

Leukocyte-mimetic liposomes possessing leukocyte membrane proteins pass through inflamed endothelial cell layer by regulating intercellular junctions

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1 **Abstract**

2 Nanoparticles such as liposomes have been applied for the treatment of various diseases
3 such as cancer and inflammatory diseases by utilizing the enhanced permeability and retention effect.
4 However, their entry into inflammation sites is still limited since passive delivery of nanoparticles is
5 often hampered by the presence of endothelial barriers. As leukocytes can pass through the inflamed
6 endothelium via utilizing membrane protein functions, we hypothesized that incorporating leukocyte
7 membrane proteins onto liposomal membranes may impart leukocyte-mimicking functions to
8 liposomes, allowing for their adherence to and active passage through the inflamed endothelium.
9 Herein, we developed leukocyte-mimetic liposomes (LM-Lipo) by leukocyte membrane protein
10 transfer and evaluated their function *in vitro*. Transfer of membrane proteins from human leukemia
11 cells onto liposomal membranes allowed for significant association of the liposomes with inflamed
12 human endothelial cells, and subsequent passage through inflamed endothelial cell layer. The
13 confocal images showed that LM-Lipo significantly induced vascular endothelial-cadherin
14 displacement. These results indicate that LM-Lipo adhered to and regulated intercellular junctions of
15 inflamed endothelial cell layer, resulting in passage through the layer, by mimicking the function of
16 leukocytes. Furthermore, it is suggested that liposomes possessing leukocyte-like functions could be
17 useful for drug delivery to inflammation sites by overcoming endothelial barriers.

18

19 **Keywords**

20 Liposome; Leukocyte; Intermembrane protein transfer; Inflamed endothelial cell layer; Vascular
21 endothelial-cadherin; Inflammatory disease

22

23 **Abbreviation:** ATRA, all-trans retinoic acid; CD11a, lymphocyte function-associated antigen-1;
24 CD11b, macrophage antigen-1; DCP, dicetylphosphate; DiIC₁₈,
25 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; DMPC,
26 dimyristoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; EPC, egg

- 1 phosphatidylcholine; F-actin, filamentous actin; ICAM-1, intercellular adhesion molecule-1; HL-60,
- 2 human promyelocytic leukemia cell; HUVEC, human umbilical vein endothelial cell; TNF- α , tumor
- 3 necrosis factor-alpha; VE-cadherin, vascular endothelial-cadherin
- 4

1 **1. Introduction**

2 To develop new therapies for the treatment of a variety of diseases, delivery of drugs using
3 nanoparticle drug delivery system (DDS), such as liposomes, has been broadly employed (Oku,
4 2017). For example, application of liposomes for cancer therapy is a well-known approach (Miao et
5 al., 2015), and our previous studies demonstrated the applicability of liposomes for the treatment of
6 cerebral ischemia/reperfusion injury, one of the inflammatory diseases (Fukuta et al., 2017; Ishii et
7 al., 2012). The therapeutic approaches for cancer and certain inflammatory diseases are based on
8 selective accumulation of liposomes in the diseased sites by passive extravasation through leaky
9 blood vessels by the enhanced permeability and retention effect (Maeda et al., 2000). Also, to
10 increase the accumulation amount and therapeutic efficacy of nanoparticles in the diseased sites,
11 their modification with specific targeting ligands (e.g., antibodies and peptides) has been broadly
12 reported (Byrne et al., 2008; Nishikawa et al., 2012; Zhao et al., 2016). However, nanoparticle entry
13 into the sites of inflammation-related diseases is still limited since delivery of such nanoparticles to
14 the diseased area is dependent on passive process and is often hampered by the presence of
15 biological barriers including endothelial cells (Anselmo et al., 2015; Blanco et al., 2015). Thus, to
16 achieve more secure drug delivery into the inflammation sites, functionalization of nanoparticles to
17 allow for their active passage through the inflamed endothelial barriers is needed.

18 In recent years, focus has been on application of circulatory cells (e.g., red blood cells,
19 leukocytes and platelets) as functional elements of drug carriers for the development of novel
20 nanoparticles that utilize the properties of those cells, namely, biomimetic DDS (Anselmo and
21 Mitragotri, 2014; Luk and Zhang, 2015). In particular, leukocytes are known to bind to inflamed
22 endothelial cells via certain adhesion molecules, including intercellular adhesion molecule-1
23 (ICAM-1), and migrate to the site of inflammation, which leads to further pathological progression
24 of inflammatory diseases via release of inflammatory mediators (Iadecola and Anrather, 2011;
25 Vestweber, 2015). Indeed, it was reported that synthetic nanoparticles decorated with leukocyte
26 membranes exhibit the ability to target inflamed tumor endothelium and to migrate into the diseased

1 area (Kang et al., 2017; Parodi et al., 2013). Based on these findings, we hypothesized that
2 incorporation of leukocyte membrane proteins onto liposomal membranes may allow them to obtain
3 leukocyte-mimicking functions, and facilitate the ability of the resultant liposomes to adhere to and
4 actively pass through the inflamed endothelium around the diseased site, similar to leukocytes.

5 As mentioned above, imparting biomimetic properties to nanoparticles is expected to be an
6 attractive strategy for targeted delivery to inflammation sites beyond the inflamed endothelial barrier.
7 However, for bringing about biomimetic properties to nanoparticles, complicated processes, loss of
8 protein activities, and difficulty in controlling physical properties (e.g., size and homogeneity) have
9 been mentioned to be improved (Millan et al., 2004; Molinaro et al., 2016). It was previously
10 reported that various membrane proteins can be spontaneously transferred to liposomal membranes
11 without the need for solubilization and protein reconstitution steps using detergents, organic solvents,
12 sonication, etc., a phenomenon known as intermembrane protein transfer (Huestis and Newton, 1986;
13 Newton and Huestis, 1988). Since the transferred proteins retain their native activity and orientation
14 in the lipid bilayer, intermembrane protein transfer was employed as a protein reconstitution method
15 to provide functions to liposomes as well as other materials (Kogure et al., 1997b; Okumura et al.,
16 1994). For example, a cancer vaccine was prepared by reconstitution of tumor antigens onto
17 liposomes via intermembrane protein transfer, and was subsequently demonstrated to result in
18 successful tumor regression in tumor model mice (Shibata et al., 1991). We previously also reported
19 that transfer of an influenza virus fusion protein, namely hemagglutinin, to erythrocyte ghosts
20 enabled the ghosts to fuse with cells, resulting in delivery of entrapped proteins into the cells
21 (Kogure et al., 2000). Hence, this protein reconstitution method is considered a promising approach
22 for easily imparting leukocyte-mimicking properties onto liposomes.

23 As one of the mechanisms of leukocyte passage through the inflamed endothelium
24 following binding to adhesion molecules, interaction of membrane proteins, namely lymphocyte
25 function-associated antigen-1 (LFA-1; CD11a) and macrophage antigen-1 (Mac-1; CD11b), with
26 ICAM-1 was reported (Diamond et al., 1991; van Buul et al., 2007). When leukocytes adhere to

1 inflamed endothelium via binding of both CD11a and CD11b to ICAM-1, activation of the ICAM-1
2 signaling pathway is induced, leading to an increase in permeability of the endothelial cells and
3 leukocyte infiltration into the inflammatory sites (Etienne-Manneville et al., 2000; Palomba et al.,
4 2016). Therefore, transfer of leukocyte membrane proteins including both CD11a and CD11b onto
5 liposomal membranes is considered to enable the liposomes to exhibit the ability to not only adhere
6 to but also pass through the inflamed endothelium via interactions with ICAM-1. To this end, we
7 used a human promyelocytic leukemia cell line, namely HL-60 cell, as a donor of leukocyte
8 membrane proteins in this study, since HL-60 cells constitutively express CD11a. Additionally,
9 CD11b expression can be induced by differentiation into neutrophil-like cells by the treatment with
10 all-trans retinoic acid (ATRA) (Trayner et al., 1998). For evaluating the function of liposomes, we
11 used tumor necrosis factor- α (TNF- α)-treated human umbilical vein endothelial cells (HUVECs) as a
12 model of inflamed endothelium since treatment with TNF- α was reported to induce ICAM-1
13 expression in HUVECs (Ramana et al., 2004).

