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1 **Supplementary materials**
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3 Endothelial Cell Membrane-Based Biosurface for Targeted Delivery to Acute 4 Injury: Analysis of Leukocyte-Mediated Nanoparticle Transportation

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19 ## Experimental section

20 **Materials**

21 The human umbilical vein endothelial cells (HUVEC), the endothelial cell culture medium
22 (ECM), fetal bovine serum (FBS), penicillin/streptomycin (P/S) and endothelial cell growth
23 supplement (ECGS) were purchased from ScienCell (CA, USA). HL-60 and THP-1 cells were
24 obtained from the China Center for Type Culture Collection (China). Trypsin and DPBS were
25 obtained from Gibco (ThermoFisher, MA, USA). The human TNF-α was bought from PeproTech
26 (NJ, USA). The PLGA (50:50) (RG502H) and collagenase I came from Sigma. DiI, DiD and DiR for
27 the labeling of nanoparticles, and 4',6-diamidino-2-phenylindole (DAPI) was obtained from
28 the ThermoFisher (Waltham, MA). 4% polyformaldehyde (PFA) was purchased from the
29 BOSTER (Wuhan, China). The heparin sodium was from Solarbio (Beijing, China). The acetone
30 was bought from Beijing Tong Guang Fine Chemicals Company (Beijing, China). All of the
31 reagents for flow cytometry analysis such as the red blood cells (RBC) lysis buffer, fluorescence
32 labelled antibodies, the fixation buffer and Zombie Violet^{TM} Fixable Viability Kit were
33 purchased from Biolegend (CA, USA). The ethylene diamine tetraacetic acid (EDTA) and
34 primers were obtained from Sangon Biotech (Shanghai, China) respectively. Other chemicals
35 and instruments used were specified in each experiment.

36 **Activation of endothelial cells**

37 The HUVECs were incubated with ECM complete medium supplemented with 2% FBS, 1% P/S
38 and 1% ECGS and were activated by TNF-α with concentrations of 0, 10 or 100 ng/mL for 6 h.
39 After washed by DPBS for 2 times, the cells were lysed by the Trizol reagent (ThermoFisher,
40 MA, USA) and the mRNA was isolated and reverse transcribed with TransScript First-Strand
41 cDNA Synthesis SuperMix (Transgene, China, Beijing). The gene expression levels were
42 analyzed using the SYBR Green real-time PCR kit (Transgene, China, Beijing) and quantified

43 with the Bio-Rad CFX96 Real time PCR System (Bio-Rad, Hercules, CA). The gene expression
44 levels were normalized to GAPDH values respectively and the relative expression was
45 measured by the comparative CT method. Primers used were shown in **Table S1**. To
46 investigate the adhesion of monocytes to the activated endothelium, the HUVECs were
47 incubated in 96-well plate with density of 20,000 cells per well for 24 h. As described before,
48 endothelial cells were stimulated with or without 10 ng/mL of TNF-α for 6 h. After the
49 stimulation, 80,000 cells of THP-1 cells in RPMI complete medium were added to each well of
50 endothelial cells. Following 1h of co-incubation, the cells were washed by DPBS for 3 times
51 and the final THP-1 cells left in the wells were observed by microscopy. The images with 400X
52 magnification were randomly taken from 3 duplicate wells, the cell number of THP-1 cells in
53 the plate were counted and the average THP-1 numbers were analyzed for each experiment
54 group.

55 **Table S1.** The sequences of primers used in this study.

	Forward	Reverse
ICAM-1	ATGCCCAGACATCTGTGTCC	GGGGTCTCTATGCCCAACAA
VCAM-1	TTTGACAGGCTGGAGATAGACT	TCAATGTGTAATTTAGCTCGGCA
E-selectin	CAGCAAAGGTACACACACCTG	CAGACCCACACATTGTTGACTT
P-selectin	ATGGGTGGGAACCAAAAAGG	GGCTGACGGACTCTTGATGTAT
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

56

57 **Fabrication and characterization of nanoparticles**

58 To isolate the endothelial cell membranes, the cells were scraped and collected in DPBS with
59 5 mM EDTA. Cell membranes from about 30 million to 50 million cells were isolated by the
60 plasma membrane protein isolation kit (SM-005) from Invent Biotechnologies (MN, USA) and
61 stored in DPBS at -80°C before using. The membrane vesicles derived from the activated or
62 non-activated ECs were analyzed by label-free protein mass spectrometry to identify and
63 compare the relative abundance of proteins. Briefly, after treated by the lysing buffer

