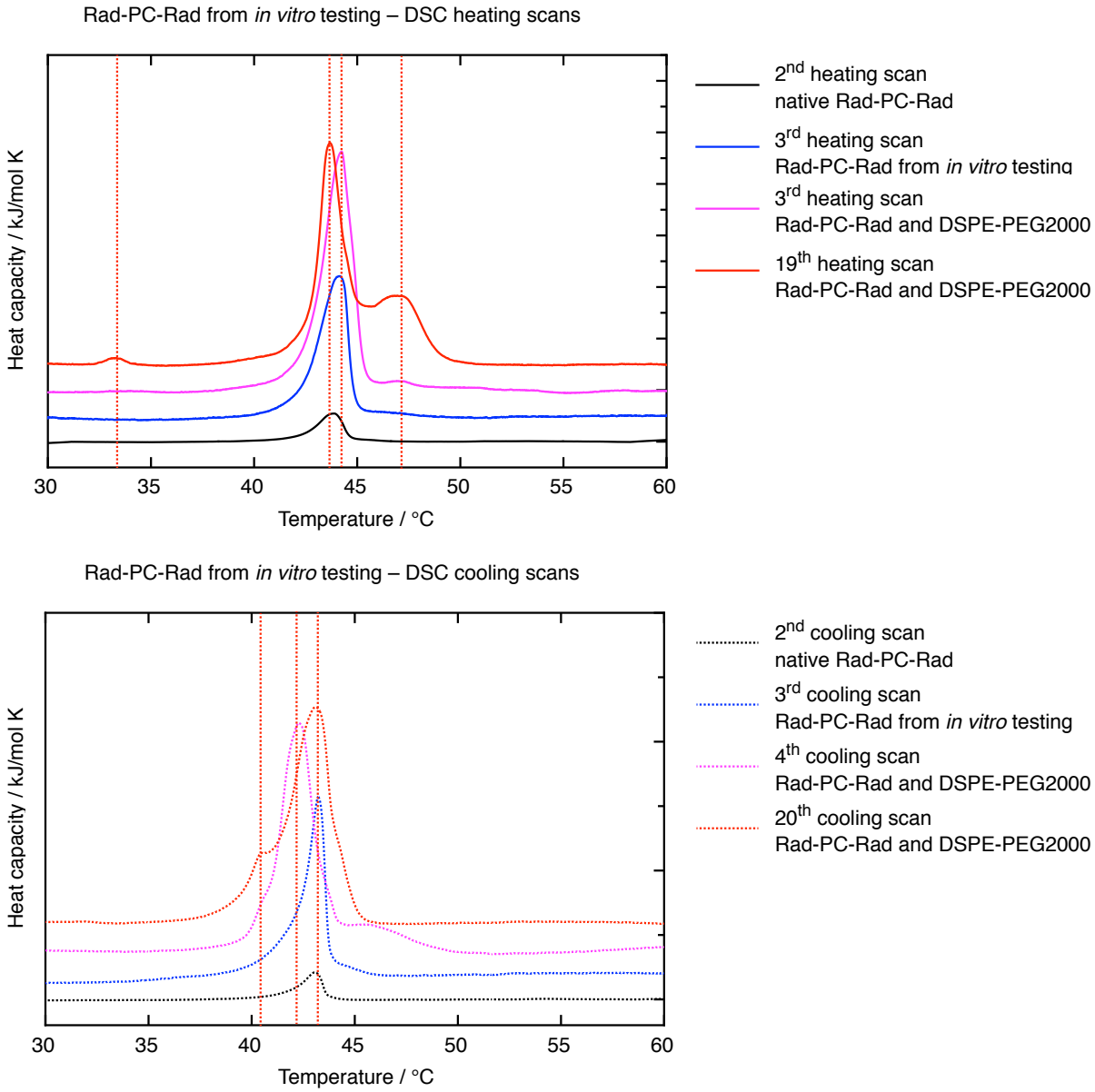

Figure S1. DSC heating and cooling curves measured for Rad-PC-Rad and Rad-PC-Rad/DSPE-PEG vesicles.