14 In the present study, we aimed to develop liposomes possessing leukocyte-mimicking
15 functions, namely leukocyte-mimetic liposomes (LM-Lipo), via transfer of membrane proteins from
16 HL-60 cells. Then, by using TNF- α -treated HUVECs as a model of inflamed endothelium, we
17 investigated the ability of LM-Lipo to adhere to and actively pass through inflamed endothelial cell
18 layer *in vitro*.

19

1 **2. Material and Methods**

2

3 **2.1. Cell cultures**

4 The human promyelocytic leukemia cell line, HL-60, and human umbilical vein endothelial
5 cells (HUVECs) were purchased from DS Pharma Biomedical Co., Ltd (Osaka, Japan) and
6 PromoCell GmbH (Heidelberg, Germany), respectively. HL-60 cells were cultured in RPMI-1640
7 medium (Nacalai tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum, 100 U/mL
8 penicillin (Gibco, MA, USA), and 100 µg/mL streptomycin (Gibco). HUVECs were cultured in
9 endothelial growth medium (EGM-2, PromoCell GmbH) composed of endothelial basal medium
10 (EBM-2, PromoCell GmbH), 30 µg/mL gentamicin sulfate (30 µg/mL)/amphotericin B (15 ng/mL)
11 (GA-1000; Lonza, Tokyo, Japan) and endothelial cell growth medium-2 supplement pack
12 (PromoCell GmbH). HUVECs used in the present study were between passage 3 and 6. The cells
13 were cultured at 37°C in a 5% CO₂ incubator.

14

15 **2.2. Differentiation of HL-60 cells**

16 HL-60 cells (1.0 x 10⁶ cells/3 mL) seeded onto a 60-mm dish were incubated in RPMI-1640
17 medium containing 1 µM all-trans retinoic acid (ATRA; Wako Pure Chemical, Osaka, Japan)
18 dissolved in dimethyl sulfoxide (DMSO; Wako Pure Chemical). The final concentration of DMSO in
19 the media was 0.1%. For the non-differentiated group, cells were incubated with 0.1% DMSO. After
20 incubation for 0, 24, 48, 72, 96, and 120 h, the cells were harvested and washed with
21 phosphate-buffered saline (PBS). The cells were then incubated with Fc receptor blocking solution
22 (Human TruStain FcX™; Biolegend, San Diego, CA, USA) at 4°C for 10 min, followed by with
23 Alexa Fluor 488-conjugated anti-human CD11b antibody (Biolegend) at 4°C for 30 min. After
24 washing the cells twice with PBS, the proportion of CD11b-positive cells, namely
25 neutrophil-differentiated HL-60 cells, was measured by flow cytometry (Gallios; Beckman Coulter,
26 CA, USA).

1 **2.3. Western blotting**

2 Anti-CD11a rabbit monoclonal antibody (ab52895; Abcam, Cambridge, UK), anti-CD11b
3 rabbit monoclonal antibody (ab133357; Abcam), anti- β -actin mouse monoclonal antibody (sc-47778;
4 Santa Cruz Biotechnology, CA, USA), horse-radish peroxidase (HRP)-conjugated anti-rabbit IgG
5 polyclonal antibody (A24531; Thermo Fisher Scientific, Waltham, MA, USA), and HRP-conjugated
6 anti-mouse IgG rabbit polyclonal antibody (ab97046; Abcam) were purchased from the sources
7 indicated. To examine the expression of CD11a and CD11b in HL-60 cells, the media of the cells
8 cultured in the presence of 1 μ M ATRA or 0.1% DMSO for 96 h were removed following
9 centrifugation at 1,000 g for 5 min at 4°C. The cells were washed with PBS, centrifuged, and the
10 supernatant was subsequently removed. This cycle was repeated three times. The cells were then
11 lysed with lysis buffer comprised of 1% Triton-X100, 10 mM Tris (pH 7.5), 50 μ g/mL aprotinin, 200
12 μ M leupeptin, 2 mM phenylmethylsulfonyl fluoride, and 100 μ M pepstatin A, followed by
13 determination of protein concentration using a bicinchoninic acid (BCA) Protein Assay Reagent Kit
14 (Pierce Biotechnology, Rockford, IL, USA). The resultant samples (10 μ g protein) were subjected to
15 10% SDS-PAGE, and the proteins were electrophoretically transferred to a polyvinylidene difluoride
16 (PVDF) membrane (Bio-Rad, Hercules, CA, USA). The PVDF membrane was incubated with 3%
17 bovine serum albumin (BSA) dissolved in Tris-HCl-buffered saline containing 0.1% Tween20 (pH
18 7.4) for 1 h at 37°C, followed by incubation with anti-CD11a at a dilution of 1:5000, anti-CD11b
19 antibody (1:1000) or anti- β -actin antibody (1:20000) for 24 h at 4°C, respectively. The PVDF
20 membrane was then incubated with a secondary antibody at a dilution of 1: 2000 for rabbit IgG or
21 1:50000 for mouse IgG for 1 h at 37°C. Each protein was detected using a LAS-4000 mini system
22 (Fuji Film, Tokyo, Japan) after incubation with a chemiluminescent substrate reagent (ECL prime;
23 GE Healthcare, Little Chalfont, UK).

24

25 **2.4. Preparation of liposomes**

26 Egg phosphatidylcholine (EPC) and dicetylphosphate (DCP) were purchased from

1 Sigma-Aldrich (Tokyo, Japan). Dioleoylphosphatidylethanolamine (DOPE) and
2 dimyristoylphosphatidylcholine (DMPC) were purchased from NOF Corporation (Tokyo, Japan).
3 Liposomes composed of EPC/DCP (7/3 molar ratio), EPC/DCP/DOPE (3.5/3/3.5 molar ratio),
4 DMPC/DCP (7/3 molar ratio), and DMPC/DCP/DOPE (3.5/3/3.5 molar ratio) were prepared by the
5 thin-film method. The above lipids dissolved in chloroform were added to test tubes and dried using
6 nitrogen gas. To prepare fluorescence-labeled liposomes, 1,
7 1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI₁₈; Thermo Fisher Scientific,
8 Waltham, MA, USA) dissolved in chloroform was added to the initial lipid solution at a
9 concentration of 1 mol% of total lipid. The lipid film was hydrated with 0.3 M sucrose phosphate
10 buffer (pH 7.4), and the liposomal suspensions were freeze-thawed for three cycles using dry-ice
11 ethanol. The size of liposomes was then adjusted by extrusion through polycarbonate membrane
12 filters with 100-nm pores (Nuclepore, Cambridge, MA, USA). The particle size and ζ -potential of the
13 liposomes were measured with a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK).

14

15 **2.5. Intermembrane protein transfer**

16 After culturing HL-60 cells in the presence of 1 μ M ATRA or 0.1% DMSO for 96 h, the
17 media were removed following centrifugation at 1,000 g for 5 min at 4°C. The cells were washed
18 with PBS and centrifuged, followed by removal of the supernatant. This cycle was repeated three
19 times. Thereafter, the liposomal suspensions (1 mM as total lipids, 1 mL) were added and incubated
20 with the cells (5×10^6 cells/1 mL of 1 mM liposomes) in a 35-mm dish for 90 min at 37°C with
21 shaking. As a control, 0.3 M sucrose phosphate buffer was also incubated with the cells. The
22 incubated liposomes and 0.3 M sucrose phosphate buffer were harvested, centrifuged at 2,000 g for 1
23 min, and the supernatants were recovered and centrifuged to remove the cells. This cycle was
24 repeated five times. The recovered samples were used in the following experiments.