64 solutions (6% SDS, 200 mM DTT, 200 mM Tris-HCl, pH 7.6.) at 95°C for 5 min, the membrane
65 vesicles were loaded for the SDS-PAGE electrophoresis and the Coomassie-stained total
66 aggregated proteins from each sample were cut out of the gel which was further de-stained
67 with a solution of 100 mM ammonium bicarbonate in 50% acetonitrile. After the dithiothreitol
68 reduction and iodoacetamide alkylation, the proteins were digested with porcine trypsin
69 (Promega, Madison, WI) overnight at 37 °C. The resulting peptides were loaded to Thermo
70 Orbitrap Fusion Lumos after extracted from the gel pieces with 80% acetonitrile and 0.1%
71 formic acid (FA), dried in a vacuum centrifuge concentrator and resuspended in 0.1% FA. The
72 full MS spectra were acquired in the Orbitrap mass analyzer. The MS data were aligned with
73 the Escherichia coli Reviewed Swiss-Port database by the Proteome Discoverer 2.2 software.
74 Three independent samples were utilized for each group. The protein gel electrophoresis was
75 done to investigate the protein profiles of membrane vesicles and membrane-coated
76 nanoparticles. Briefly, the endothelial cell membrane related materials with 20 μg of proteins
77 were denaturalized by adding SDS-PAGE protein loading buffer (Beyotime, Shanghai, China)
78 and heated in boiling water for 5 min. The samples were added to the prepared gel with 4%
79 stacking gel and 12% resolving gel and the electrophoresis was run at 80 V for 30 min in the
80 stacking gel and 120 V for 50 min in the resolving gel. After the staining with the Commassie
81 Blue staining solution for 30 min at room temperature and decolorization with deionized
82 water, the gel was observed under the Gel Doc™ XR+ system (Bio-Rad, CA, USA).
83 To label the membrane vesicles with DiI, DiD or DiR, the membranes were incubated with 5
84 μM of dyes at 37°C for 15 min before the final washing step as the isolation kit described.
85 PLGA nanoparticles were prepared by a nanoprecipitation method in which 10 mg PLGA in
86 acetone was added dropwise into 3 mL water with stirring and evaporated at room
87 temperature overnight. The particle solution was purified using ultracentrifuge tubes with 10

88 k MWCO and was finally stored in water. To fabricate DiI, DiD or DiR labeled PLGA
89 nanoparticles, 10 µg of dye material was added into the PLGA acetone solution before
90 dropping into the water. The PLGA-PEG nanoparticles with or without labeling were fabricated
91 by the same protocol as PLGA nanoparticles. When PLGA nanoparticles and cell membranes
92 at weight ratio of 2:1 were co-extruded through an Avanti-mini extruder (Avanti Polar Lipids,
93 Inc, AL, USA), the cell membrane coated PLGA nanoparticles could be fabricated. Extra cell
94 membranes were excluded by centrifugation at 3000 g and the final hybrid nanoparticles were
95 stored in DPBS at 4°C. To observe the morphologies of nanoparticles, the bare PLGA (P)
96 nanoparticles with 0.5 mg/mL concentration were directly added onto the copper grids and
97 the membrane-containing materials were fixed by 4% PFA and 2% glutaraldehyde successively
98 before the addition of the uranyl acetate on to the copper grids. The dried copper grids were
99 imaged under the transmission electron microscope (TEM) imaging system (FEI Tecnai T20,
100 OR, USA) at 200 kV. The hydrodynamic size and zeta-potential of 0.5 mg/mL of nanoparticles
101 in water were analyzed by the dynamic light scattering (DLS) using a Zetasizer Nano ZSP system
102 (Malvern Panalytical, Malvern, UK).