4. Complement immunoassay

The activation of the complement system is concentration- and time-dependent. Therefore, identification of the proper lipid concentration would help to avoid the appearance of unexpected side reactions. However, the applied concentration should be still therapeutically relevant. Therefore, before the conduction of the complement assay, we had to perform preliminary tests in order to find the appropriate lipid concentration for further studies. We tested two lipid formulations – R3 and R4. We started from the maximum concentration that we obtained after liposome preparation, which was 10 mg/mL. Those samples were diluted two, four and eight times. The observed results are presented in Figure S2. PEG-free sample R3 demonstrated an increase in complement activation, with increasing the lipid concentration. While PEG-containing sample R4 has shown slight decrease of SC5b-9 level with increasing the lipid concentration. Based on this preliminary result, we have decided to test Rad-PC-Rad liposomal formulations at two concentrations: 10 and 5 mg/mL.

Figure S3 demonstrates the level of complement fragments, induced by incubation with Rad-PC-Rad liposomes at higher and lower concentrations ('d' indicates 'diluted' samples). As it was initially observed by a preliminary test, PEG-free liposomal formulations, R1 and R3, show a statistical significance in the complement activation between concentrated and diluted samples (Figure 3B, D); while PEG-containing formulations, R2 and R4, show no statistical difference. This phenomenon was partially true in case of C3a and C5a, because the observed differences were too small to be detected statistically. No differences were observed in the concentration of C4d fragment, as the lectin pathway is not involved in the activation of the complement system.

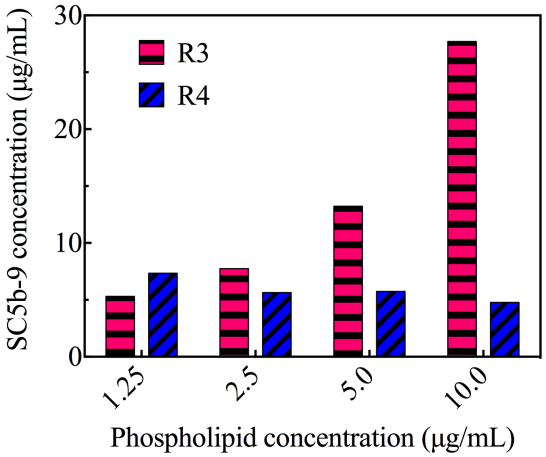

Figure S2. The level of SC5b-9 protein complex after incubation of one human sera with R3 and R4 liposomal suspensions at four phospholipid concentrations: 1.25, 2.5, 5.0 and 10 μg/mL. Incubation was performed for a period of 40 min at 37 °C.