25

26

1 **2.6. Observation of protein transfer onto liposomes**

2 To prepare the liposome samples for BCA assay and SDS-PAGE, 1 mL of the recovered
3 liposomes (1 mM) following intermembrane transfer were ultracentrifuged at 112,500 *g* for 60 min at
4 4°C (Optima L-90K; Beckman Coulter, Tokyo, Japan). The pellets of the liposomes were then
5 resuspended in 100 µL of 0.3 M sucrose phosphate buffer to yield a liposome concentration of 10
6 mM. To prepare the control sample, the incubated and recovered 0.3 M sucrose phosphate buffer was
7 also ultracentrifuged as described above. For determining protein concentration of the resultant
8 liposomes, the liposomes were solubilized in 1% Triton-X100, and then protein concentration of
9 each liposomal sample was measured by using a BCA Protein Assay Reagent Kit (Pierce
10 Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. In the case of
11 SDS-PAGE, the resultant samples (0.2 µmol as lipid concentration for the liposome samples or 10 µg
12 protein for the cellular samples) were exposed to 10% SDS-PAGE. The proteins were then
13 electrophoretically transferred to a PVDF membrane following SDS-PAGE. The PVDF membrane
14 was then incubated with 3% BSA in Tris-buffered saline containing 0.1% Tween®20, each primary
15 antibody, and secondary antibody, as described above. Finally, the proteins were incubated with ECL
16 prime, and then detected with a LAS-4000 mini system. Quantification of signal intensities of each
17 band in the images of Western blotting was performed by using an image-analysis system (ImageJ;
18 National Institutes of Health, Bethesda, MD, USA). To calculate transfer efficiency of CD11a and
19 CD11b onto liposomes, proportions of CD11a and CD11b in HL-60 cells were determined by silver
20 staining (Silver Stain 2 Kit Wako, Wako Pure Chemical) following SDS-PAGE.

21

22 **2.7. Evaluation of liposome association with HUVECs**

23 HUVECs were seeded onto a 35-mm glass bottom dish at a density of 1×10^5 cells/dish and
24 incubated overnight. The cells were then treated with TNF- α (10 ng/mL in EGM-2) for 16 h. DiIC₁₈
25 (DiI)-labeled liposomes composed of EPC/DCP/DOPE (3.5/3/3.5 molar ratio) or DMPC/DCP/DOPE
26 (3.5/3/3.5 molar ratio) were incubated with non-differentiated or differentiated HL-60 cells as

1 described above (see Intermembrane protein transfer). Liposomes mixed in EBM-2 at a
2 concentration at 0.5 mM (as total lipids) were added onto TNF- α -treated HUVECs for 3 h at 37°C.
3 After removing the liposomes and washing twice with PBS, the cells were fixed with 4%
4 paraformaldehyde (PFA) for 1 h at 37°C. The cells were then washed with PBS three times, and then
5 incubated with PBS containing 0.1% Triton X100 for 15 min at 37°C, followed by incubation with 1
6 $\mu\text{g/mL}$ 4', 6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific) in PBS for 15 min at 37°C.
7 After washing with PBS, the fluorescence was observed using a fluorescence microscope (Axio
8 Vert.A1, Carl Zeiss, Jena, Germany).

9 To quantify the amount of the liposomes associated with HUVECs, the cells were seeded
10 onto 24-well plate at a density of 5×10^4 cells/well, and treated with TNF- α as mentioned above.
11 Following treatment with each liposomal sample for 3 h, the cells were washed with PBS and lysed
12 with 1% n-octyl- β -D-glucoside (Dojindo, Kumamoto, Japan). The fluorescence intensity of DiI was
13 determined using a Tecan Infinite M200 microplate reader (Salzburg, Männedorf, Switzerland).
14 Thereafter, the total protein content was measured using a BCA Protein Assay Reagent Kit.

15

16 **2.8. Transwell permeability assay**

17 HUVECs were seeded onto a 6.5-mm Transwell plate with 8- μm pore polyester membrane
18 inserts (Costar, Corning, Kennebunk, ME, USA) at a density of 2×10^5 cells/insert. The inserts were
19 pre-incubated with 0.1% gelatin in PBS for 1 h at 37°C, followed by EGM-2 overnight before
20 seeding. At 48 h after seeding, the media on both the upper and bottom compartments were removed,
21 and EGM-2 containing TNF- α (10 ng/mL) was then added to both compartments. At 16 h after
22 incubation with TNF- α , the cells were treated with the liposomes (50 nmol as lipid dose/insert)
23 incubated with non-differentiated or differentiated HL-60 cells, which liposomes were mixed in
24 EBM-2 at a concentration of 0.5 mM. At 3 h after incubation, the media in the bottom compartment
25 was collected and the DiI fluorescence was measured using a Tecan Infinite M200 microplate reader
26 (excitation: 549 nm, emission: 592 nm).

1 **2.9. Actin staining and immunostaining**

2 HUVECs were seeded onto a 35-mm glass bottom dish coated with 0.1% gelatin and
3 incubated overnight. The cells were treated with TNF- α and liposomes as described above (see
4 Evaluation of liposome association with HUVECs). For filamentous actin (F-actin) staining, cells
5 were treated with liposomes for 3 h at 37°C, washed with PBS, and subsequently fixed in 4% PFA
6 for 10 min at 37°C following liposomal incubation. After washing twice with PBS, HUVECs were
7 permeabilized with PBS containing 0.1% Triton-X100 for 5 min at 37°C. The cells were then
8 incubated in PBS containing 1% BSA for 20 min at 37°C, followed by incubation with Alexa Fluor
9 488 phalloidin in PBS (1:40; Thermo Fisher Scientific) for 20 min at room temperature. After
10 washing with PBS, the cells were stained with DAPI, and the fluorescence was observed by confocal
11 laser scanning microscopy (LSM700, Carl Zeiss).

12 For immunostaining of vascular endothelial-cadherin (VE-cadherin), cells were washed with
13 PBS, fixed with 4% PFA for 20 min at 37°C, and permeabilized in PBS containing 1% BSA and
14 0.1% TritonX-100 for 20 min. The cells were then incubated with rabbit anti-VE-cadherin antibody
15 (2.5 $\mu\text{g}/\text{mL}$; ab33168; Abcam) for 1 h at 37°C, followed by washing with PBS containing 1% BSA
16 and then incubation with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200; ab150077; Abcam)
17 for 1 h at 37°C. After washing, the nuclei were stained with 1 $\mu\text{g}/\text{mL}$ DAPI solution for 15 min at
18 37°C. The fluorescence of the cells was monitored using a confocal laser scanning microscope. To
19 evaluate level of VE-cadherin expression, the experiments were performed 5 times independently,
20 and fluorescence intensities of VE-cadherin positive areas between cell borders were analyzed for
21 total 40 or more images per each group of HUVECs using an image-analysis system ImageJ.

22

23 **2.10. Statistical analysis**

24 Statistical differences were evaluated by one-way analysis of variance (ANOVA) with the
25 Tukey *post-hoc* test. Data were presented as mean \pm S.D.

26

1 **3. Results**

2

3 **3.1. Differentiation of HL-60 cells into neutrophil-like cells by ATRA**

4 We firstly evaluated the differentiation of HL-60 cells into neutrophil-like cells by ATRA
5 treatment for the preparation of donor cells to transfer the leukocyte membrane proteins CD11a and
6 CD11b onto liposomal membranes. Flow cytometry results showed that cultivation of HL-60 cells in
7 the presence of 1 μ M ATRA significantly increased CD11b-positive cells in a time-dependent
8 manner following initiation of ATRA treatment, whereas CD11b-positive cells did not increase in
9 HL-60 cells cultured in the absence of ATRA (Figs. 1A and B). Since the percentage of
10 CD11b-positive cells approached nearly 80% at 96 h after ATRA treatment and remained unchanged
11 at 120 h (Fig. 1B), the differentiation of HL-60 cells into neutrophil-like cells was certainly achieved
12 by the ATRA treatment for 96 h. Western blotting showed that HL-60 cells treated with ATRA for 96
13 h expressed both CD11a and CD11b, whereas those not treated with ATRA did not express CD11b
14 (Fig. 1C). Based on these results, we decided to use those cells treated with ATRA for 96 h as
15 neutrophil-differentiated cells, i.e., as donor cells of leukocyte membrane proteins, in subsequent
16 experiments. On the other hand, the expression level of CD11a was significantly reduced to
17 approximately 60% by the treatment with ATRA (Fig. 1D and Supplementary Fig. 1).