103 **Cellular uptake of nanoparticles *in vitro***

104 To first investigate the adhesive of the membrane materials, membranes derived from
105 different cell resources (RAW246.7, J477.A and COS7 cells) were isolated and extruded
106 through the 400-nm pore size mini-extruder and the membrane vesicles dyed with DiI were
107 incubated with THP-1 cells for 1 h or 4 h. The flow cytometry analysis was used to quantify the
108 uptake efficiency of nano-vesicles. To further study the interaction of membrane coated
109 nanoparticles with leukocytes *in vitro*, the polymer nanoparticles were dyed with DiI and the
110 EM-P, P and PEG-P nanoparticles with the same fluorescence intensity were incubated with
111 THP-1 monocytes and HL-60 neutrophils for 1 h respectively. After washed by DPBS and fixed

112 by 4% PFA, the fluorescence intensity of cells was quantified by the flow cytometry analysis.
113 To observe the uptake of nanoparticles in cells, the membrane materials were dyed with DiD
114 and the P or PEG-P were dyed with DiI. After the incubation, washing and fixation, the cells
115 were observed under the confocal imaging system (Nikon A1R, Tokyo, Japan).

116 **Delivery efficiency in the acute lung inflammation model of mice**

117 This study was performed in strict accordance with the eighth edition of Guide for the Care
118 and Use of Laboratory Animals (Guide, NRC 2011) and was approved by the Institutional
119 Animal Care and Use Committee of Peking University (Beijing, China) which is fully accredited
120 by the AAALAC International. 6~8 weeks old BALB/c mice weighing about 15~18 g were used.
121 After the anesthetization, the mice were put in supine position and intranasally administrated
122 by1 μg/μL LPS in DPBS with LPS dose of 2 mg/kg. After the stimulation by LPS for 4 h, the
123 nanoparticles stained with DiR were intravenously injected with dose of 1 mg/kg of P and the
124 doses of other nanoparticles were adjusted to the same fluorescence intensity as P groups.
125 The organs were extracted after 24 h of administration and the fluorescence intensities were
126 detected by a live imaging system (IVIS Lumina Series III, PerkinElmer, MA, USA). The total
127 fluorescence intensities of organs were used to represent the content of nanoparticles and
128 the relative contents of nanoparticles in the lung to liver and spleen respectively were used to
129 indicate the delivery efficiency of nanoparticles *in vivo*.

130 **Cell distribution of nanoparticles in blood and in inflamed lung**

131 After the intravenous injection of DiI-labeled nanoparticles with the same dose in the study of
132 delivery efficiency, the blood at different time points and lung tissue at 24 h following injection
133 were extracted and the cell distribution of nanoparticles in leukocytes, mainly the neutrophils,
134 monocytes, macrophages, B cells and T cells, were analyzed. The erythrocytes were excluded
135 from the blood samples and the lung tissues were cut into pieces, digested by 3 mL of 3 mg/mL

136 collagenase D in RPMI medium at 37°C for 30 min and filtrated through 70 μm-cell filters to
137 obtain single cell suspensions. The cells were stained by the cocktail of antibodies
138 (PERCP/CY5.5-Ly6G (1A8), FITC-CD11b (M1/70), PE/CY7-Ly-6C (HK1.4), Alexa Fluor® 700-CD3 (17A2),
139 APC/Fire 750-CD19 (6D5), APC-F4/80 (BM8)) according to the protocol of antibody staining
140 provided by Biolegend (CA, USA). The anti-mouse CD16/32 was used as Fc blocker to exclude
141 the unspecific conjugation of antibodies. The True-Stain Monocyte Blocker™ from Biolegend
142 was used to exclude the unspecific identification of monocytes. After labeling with Zombie
143 Violet™ Fixable Viability Kit to exclude dead cells, the cells were fixed by Fixation Buffer
144 obtained from Biolegend and used for flow cytometry analysis.

145 **Investigation of nanoparticles in the zebrafish model**

146 According to protocols approved by the Peking University Animal Care and Use Committee,
147 zebrafish were raised and handled in a facility that is fully accredited by the AAALAC. At one
148 day post fertilization (dpf), the embryos were treated with 0.002% Phenylthiourea (PTU)
149 (Sigma, MO, USA) to prevent pigment synthesis. 3-day old Tg(*coro1a*:EGFP) zebrafish in which
150 leukocytes specifically expressed EGFP were put into a 6-mm dish and the tail fin of zebrafish
151 were cut by scalpel. Immediately after the amputation of tail fin, the zebrafish were
152 anesthetized with 0.01% tricaine (Sigma, MO, USA) and embedded in 1 % ultrapure low
153 melting point agarose (ThermoFisher) gel on a cover slip. The DiI labeled nanoparticles with
154 concentration of 2 μg/μL were injected into the zebrafish through the post cardinal vein (PCV)
155 by a microinjection system under a microscope. The outer diameter (OD) and inner diameter
156 (ID) of the vitric injection needle was 1 mm and 0.58 mm respectively and the volume of
157 nanoparticle solution was estimated to be about 10 nL per zebrafish. After the injection,
158 zebrafish were observed under a confocal system (Nikon A1R, Tokyo, Japan) and the real time
159 imaging of the nanoparticle distribution in the injured tail area of zebrafish were taken within