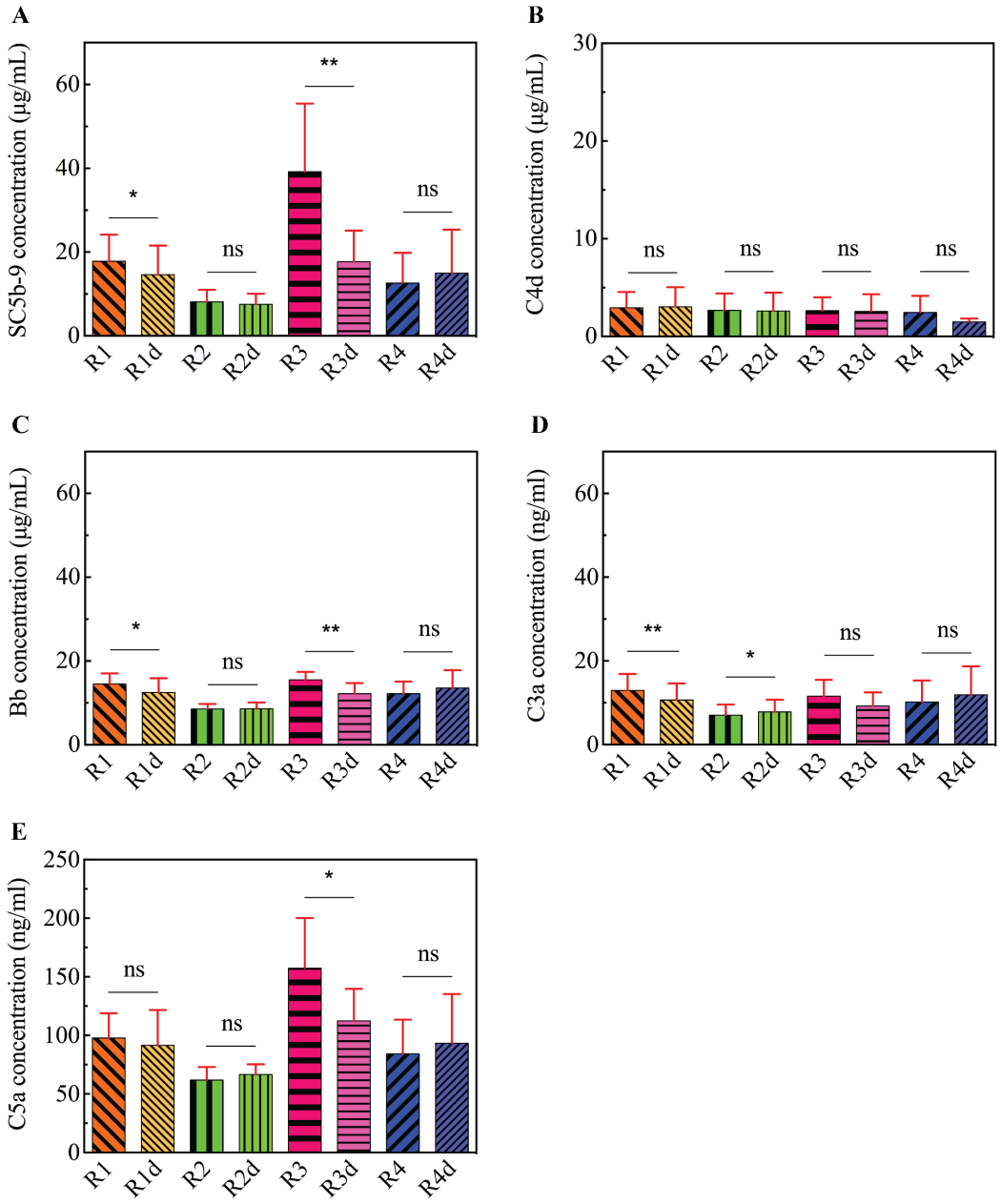

Figure S3. The level of SC5b-9 (A), C4d (B), Bb (C), C3a (D) and C5a (E) complement proteins after incubation of Rad-PC-Rad samples at two phospholipid concentrations: 10 µg/mL (R1, R2, R3, R4) and 5 µg/mL (R1d, R2d, R3d, R4d). Incubation was performed for a period of 40 min at 37 °C for all samples. Sera of donor #5 was excluded from the graph to better distinguish the contrast in complement activation between Rad-PC-Rad liposomal samples with and without DSPE-PEG. The data are represented as mean values with error bars derived from the standard deviation among six donors. The significance of differences among the corresponding groups was determined by paired T-test. *P*-values lower than 0.05 were considered as statistically significant.

To further proceed with the analysis, we examined the level of complement activation over time.