18

19 **3.2. Intermembrane transfer of leukocyte membrane proteins from HL-60 cells onto liposomes**

20 Using both differentiated and non-differentiated HL-60 cells, we examined the
21 intermembrane protein transfer of membrane proteins onto liposomal membranes. Since the protein
22 transfer was previously reported to be facilitated by increasing membrane fluidity of recipient
23 liposomes (Waters et al., 1996), we prepared liposomes composed of EPC (containing unsaturated
24 phospholipids) and liposomes composed of the saturated phospholipid DMPC to confirm the
25 influence of membrane fluidity on protein transfer efficiency. Moreover, as previous studies reported
26 that the efficiency of protein transfer to liposomes is increased by the presence of phase separation

1 via incorporation of the amphiphilic compound DCP into the liposomes (Kogure et al., 1997a;
 2 Kogure et al., 1999), we also prepared liposomes containing DCP. In addition, we incorporated the
 3 fusogenic phospholipid DOPE, because intermembrane protein transfer is induced by partial fusion
 4 between cellular membranes and liposomal membranes (Huestis and Newton, 1986; Newton and
 5 Huestis, 1988). Particle size, polydispersity index, and ζ -potential of liposomes used in the present
 6 study are shown in Table 1. The particle size and ζ -potential of each of the prepared liposomes was
 7 approximately 100-150 nm and -10 mV, respectively. To determine total protein concentration in the
 8 recovered liposome suspensions, BCA assay was performed for each liposome suspension incubated
 9 with differentiated or non-differentiated HL-60 cells. The results showed that the protein
 10 concentration tended to increase by incorporating DOPE in both EPC- and DMPC-based liposomes,
 11 and the concentrations were higher in the case of liposomal suspension been incubated with
 12 differentiated HL-60 cells (ATRA (+)) than that with non-differentiated cells (ATRA (-)) (Fig. 2). On
 13 the other hand, the particle sizes of the liposomes increased and the ζ -potentials became more
 14 negatively charged after incubation with HL-60 cells (Table 1).

15

16 **Table 1.** Physicochemical properties of liposomes before and after intermembrane protein transfer.

	Particle size (d. nm)		Polydispersity index		ζ -Potential (mV)	
	Before	After	Before	After	Before	After
EPC/DCP -Lipo	113.9 ± 12.2	126.0 ± 18.0	0.26 ± 0.11	0.24 ± 0.03	-9.7 ± 1.0	-13.7 ± 2.5
EPC/DCP/ DOPE-Lipo	109.6 ± 12.2	150.7 ± 14.4	0.21 ± 0.09	0.35 ± 0.08	-11.3 ± 3.6	-16.0 ± 1.8
DMPC/DCP -Lipo	145.0 ± 28.0	185.0 ± 61.1	0.37 ± 0.11	0.49 ± 0.13	-9.8 ± 1.4	-13.3 ± 1.4
DMPC/DCP/ DOPE-Lipo	119.2 ± 12.2	140.3 ± 17.1	0.24 ± 0.12	0.35 ± 0.08	-10.9 ± 2.7	-15.1 ± 2.4

17

18

1 Next, transfer of CD11a and CD11b onto liposomes was evaluated by Western blotting. The
2 results of Western blotting showed that the presence of CD11a was observed in liposomal samples
3 incubated with HL-60 cells (Fig. 3A). Importantly, CD11b was only observed in those liposomes
4 incubated with ATRA-treated neutrophil-differentiated HL-60 cells (Fig. 3B). Considering the
5 differences in liposome composition, in the groups of liposomes containing DOPE, both CD11a and
6 CD11b were more clearly detected compared with the groups of liposomes containing only DCP
7 (EPC/DCP-Lipo and DMPC/DCP-Lipo). However, neither CD11a nor CD11b was observed upon
8 incubation of the cells with 0.3 M sucrose phosphate buffer. Quantitative data of Western blotting
9 indicated that transfer amount of CD11a was significantly higher in the group of
10 EPC/DCP/DOPE-Lipo compared with EPC/DCP-Lipo and DMPC/DCP-Lipo, and also higher than
11 that of DMPC/DCP/DOPE-Lipo (Figs. 3C and D). In the case of CD11b, its transfer amount tended
12 to be higher in the groups of liposomes containing DOPE (Fig. 3E). Since proportions of CD11a and
13 CD11b in HL-60 cells were indicated to be approximately 1 % from silver staining following
14 SDS-PAGE (data not shown), transfer efficiency of HL-60 cell membrane proteins, and that of
15 CD11a and CD11b onto liposomes was estimated as shown in Supplementary Table 1. The data also
16 indicated that incorporation of DOPE into liposomes could increase transfer efficiency of both
17 CD11a and CD11b. Based on these results, it was indicated that transfer of leukocyte membrane
18 proteins CD11a and CD11b onto liposomes was achieved by intermembrane protein transfer. The
19 resultant liposomes possessing leukocyte membrane proteins were designated as “leukocyte-mimetic
20 liposomes (LM-Lipo)”. EPC/DCP/DOPE-Lipo (EPC/DO-Lipo) and DMPC/DCP/DOPE-Lipo
21 (DMPC/DO-Lipo) were used in subsequent experiments.

22

23 **3.3. Association of leukocyte-mimetic liposomes (LM-Lipo) with TNF- α -treated HUVECs**

24 Next, association of LM-Lipo with inflamed endothelial cells was investigated using
25 TNF- α -treated HUVECs as an *in vitro* model, since it has been reported that treatment with TNF- α
26 can induce ICAM-1 expression in HUVECs (Ramana et al., 2004). We confirmed that expression of

1 ICAM-1 was time-dependently and significantly increased at 16 h after TNF- α treatment, at which
2 point ICAM-1 expression became the highest under the experimental conditions (Supplementary Fig.
3 2). By using fluorescence (DiI)-labeled liposomes, we evaluated the association of LM-Lipo with
4 TNF- α -treated HUVECs using a fluorescence microscope. The results showed that the DiI
5 fluorescence of HUVECs treated with leukocyte-mimetic EPC/DO-Lipo (LM-EPC/DO-Lipo) or
6 DMPC/DO-Lipo (LM-DMPC/DO-Lipo) was stronger than plain liposomes regardless of whether
7 HL-60 cells had been differentiated (ATRA (+)) or not (ATRA (-)) (Figs. 4A, B, D, and E).
8 Importantly, higher fluorescence intensities were observed for TNF- α -treated HUVECs in both the
9 LM-EPC/DO-Lipo and LM-DMPC/DO-Lipo groups compared with untreated HUVECs. The
10 confocal images also showed that DiI fluorescences in TNF- α -treated HUVECs incubated with
11 LM-EPC/DO-Lipo (ATRA (-)) and ATRA (+)) or LM-DMPC/DO-Lipo (ATRA (+)) were higher than
12 plain liposomes (Supplementary Fig. 3). Also, some areas with higher fluorescence than other areas
13 were observed, which were considered to be resulted from the liposomes localized in endosomes.
14 The DiI fluorescence was also determined by lysing the cells, and the results indicated that the
15 fluorescence intensity of the cells treated with LM-Lipo was significantly higher than that with plain
16 liposomes in TNF- α -treated HUVECs (Figs. 4C and F). Moreover, LM-EPC/DO-Lipo incubated
17 with non-differentiated (LM-EPC/DO-Lipo ATRA (-)) or differentiated HL-60 cells
18 (LM-EPC/DO-Lipo ATRA (+)) and LM-DMPC/DO-Lipo (ATRA (+)) had significantly higher
19 affinity to TNF- α -treated HUVECs compared with untreated ones, suggesting that those negatively
20 charged liposomes attained leukocyte-mimetic properties to facilitate their adherence to inflamed
21 endothelial cells. Because LM-DMPC/DO-Lipo showed lower affinity to TNF- α -treated HUVECs
22 than LM-EPC/DO-Lipo, LM-EPC/DO-Lipo were used in subsequent experiments.

23

24 **3.4. Passage of leukocyte-mimetic liposomes through TNF- α -treated HUVECs**

25 Next, the ability of LM-Lipo to pass through inflamed endothelial cell layer was evaluated
26 using TNF- α -treated HUVECs seeded onto Transwell inserts. DiI-labeled LM-Lipo were added onto

1 the upper compartments of the TNF- α -treated HUVECs cultured on the inserts, and the DiI
2 fluorescence of the media in the bottom compartments was measured 3 h after liposomal treatment.
3 The results showed that there was almost no difference between the groups of cells treated with
4 EPC/DO-Lipo or LM-EPC/DO-Lipo (ATRA (-)), whereas the fluorescence intensity in the bottom
5 compartment was higher in the LM-EPC/DO-Lipo (ATRA (+))-treated group compared with that in
6 the EPC/DO-Lipo-treated group (Fig. 5). These results suggest that LM-EPC/DO-Lipo (ATRA (+))
7 not only adhered to but also passed through inflamed endothelial cell layer.