160 2 h. Following the real time imaging, the zebrafish were recovered from the embedded gel
161 and put into dishes with E2 water and incubated in the zebrafish incubator. 24 h post the
162 injection, the zebrafish were anesthetized and embedded again in 1 % agarose gel and the
163 whole-body imaging of zebrafish were taken. Specifically, 3D imaging with Z dimension
164 ranging from the top to bottom where the fluorescence signal of nanoparticles appeared and
165 disappeared respectively were taken at the head, main body and the tail of zebrafish
166 respectively. The Z dimension pictures of each body part were stacked and the stacked images
167 from the head, main body and tail were jointed into one picture to view the whole body of
168 zebrafish. According to the structure of the tissue, the areas of heart (H), liver (L), pancreas
169 (P), gall bladder (GB), gastrointestinal (GI) tract and tail (T) were marked out. The injured tail
170 areas were indicated as box with length of 500 µm distance to the apex of the tail.

171 **Statistical analysis**

172 Statistical comparisons between two groups were carried out using two tailed Student's t-test
173 analysis of variance. The comparisons above three groups were analyzed by one-way ANOVA
174 analysis followed by Tukey's multiple comparison test. All statistical tests were conducted
175 using GraphPad Prism software. $p < 0.05$ was considered to be statistically significant.

176

177 **Table S2.** Mass spectrum analysis of proteins in EM and NEM.

	EM	NEM
Protein name	**PSM**	**PSM**
Intercellular adhesion molecule 1 (ICAM-1)	176	15
Vascular cell adhesion protein 1 (VCAM-1)	171	ND
E-selectin	84	ND
Leukocyte surface antigen CD47 (CD47)	11	13

178 The values of peptide spectrum match (PSM) indicated the relative abundance of proteins.

179 Comparing to the non-activated endothelial cell membrane (NEM), the activated endothelial

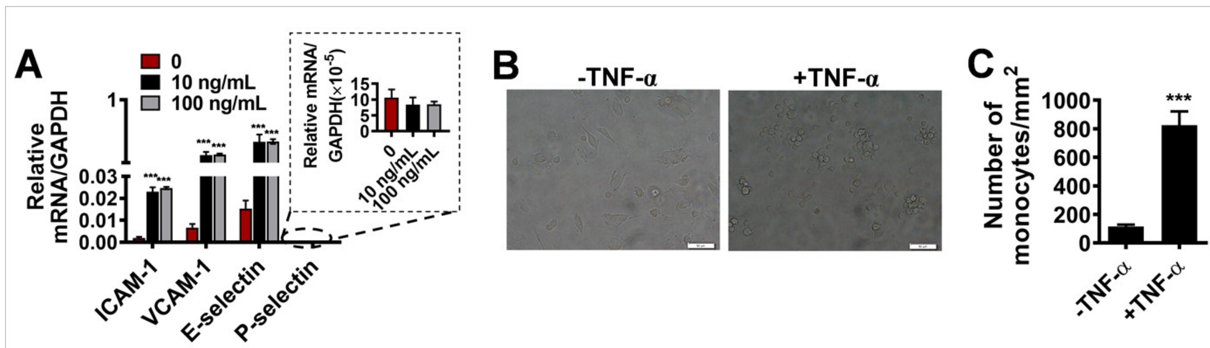
180 cell membrane (EM) contained adhesion molecules with much higher PSM values, suggesting

181 much higher abundance of these molecules in EM materials. The 'stealth' molecule, CD47, was

182 also detected in these membrane materials with similar contents. (ND, non-detected)