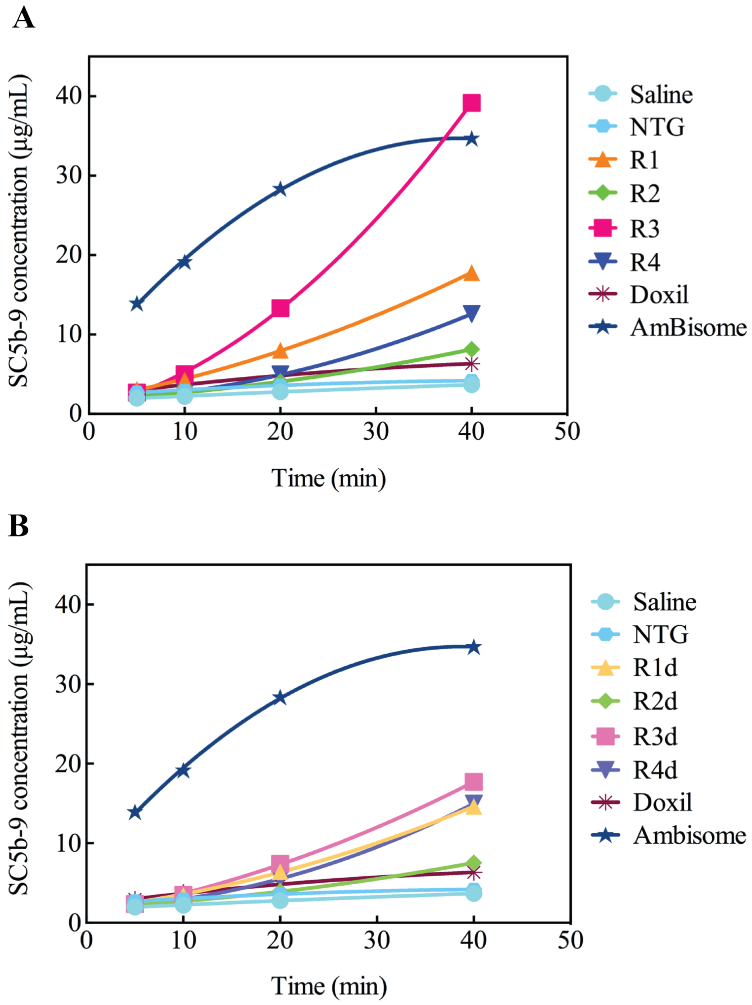

Figure S4. The level of TCC at various time points. SC5b-9 concentration from six human sera incubated at 37 °C with the negative controls, Rad-PC-Rad liposomes at (A) higher lipid content (10 mg/mL) and at (B) lower lipid content (5 mg/mL), Doxil and AmBisome. The reaction was terminated after 5, 10, 20, and 40 minutes. The data are shown as the mean value among the five donors. The serum of donor #5 was omitted for clarity.

Table S3 provides the concentration values of complement proteins. Human sera from six independent donors were incubated for a period of 40 minutes at a temperature of 37 °C with saline, nitroglycerin (NTG), Rad-PC-Rad liposomal suspensions of selected composition (R1, R2, R3, R4), Doxil® and AmBisome®.

Table S3. The concentration of SC5b-9, C4d, Bb, C3a and C5a complement proteins.