8

9 **3.5. Influence of leukocyte-mimetic liposomes on cytoskeleton and intercellular adhesion** 10 **junctions of inflamed endothelial cells**

11 To analyze the mechanism of passage of LM-EPC/DO-Lipo (ATRA (+)) through the
12 TNF- α -treated HUVEC layer, we investigated the influence of liposomal treatment on the
13 cytoskeleton and intercellular adhesion junctions of inflamed endothelial cells. Confocal images of
14 F-actin staining showed that the treatment with LM-EPC/DO-Lipo, especially LM-EPC/DO-Lipo
15 (ATRA (+)), induced intracellular morphological changes of F-actin in TNF- α -treated HUVECs
16 compared with non-treated HUVECs (Figs. 6A-D). As shown in Figure 6D, in the HUVECs treated
17 with LM-EPC/DO-Lipo (ATRA (+)), the state of F-actin was changed and became sharp-shaped
18 structures, and intracellular fluorescence derived from F-actin (Alexa Fluor 488 phalloidin)
19 decreased compared with control (TNF- α (-)) and EPC/DO-Lipo groups.

20 We then evaluated the effect of treatment with LM-Lipo on the state of the intercellular
21 adhesion protein, VE-cadherin, in TNF- α -treated HUVECs. Confocal images showed that expression
22 of VE-cadherin and cell borders were clearly observed in normal HUVECs (Fig. 7A). Although the
23 apparent formation of intercellular junctions was observed in TNF- α -treated cells treated with plain
24 EPC/DO-Lipo (Fig. 7B), the fluorescence signals derived from VE-cadherin expression was
25 decreased in the LM-EPC/DO-Lipo (ATRA (-))-treated cells, and the intercellular adhesions were
26 also slightly compromised (Fig. 7C). Importantly, treatment with LM-EPC/DO-Lipo (ATRA (+))

1 markedly perturbed VE-cadherin expression and the formation of intercellular junctions compared to
2 the other groups (Fig. 7D). The quantification data also indicated that the level of VE-cadherin
3 expression in TNF- α -treated HUVECs incubated with LM-EPC/DO-Lipo (ATRA (+)) was
4 significantly lower than the HUVECs incubated with plain EPC/DO-Lipo, and tended to be
5 decreased compared with the cells treated with LM-EPC/DO-Lipo (ATRA (-)) (Fig. 7E). On the
6 other hand, the expression of VE-cadherin was almost not changed by the treatment with
7 EPC/DO-Lipo in the absence of TNF- α (Supplementary Figs. 5A and B). We also confirmed that
8 HL-60 cells could induce morphological change of F-actin in TNF- α -treated HUVECs and the state
9 of F-actin became sharp-shaped structures (Supplementary Fig. 4). In addition, the expression of
10 VE-cadherin decreased in the TNF- α -treated HUVECs incubated with HL-60 cells, especially with
11 ATRA-treated (differentiated) HL-60 cells, in comparison to HUVECs treated with TNF- α alone
12 (Supplementary Figs. 5C and D), as reported previously in leukocytes (Isac et al., 2011; Vestweber,
13 2012). These results suggest that the LM-EPC/DO-Lipo (ATRA (+)) regulates intercellular junctions
14 of the TNF- α -treated HUVECs by exerting functions of both CD11a and CD11b derived from
15 neutrophil-differentiated HL-60 cells.

16

1 **4. Discussion**

2 Since accumulation of nanoparticles into the sites of tumor and inflammation is dependent
3 on passive process, design of nanoparticles which can actively overcome the inflamed endothelium
4 and accumulate in the diseased area is required (Anselmo et al., 2015; Blanco et al., 2015).
5 Leukocytes are known to adhere to and pass through the inflamed endothelium via the interaction of
6 their membrane proteins and adhesion molecules expressed on the endothelial cells
7 (Etienne-Manneville et al., 2000; Vestweber, 2015). Thus, we hypothesized that incorporation of
8 leukocyte membrane proteins onto liposomes could enable the liposomes to mimic the function of
9 leukocytes, and thus allow for their active passage through the inflamed endothelium. Herein, we
10 employed intermembrane protein transfer as a protein reconstitution method to impart nanoparticles
11 with biomimetic properties, since this method is reported to be able to transfer cellular membrane
12 proteins onto liposomal membranes with native orientation and activities (Huestis and Newton, 1986;
13 Newton and Huestis, 1988). In the present study, we used liposomes containing the amphiphilic
14 compound DCP because it was previously revealed that incorporation of DCP into liposomes induces
15 phase separation of the liposomal membrane, resulting in increase in the efficiency of membrane
16 protein transfer (Kogure et al., 1997a; Kogure et al., 1999). Similar to the previous studies, results
17 shown in Fig. 3 confirmed the transfer of the membrane proteins of neutrophil-differentiated HL-60
18 cells, CD11a and CD11b, onto liposomes containing DCP. Additionally, the results of Western
19 blotting showed that the transfer was more clearly observed in the group of liposomes composed of
20 EPC than liposomes composed of DMPC (Figs. 3A and B). These results are consistent with a
21 previous report, which showed that protein transfer is facilitated by increasing the membrane fluidity
22 of the liposomes (Waters et al., 1996). In particular, when the fusogenic unsaturated phospholipid
23 DOPE was incorporated into liposomes, higher transfer efficiency was observed both in EPC- and
24 DMPC-based liposomes (Figs. 2 and 3). In fact, the amounts of total proteins, CD11a, and CD11b in
25 the liposome suspensions collected after incubation with the cells tended to be higher both in EPC-
26 and DMPC-based liposomes containing DOPE (Supplementary Table. 1). These results suggest that

1 incorporation of DOPE into liposomes could increase the efficiency of intermembrane protein
2 transfer.

3 As shown in Fig. 1D and supplementary Fig. 1, differentiation of HL-60 cells by ATRA
4 treatment significantly reduced CD11a expression levels to approximately 60% of non-differentiated
5 HL-60 cells, although CD11b expression was induced by ATRA treatment (Figs. 1A-C). On the other
6 hand, the amount of CD11a transferred onto liposomes from ATRA-treated differentiated HL-60 cells
7 were larger than or almost same as that from non-differentiated cells (Supplementary Table 1),
8 despite the CD11a expression level in differentiated HL-60 cells been lower than that in
9 non-differentiated cells. From these results, it is considered that transfer efficiency of cellular
10 membrane proteins via intermembrane protein transfer would be changed by the state of donor cells.

11 Leukocytes are known to adhere to inflamed endothelium and infiltrate into the site of
12 inflammation by utilizing the functions of their carbohydrate chains and membrane proteins. In
13 particular, the membrane proteins CD11a and CD11b, which were shown to be expressed in
14 ATRA-induced neutrophil-differentiated HL-60 in our present study (Fig. 1) as well as in previous
15 reports (Gee et al., 2012), have been reported to interact with ICAM-1 (Diamond et al., 1991; van
16 Buul et al., 2007). Thus, to evaluate the leukocyte-mimicking ability of LM-Lipo, we first examined
17 the association of the liposomes with inflamed endothelial cells using TNF- α -treated HUVECs,
18 which express ICAM-1 as shown in Supplementary Fig. 2. The results showed that LM-Lipo
19 significantly associated with the TNF- α -treated HUVECs compared with plain liposomes (Fig. 4). In
20 addition, the liposomes showed significantly higher affinity to TNF- α -treated HUVECs than to
21 untreated HUVECs (Fig. 4). Since CD11a has been reported to be involved in leukocyte adhesion to
22 inflamed endothelium, it is considered that liposomes incubated with non-differentiated HL-60 cells
23 could also significantly associate with TNF- α -treated HUVECs. These results suggest that liposomes
24 possessing leukocyte membrane proteins could attain leukocyte-mimetic functions and selectively
25 adhere to inflamed endothelial cells via ICAM-1. Moreover, it was confirmed that the transferred
26 membrane proteins CD11a and CD11b could retained their functionalities and native orientations on

1 the liposomal membranes.