183
184

185

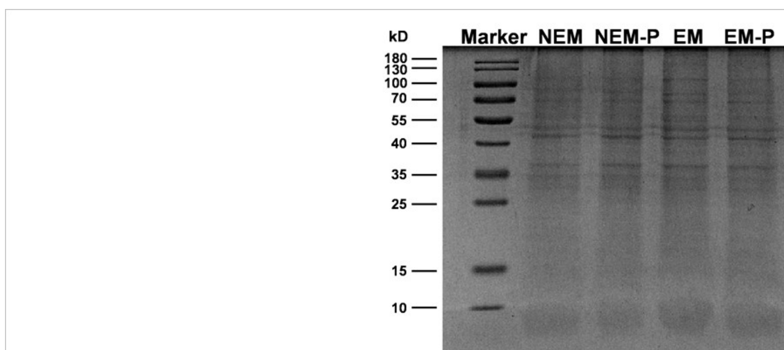


186 **Fig. S1** A) mRNA expression level of adhesion molecules in HUVECs with 0, 10 ng/mL or 100

187 ng/mL TNF-α stimulation. B) Microscopy images and C) the quantitative number of monocytes

188 adhere to HUVEC with or without TNF-α activation. (Mean ± SD, n=3; *** p<0.001)

189



190

191 **Fig. S2** Gel electrophoresis for protein profiles of non-activated endothelial cell membrane

192 vesicles (NEM) and activated endothelial cell membrane vesicles (EM) and their corresponding

193 membrane-coated PLGA nanoparticles (NEM-P and EM-P). The protein profiles in EM-P and

194 NEM-P were consistent with the profiles in the EM and NEM respectively, suggesting the

195 successful preservation and transfer of membrane proteins to the membrane-coated

196 nanoparticles.

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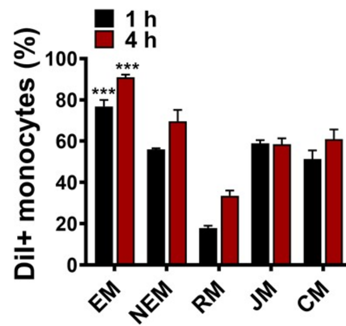
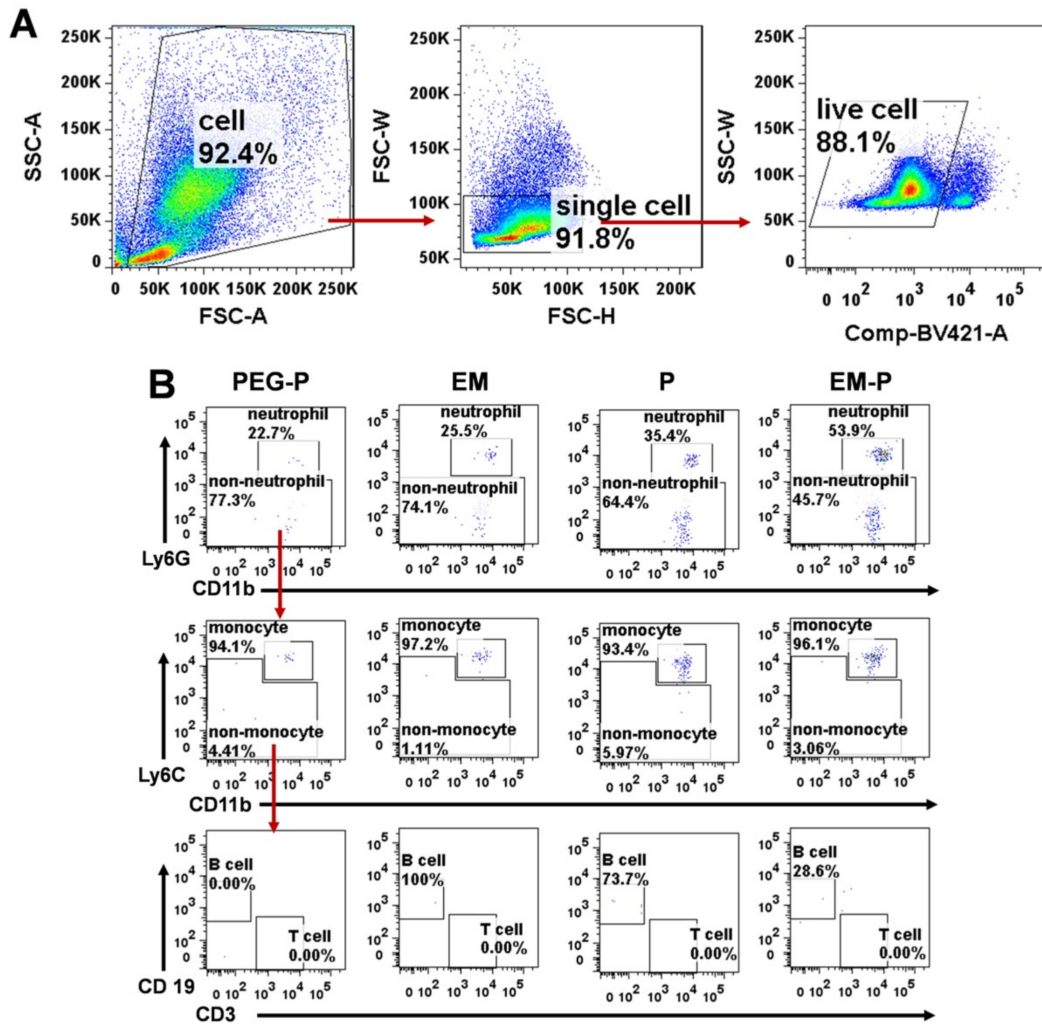