Complement protein	Donors	Treatment samples								
		Saline	NTG	R1	R2	R3	R4	Doxil®	AmBisome®	Zymosan
SC5b-9 (μg/mL)	#1	3.81	4.69	14.16	6.53	39.48	7.11	7.52	17.30	199.52
	#2	3.33	3.74	10.19	6.22	16.04	7.98	6.27	22.02	198.94
	#3	2.50	2.96	25.30	8.06	58.67	22.68	4.86	36.95	177.28
	#4	3.35	3.67	15.94	6.74	32.43	7.17	4.73	45.19	182.59
	#5	5.99	7.66	169.08	158.85	166.79	150.99	8.30	47.91	171.66
	#6	5.40	5.92	23.39	13.12	49.23	17.97	8.36	51.90	252.54
C4d (μg/mL)	#1	1.49	2.01	1.83	1.49	1.93	1.61	1.55	1.35	1.78
	#2	1.64	1.75	2.44	2.16	2.33	1.85	2.52	2.42	3.00
	#3	1.42	1.72	2.06	1.88	1.65	1.42	1.33	2.16	2.25
	#4	2.15	3.39	2.63	2.12	2.25	1.92	2.26	3.19	3.42
	#5	5.42	6.63	1.58	2.59	3.18	4.28	4.57	5.96	7.04
	#6	6.80	6.52	5.77	5.73	5.03	5.49	2.50	2.27	3.88
Bb (μg/mL)	#1	7.02	7.89	13.39	7.07	16.50	10.27	13.08	19.20	40.39
	#2	6.00	7.04	15.48	7.77	12.87	10.15	10.39	24.58	44.80
	#3	5.03	5.00	16.50	8.56	17.67	15.00	8.77	24.44	29.00
	#4	5.92	6.83	10.63	9.03	14.31	9.91	17.93	27.53	33.76
	#5	7.69	9.91	59.83	30.94	41.79	33.71	10.15	30.56	36.87
	#6	7.82	9.35	16.59	10.25	16.23	15.67	13.39	34.53	51.03
C3a (ng/mL)	#1	4.43	4.66	11.01	4.37	10.46	6.42	6.01	12.08	18.51
	#2	2.48	3.12	10.23	4.25	7.49	6.16	7.39	15.63	18.80
	#3	5.12	5.38	18.98	9.58	17.62	18.69	11.28	26.09	21.27
	#4	4.62	7.23	9.72	8.97	12.87	8.48	8.89	25.46	20.98
	#5	7.74	12.81	24.20	5.39	15.51	14.74	11.86	25.98	14.60
	#6	5.55	8.14	14.84	7.88	9.38	11.09	10.76	6.53	20.61
C5a (ng/mL)	#1	70.36	82.12	110.56	70.54	218.23	66.71	48.75	152.44	1471.22
	#2	56.86	50.12	88.96	66.26	115.40	92.51	69.72	171.31	1488.54
	#3	26.96	20.22	111.75	52.49	183.86	108.38	30.61	141.23	748.25
	#4	29.61	29.06	64.52	47.75	141.65	42.46	33.43	192.78	759.19
	#5	32.51	42.00	587.64	677.53	561.06	561.41	45.10	204.00	791.10
	#6	38.27	35.08	113.02	72.45	128.16	110.84	59.05	231.35	1344.49