2 In the present study, LM-EPC/DO-Lipo (ATRA (+)) demonstrated an ability to pass through
3 TNF- α -treated HUVEC layer cultured onto a Transwell plate compared with plain EPC/DO-Lipo
4 (Fig. 5). As one of the mechanisms of leukocyte extravasation across inflamed endothelium via
5 binding to adhesion molecules, it has been demonstrated that interaction of CD11a and CD11b on
6 leukocytes with ICAM-1 on endothelial cells induces activation of the ICAM-1 signaling pathway
7 (Palomba et al., 2016). This activation leads to a subsequent increase in intracellular calcium
8 concentration, protein kinase C- α activation, and phosphorylation of VE-cadherin, which results in
9 displacement of VE-cadherin and formation of gaps between the inflamed endothelial cells
10 (Sandoval et al., 2001; Turowski et al., 2008). It has also been reported that when leukocytes pass
11 through inflamed endothelium via the paracellular route, actin depolymerization is induced in
12 addition to displacement of intercellular junction proteins (Mamdouh et al., 2009). In fact, the
13 displacement of VE-cadherin and decrease in its expression level, and change of actin cytoskeleton
14 were seen in TNF- α -treated HUVECs incubated with LM-Lipo possessing both CD11a and CD11b
15 (Figs. 6 and 7). Based on these results, it is suggested that interaction of both CD11a and CD11b on
16 LM-Lipo with ICAM-1 resulted in activation of the ICAM-1 signaling pathway by exerting their
17 functions. This signal activation subsequently induced displacement of VE-cadherin and increased
18 the permeability between the cells, resulting in passage of LM-Lipo through the inflamed HUVEC
19 layer.

20 Liposomes could attain leukocyte-mimicking abilities by reconstitution of leukocyte
21 membrane proteins, especially CD11a and CD11b, via intermembrane protein transfer. Since the
22 protein reconstitution method nonspecifically transfers membrane proteins onto liposomes, other
23 proteins might interact with other endothelial adhesion molecules involved in leukocyte
24 extravasation (Vestweber, 2015). However, it is possible that modification of the liposome surface
25 with both CD11a and CD11b, or peptides containing ICAM-1 recognition domains that subsequently
26 activate the ICAM-1 signaling pathway, similar to the membrane proteins, could allow for adhesion

1 to inflamed endothelial cells and perturbation of intercellular junctions, leading to passage of the
2 nanoparticles through endothelial cell layer into inflammation sites. Design of such nanoparticles
3 should be both useful and interesting for future studies.

4 LM-EPC/DO-Lipo (ATRA (+)) developed in this study exhibited leukocyte-mimicking
5 properties by regulating cellular junctions of inflamed endothelial cells and passing through the
6 endothelial cell layer. Hence, LM-Lipo has the potential to be useful in applications for the treatment
7 of inflammatory diseases, similar to leukocytes invading the inflammation sites. In previous reports,
8 leukocyte-mimetic nanoparticles were prepared via complicated steps, i.e., collection and
9 purification of cellular membrane fractions from cell lysates by density gradient centrifugation,
10 followed by their modification onto nanoparticles by chemical conjugation or physical processes
11 such as sonication and extrusion (Kang et al., 2017; Parodi et al., 2013). On the other hand, the
12 intermembrane protein transfer employed in the present study has the advantage that cellular
13 membrane proteins can be easily transferred with their native activity and orientation by only
14 incubating the liposomes with the cells (Huestis and Newton, 1986; Shibata et al., 1991). Also, our
15 present study demonstrated that it is possible to construct biomimetic DDS even by such a more
16 simple method than conventional methods reported previously. *In vivo* applications using leukocytes
17 derived from animal blood should be considered a necessary next step in demonstrating further
18 utility of LM-Lipo. Since inflammation has been reported to be involved in the pathological
19 progression of a number of diseases (Chu et al., 2015; Eltzschig and Eckle, 2011; Maeda et al., 2013),
20 application of LM-Lipo could become a hopeful approach for developing treatments for such
21 diseases.

22

1 **5. Conclusions**

2 In summary, development of LM-Lipo could be achieved by intermembrane transfer of
3 leukocyte membrane proteins onto liposomal membranes. LM-Lipo was able to associate with
4 TNF- α -treated ICAM-1-expressing HUVECs and pass through the cell layer by exerting the
5 functions of leukocyte membrane proteins. Moreover, the mechanism of passage is considered to be
6 due to the displacement of VE-cadherin accompanying change of actin cytoskeleton by interaction of
7 both CD11a and CD11b with ICAM-1. Hence, the present findings suggest that imparting
8 leukocyte-mimetic properties onto liposomes represents a promising approach to overcome
9 endothelial barriers by regulating intercellular junctions, and that LM-Lipo could be a useful DDS
10 for developing a new therapy for the treatment of inflammatory diseases.

11

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17

18 **Author contributions**

19 TF and KK designed the research. TF and SY performed the experiments. TF and TT
20 analyzed the data. TF and KK wrote the paper.

21

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7

8

9 **Figure legends**

10

11 Figure 1. Differentiation of HL-60 cells into neutrophil-like cells by treatment with ATRA.

12 (A) HL-60 cells (1.0×10^6 cells/60-mm dish) were treated with 1 μ M ATRA for 0, 24, 48, 72,
13 96, or 120 h to induce differentiation into neutrophil-like cells. In the non-differentiated group, the
14 cells were cultured in the presence of 0.1% DMSO. Following incubation, the cells were collected
15 and stained with Alexa Fluor 488-conjugated anti-CD11b antibody. Thereafter, flow cytometric
16 analysis was performed to measure the CD11b-positive cells. The white and gray histograms indicate
17 cells treated without and with ATRA, respectively. (B) The percentage of CD11b-positive
18 (neutrophil-differentiated) cells was determined from the histogram data. The data show the mean \pm
19 S.D. (n=3). (C, D) Expression of CD11b (Mac-1; C) and CD11a (LFA-1; D) in ATRA-treated and
20 non-ATRA-treated groups was determined by Western blotting after incubation for 96 h. The
21 experiments of Western blotting were performed 3 times independently.

22

23 Figure 2. Determination of protein concentration of the recovered liposome suspensions after
24 incubation with HL-60 cells.

25 Liposomes consisted of each lipid composition were incubated for 90 min with HL-60 cells
26 cultivated in the presence or absence of ATRA for 96 h. Protein concentrations of the recovered

1 liposomes incubated with non-differentiated HL-60 cells (ATRA (-)) or differentiated HL-60 cells
2 (ATRA (+)) were determined by BCA assay. The data show the mean \pm S.D. (n=4). The experiments
3 were conducted 4 times independently.

4

5 Figure 3. Transfer of leukocyte membrane proteins onto liposomes via intermembrane protein
6 transfer with HL-60 cells.

7 (A, B) Liposomes comprised of each lipid composition or 0.3 M sucrose phosphate buffer
8 were incubated with non-differentiated (ATRA (-)) or neutrophil-differentiated (ATRA (+)) HL-60
9 cells for 90 min. Each lane indicates a specific liposome (0.2 μ mol as total lipid) or cell extract of
10 differentiated HL-60 cells (10 μ g protein) that was subjected to SDS-PAGE. Western blot analysis
11 was then performed to confirm the transfer of CD11a (A) and CD11b (B) onto the liposomes. Per the
12 product datasheet, the detected molecular weights of CD11a and CD11b are approximately 180 kDa
13 and 170 kDa, respectively. The bands in the leftmost lane correspond to the Western Protein Standard
14 (MagicMarkTM XP; Thermo Fisher Scientific). (C-E) The signal intensities of the bands of CD11a
15 (C; ATRA (-), D; ATRA (+)) and CD11b (E) transferred onto liposomes were calculated by using an
16 image-analysis system Image J. The relative signal intensities of each band to that of differentiated
17 HL-60 cells are presented. The data show the mean \pm S.D. (C and D; n=3, E; n=4). Statistical
18 differences were analyzed by ANOVA with Tukey *post-hoc* test. Significant differences; * $P < 0.05$,
19 ** $P < 0.01$. The experiments were performed 3 for A, C, and D and 4 times for B and E.