200 **Fig. S3** Cellular uptake efficiency of THP-1 monocytes which were incubated with the activated
201 endothelial cell membrane vesicles(EM), non-activated endothelial cell membrane vesicles
202 (NEM), RAW 246.7 cell membrane vesicles (RM), J774A.1 cell membrane vesicles (JM) and
203 COS7 cell membrane vesicles (CM). The result showed that the EM had higher affinity to bind
204 monocytes *in vitro* comparing to other kinds of membrane vesicles. (Mean ± SD, n=3; ***
205 p<0.001).

206

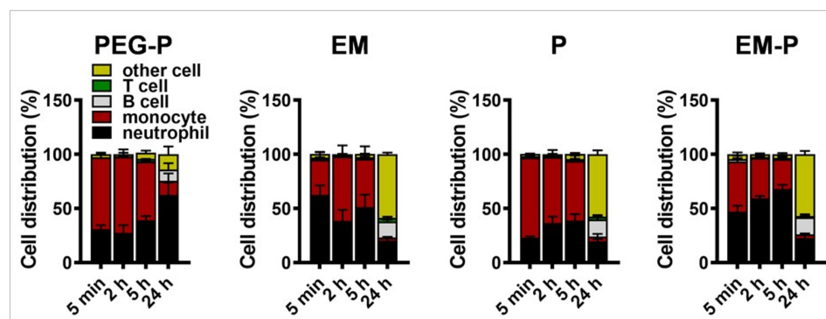


207 **Fig. S4** Examples of gates for A) the live cells and B) each kind of leukocytes in the flow

208 cytometry analysis for the blood samples.