5. Qualitative and quantitative analysis of WBCs

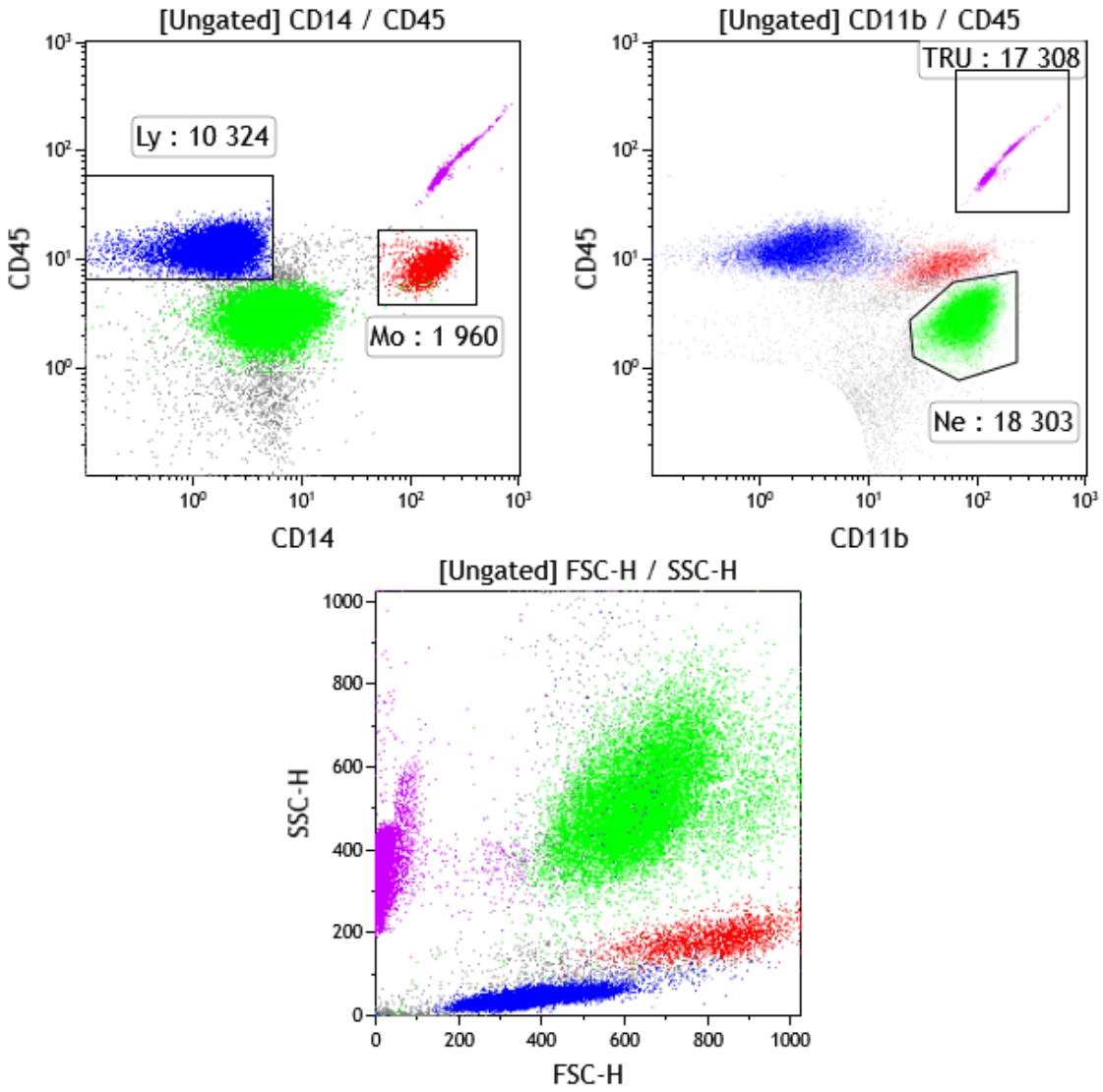

Figure S5. Gating strategy (upper panels) to identify the absolute cell concentration of samples' cells by flow cytometry. Scatter plot (lower panel, SSC/SSC) of gated cells is presented by the same color.

The cell content of samples was analyzed by flow cytometry using a FACScan instrument (BD Biosciences, USA). Before the actual tests, 50 µl of the cells were stained by antibody mixture containing fluorescein isothiocyanate (FITC) labeled anti-CD14, phycoerythrin (PE) labeled anti-CD11b and PerCP-Cy5.5 labeled anti-CD45 in TRUCount tubes (BD, cat.: 340334) for 15 min in the dark. Thereafter, WBC were resuspended in 450 µL of lysing solution (BD, cat.: 349202) for 15 min. As the employed TRUCount tubes contained a known number (48 100) of beads (gating name: TRU) measured together with cells, the exact cell concentrations of the original samples could be identified, since no centrifuge step was applied during the sample staining process. After gating, which strategy is summarized in Figure S5, the concentration and total cell number of monocytes (Mo), neutrophil granulocytes (Ne) and lymphocytes (Ly) was quantitatively determined as it is presented in Table S4. The number of WBCs was determined prior to the incubation with tested material. In case of buffy coat samples, the number of cells was determined twice: before (original) and after isolation of leukocytes. Cells were stained according to the procedure described above.

Table S4. Concentration of WBCs in the buffy coat (BC), before and after leukocytes isolation, and in whole blood (WB) samples.

	Cell concentration (cell/mL)			
	Monocytes	Neutrophils	Lymphocytes	Total cells
BC1 original	2'364'360	17'869'287	15'101'890	35'335'536
BC1 isolated cells	4'059'419	15'811'548	16'888'079	36'759'046
BC2 original	1'915'442	12'791'686	11'303'143	26'010'271
BC2 isolated cells	19'003'419	100'469'999	67'771'808	187'245'226
BC3 original	2'105'082	18'639'499	11'822'281	32'566'862
BC3 isolated cells	25'539'472	171'957'831	67'099'037	264'596'339
WB1	310'963	2'223'685	2'014'609	4'549'258
WB2	372'776	2'214'462	2'596'503	5'183'741

The viability of the cells after activation with test material and control agents was quantified by FITC labeled AnnexinV (Table S5) among (PerCP-Cy5.5 labeled) CD45 positive leukocytes. The percentage of dead cells were less than 3% after each treatment with the exception of zymosan, confirming that the observed phenomena in cytokine productions are not a consequence of different cell mortality after treatments.