20

21 Figure 4. Association of leukocyte-mimetic liposomes with TNF- α -treated inflamed HUVECs.

22 (A, B, D, E) HUVECs (1×10^5 cells/35-mm dish) were treated with TNF- α (10 ng/mL in
23 EGM) for 16 h at 37°C to induce expression of ICAM-1, as shown in Supplementary Fig. 2.
24 DiI-labeled EPC/DO-Lipo, DMPC/DO-Lipo, and LM-Lipo prepared by incubation with HL-60 cells
25 (ATRA (-) or ATRA (+)) were added onto untreated (A and D) or TNF- α -treated HUVECs (B and E),
26 and the cells were incubated for 3 h at 37°C. Thereafter, the cells were fixed, and their nuclei were

1 counterstained with DAPI. Fluorescence images were obtained by fluorescence microscopy. DiI
2 (liposome; red, upper column) and merged images (lower column) of DAPI (nuclei; blue), DiI, and
3 phase contrast are shown. Scale bars = 100 μm . (C, F) HUVECs (5×10^4 cells/well onto 24-well
4 plate) were incubated with EGM-2 containing TNF- α (10 ng/mL) for 16 h at 37°C. TNF- α -treated
5 and untreated HUVECs were then treated with the indicated DiI-labeled liposomes for 3 h at 37°C.
6 After lysis of the cells, the fluorescence intensity of DiI in the cells was measured. The relative
7 fluorescence intensity of each group to that in control (C: EPC/DO-Lipo, F: DMPC/DO-Lipo) are
8 presented. The data show the mean \pm S.D. (n=4). Statistical differences were calculated by ANOVA
9 with Tukey *post-hoc* test. Significant differences; * $P < 0.05$, # $P < 0.05$, ## $P < 0.01$ vs. DMPC/DO-Lipo,
10 ### $P < 0.001$ vs. EPC/DO-Lipo. These experiments were performed 4 times independently.

11

12 Figure 5. Passage of leukocyte-mimetic liposomes through inflamed endothelial cell layer.

13 HUVECs seeded on a Transwell plate (2×10^5 cells/inserts) were cultured for 48 h at 37°C,
14 followed by incubation with TNF- α (10 ng/mL) for 16 h at 37°C. Next, DiI-labeled EPC/DO-Lipo or
15 LM-EPC/DO-Lipo (ATRA (-) or (+)) was added (50 nmol as lipid dose/insert, 100 μL of each
16 liposome at a concentration of 0.5 mM diluted with EBM-2) onto the upper compartment of the
17 Transwell plate and incubated for 3 h at 37°C. Thereafter, the fluorescence intensity of DiI in the
18 media of the bottom compartment was measured. The relative fluorescence intensity of each group to
19 that in control (EPC/DO-Lipo) are presented. The data show the mean \pm S.D. (n = 20). Statistical
20 differences were calculated by ANOVA with Tukey *post-hoc* test. Significant difference; * $P < 0.05$ vs.
21 EPC/DO-Lipo. The experiment was performed 5 times independently, and the sum of the data of
22 each experiment is shown.

23

24 Figure 6. Effect of treatment with leukocyte-mimetic liposomes on the actin cytoskeleton in
25 TNF- α -treated HUVECs.

26 (A-D) HUVECs (1×10^5 cells) grown on 35-mm glass-bottom dishes were treated with

1 TNF- α (10 ng/mL in EGM-2 medium) for 16 h at 37°C. The cells were then treated with DiI-labeled
2 EPC/DO-Lipo or leukocyte-mimetic liposomes (LM-EPC/DO-Lipo (ATRA (-) or ATRA (+)) for 3 h
3 at 37°C. The cells were then fixed with 4% PFA, permeabilized with PBS containing 0.1%
4 TritonX-100, and incubated with 1% BSA in PBS, followed by staining with Alexa Fluor
5 488-conjugated phalloidin solution. Finally, the nuclei of the cells were stained with DAPI, and the
6 fluorescence was observed using a confocal laser scanning microscope. Images were obtained from
7 untreated (TNF- α (-)) HUVECs (A), or TNF- α -treated HUVECs treated with plain liposomes (B),
8 LM-EPC/DO-Lipo (ATRA (-)) (C), or LM-EPC/DO-Lipo (ATRA (+)) (D). Blue, green, and red
9 colors represent the individual fluorescence of DAPI (nuclei), Alexa Fluor 488 (F-actin) and DiI
10 (liposome), respectively. Scale bars = 20 μ m. The experiment was independently performed 4 times,
11 all of which showed similar profiles.

12

13 Figure 7. Influence of leukocyte-mimetic liposomes on the intercellular adhesion protein
14 VE-cadherin in inflamed endothelial cells.

15 (A-D) TNF- α -treated HUVECs were treated with each liposome according to the same
16 procedure described in the legend of Fig. 6. After removing the liposomal samples, the cells were
17 fixed with 4% PFA and permeabilized with PBS containing 1% BSA and 0.1% Triton X-100.
18 Thereafter, the cells were incubated with anti-VE cadherin antibody for 1 h at 37°C, followed by
19 incubation with Alexa Fluor 488-conjugated secondary antibody for 1 h at 37°C. After staining the
20 nuclei with DAPI, fluorescence images were obtained from untreated HUVECs (A), and
21 TNF- α -treated HUVECs treated with the indicated liposomes (B-D) by confocal laser scanning
22 microscopy. Blue and green colors show the fluorescence of DAPI (nuclei) and Alexa Fluor 488
23 (VE-cadherin), respectively. Scale bar = 20 μ m. The experiments were performed 5 times
24 independently. (E) The relative fluorescence intensity of VE-cadherin positive area between cell
25 borders to that in control was calculated from over 8 or more images per each group of HUVECs for
26 one experiment by using ImageJ. The data show the mean \pm S.D. (n=5). Statistical differences were

- 1 analyzed by ANOVA with Tukey *post-hoc* test. Significant differences; ### $P < 0.001$ vs. Control
- 2 (TNF- α (-)), * $P < 0.05$ vs. EPC/DO-Lipo (TNF- α (+)).