209

210

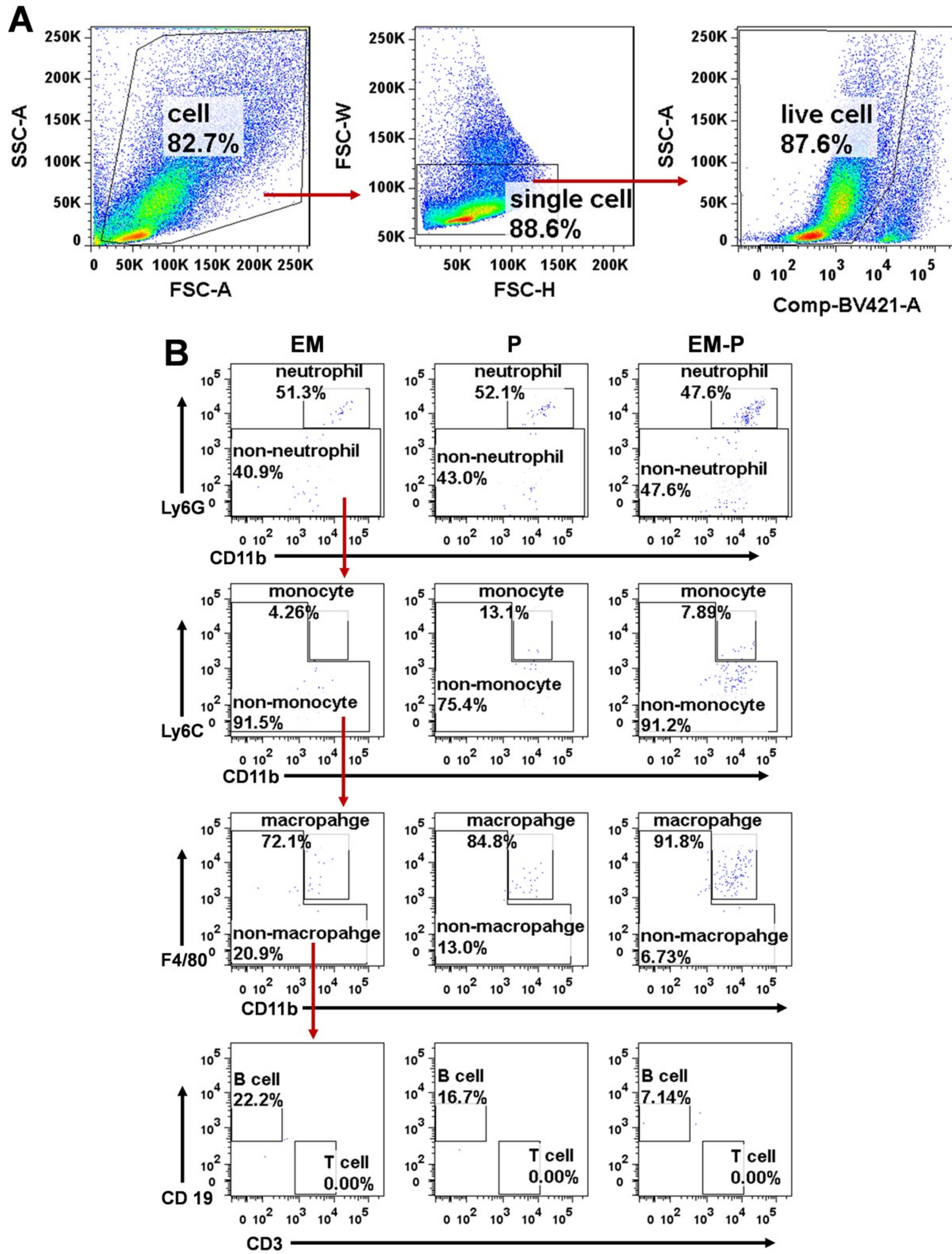


211 **Fig. S5** Statistical analysis from flow cytometry to investigate the cell distribution of

212 nanoparticles in the blood at different time points post administration. (Mean ± SEM, n=3~4).

213

214



215 **Fig. S6** Examples of gates for A) live cells and B) each kind of leukocytes in the lung by flow

216 cytometry analysis.

217

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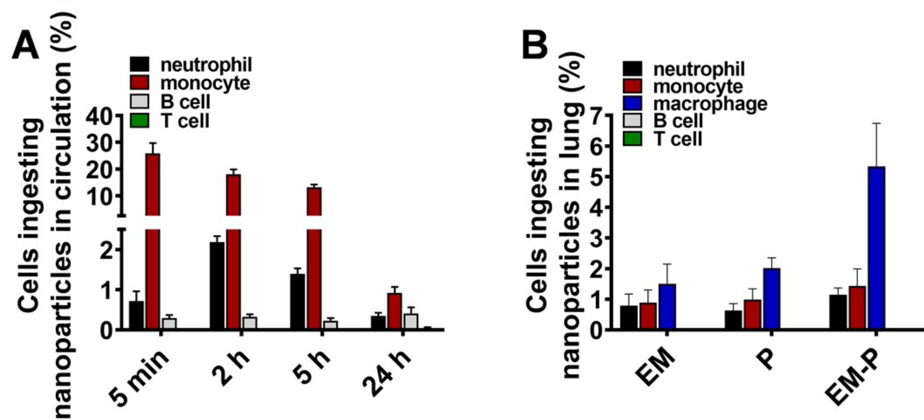

219 **Fig. S7** Percentages of each kind of leukocytes ingesting A) EM-P nanoparticles in the
220 circulation and B) EM, P and EM-P nanoparticles in the lung. The quantitative results from flow
221 cytometry analysis showed that about 2% of neutrophils in the circulation could absorb EM-P
222 (A) while only about 1% of neutrophils could take in EM-P in the lung (B). (Mean ± SEM, n=3~4)
223