Table S5. Cell viability assay of isolated leukocytes and whole blood after activation with liposomal formulations and corresponding control agents.

	Viable cells (%)						
Sample	PBS	MR5	R1	R2	R3	R4	Zymosan
BC2	98.4	98.18	98.06	98.12	98.34	98.37	64.32
WB2	97.57	98.43	97.49	97.75	97.53	97.95	60.52

6. Concentration of inflammatory cytokines

Table S6 provides the concentration values of inflammatory cytokines. WB and BC samples were incubated with R5 medium, PBS, Rad-PC-Rad liposomal suspension of selected composition (R1, R2, R3, R4) and zymozan at a temperature of 37 °C.

Table S6. The concentration of IL-6, IL-12p70, TNF-α, IL-1β, IL-8 and IL-10 inflammatory cytokines.

Cytokines	Donors	Samples						
		MR5	PBS	R1	R2	R3	R4	Zymosan
IL-6 (pg/mL)	WB1	1086.69	1950.84	15.83	1848.47	36.11	144.90	>5000.00
	WB2	98.70	86.46	0.98	81.19	14.65	86.46	>5000.00
	BC1	9.24	1.15	9.75	5.19	6.21	9.58	na
	BC2	1.32	nd	11.44	nd	nd	nd	>5000.00
	BC3	nd	nd	nd	nd	nd	nd	>5000.00
IL-12p70 (pg/mL)	WB1	1.80	2.00	3.40	1.20	1.20	2.00	20.51
	WB2	0.00	0.60	1.00	42.28	1.20	1.40	2.20
	BC1	1.20	0.80	1.00	1.20	1.00	1.40	na
	BC2	0.80	0.80	0.60	1.20	0.40	1.00	8.03
	BC3	1.00	0.80	1.00	1.40	0.80	0.60	4.20
TNF-α (pg/mL)	WB1	230.91	596.55	18.63	536.73	11.41	52.42	>5000.00
	WB2	10.83	11.80	0.50	12.19	5.57	12.97	>5000.00
	BC1	nd	nd	nd	nd	nd	nd	na
	BC2	nd	nd	nd	12.00	nd	nd	4519.33
	BC3	nd	nd	nd	nd	nd	nd	1656.93
IL-1β (pg/mL)	WB1	212.39	319.06	10.44	279.20	6.53	22.32	>5000.00
	WB2	7.24	3.33	2.14	4.16	2.14	5.93	>5000.00
	BC1	28.62	19.59	9.02	8.19	9.61	13.89	na
	BC2	7.24	7.12	11.04	8.66	16.62	7.60	>5000.00
	BC3	9.37	7.60	9.02	7.83	16.62	9.61	>5000.00
IL-8 (pg/mL)	WB1	>5000.00	>5000.00	4033.03	>5000.00	4747.50	>5000.00	>5000.00
	WB2	>5000.00	>5000.00	1497.28	>5000.00	2835.68	4565.74	>5000.00
	BC1	>5000.00	>5000.00	>5000.00	>5000.00	>5000.00	>5000.00	na
	BC2	>5000.00	>5000.00	>5000.00	>5000.00	>5000.00	>5000.00	>5000.00
	BC3	>5000.00	>5000.00	>5000.00	>5000.00	>5000.00	>5000.00	>5000.00
IL-10 (pg/mL)	WB1	nd	nd	nd	0.69	nd	nd	151.91
	WB2	nd	nd	nd	nd	nd	nd	148.40
	BC1	nd	nd	nd	nd	nd	nd	na
	BC2	nd	nd	nd	nd	nd	nd	314.32
	BC3	nd	nd	nd	nd	nd	nd	196.37

Cytokines detection limit (pg/mL): IL-6 – 2.5, IL-12p70 – 1.9, TNF-α – 3.7, IL-1β – 7.2, IL-8 – 3.6, IL-10 – 3.3. nd – not detected, na – not available.