Fig. 1

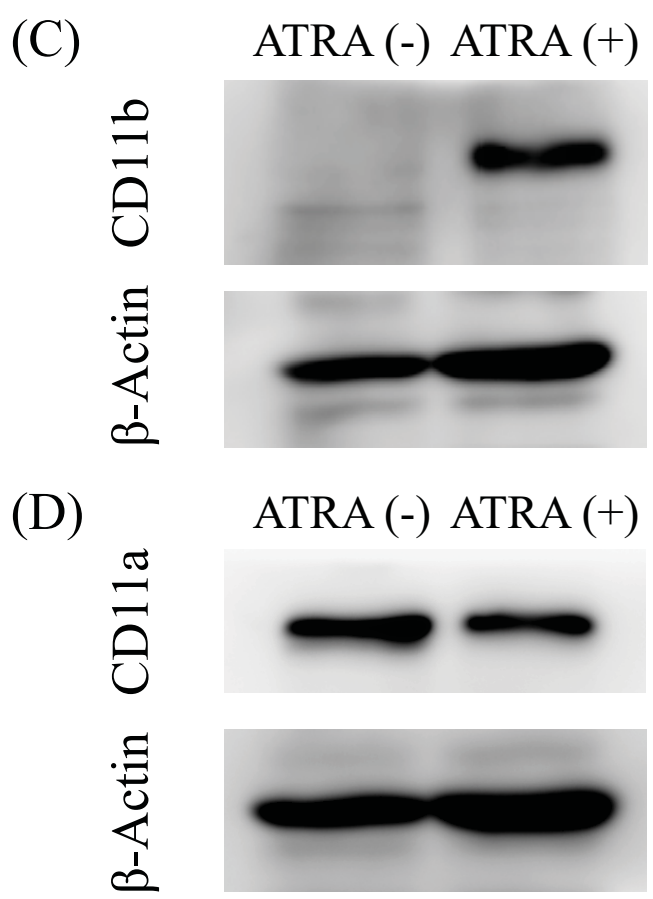
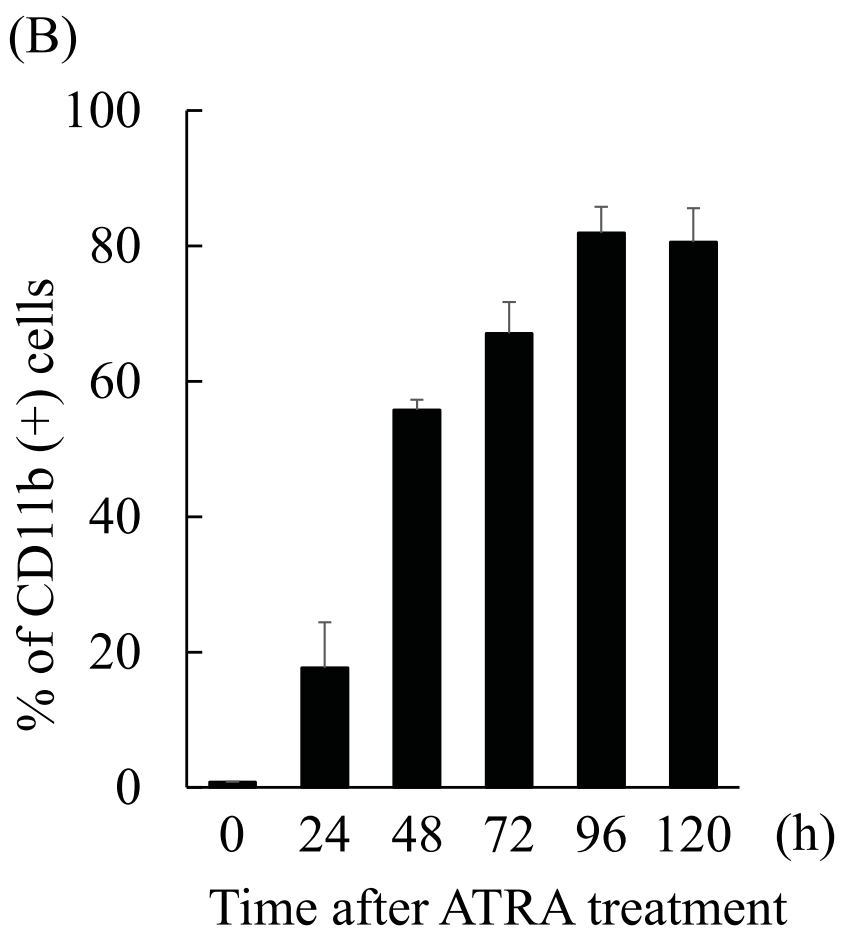
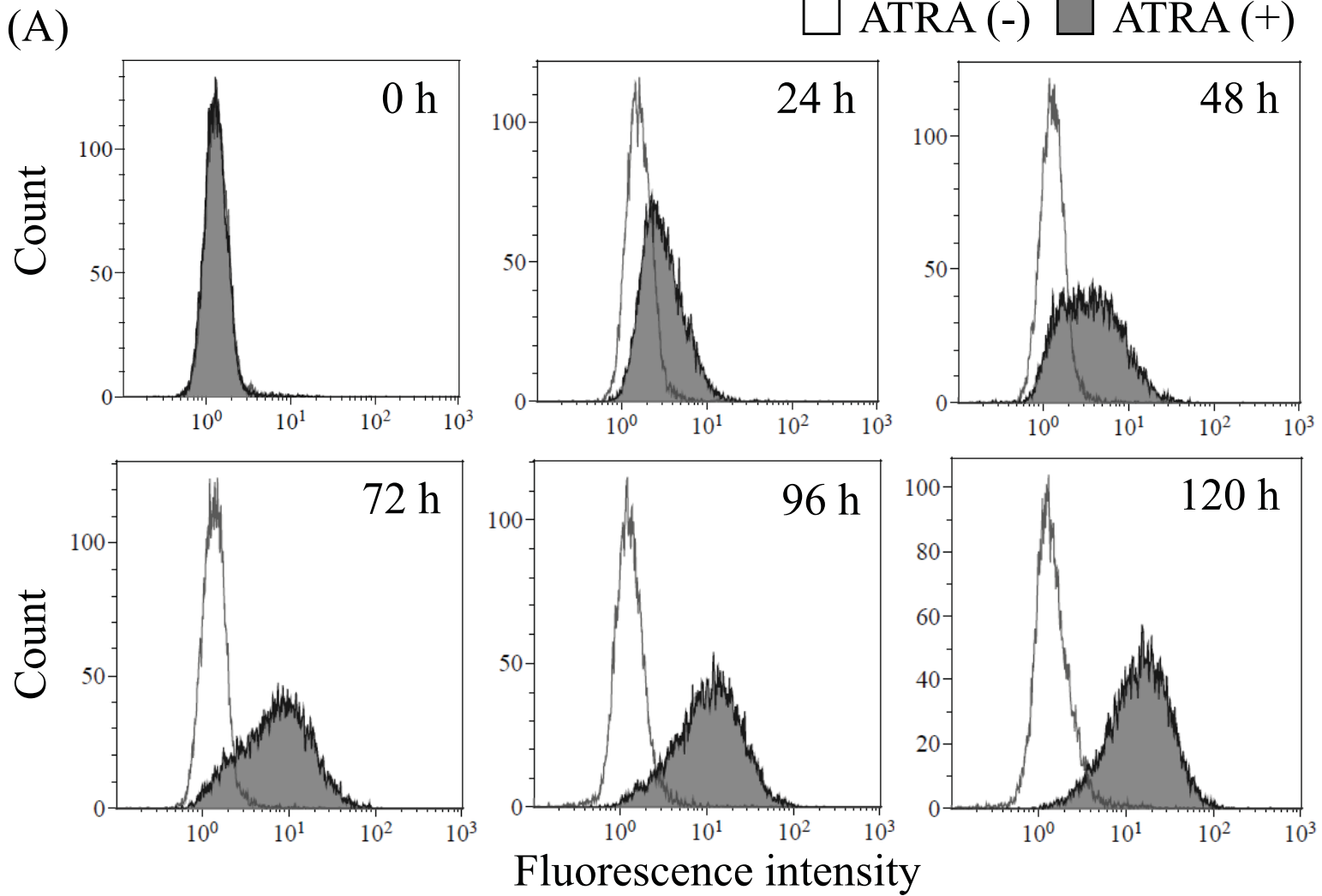


Fig. 2

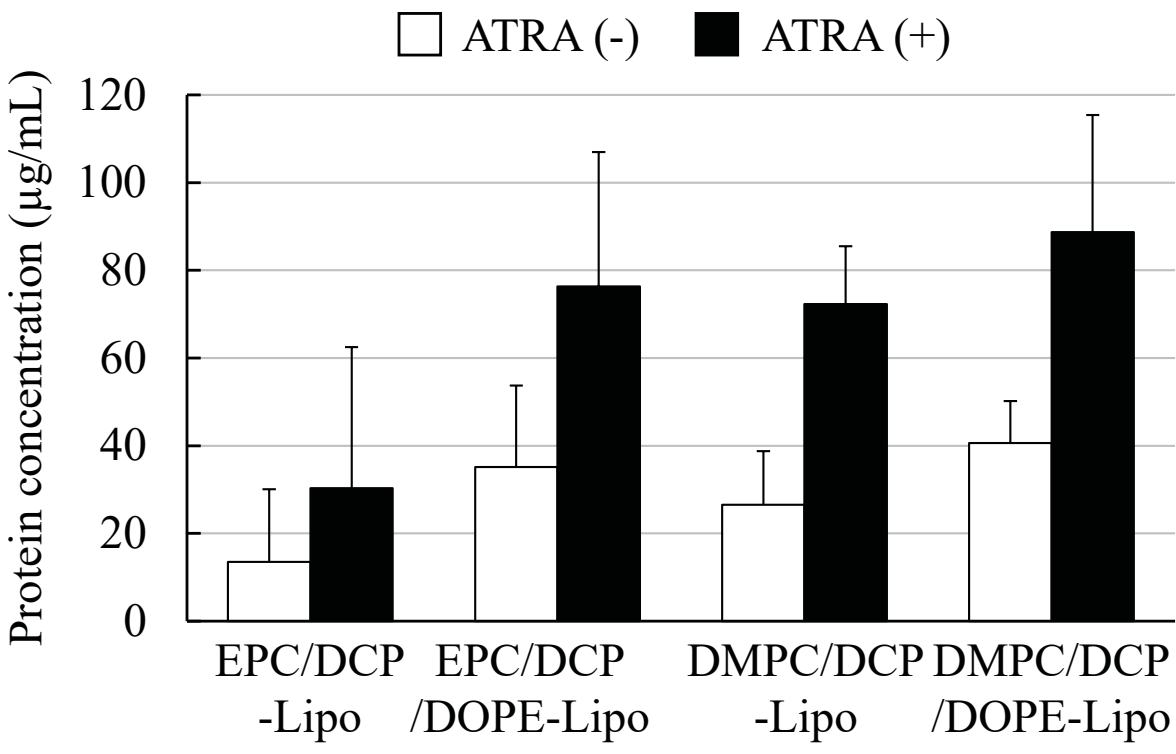


Fig. 3

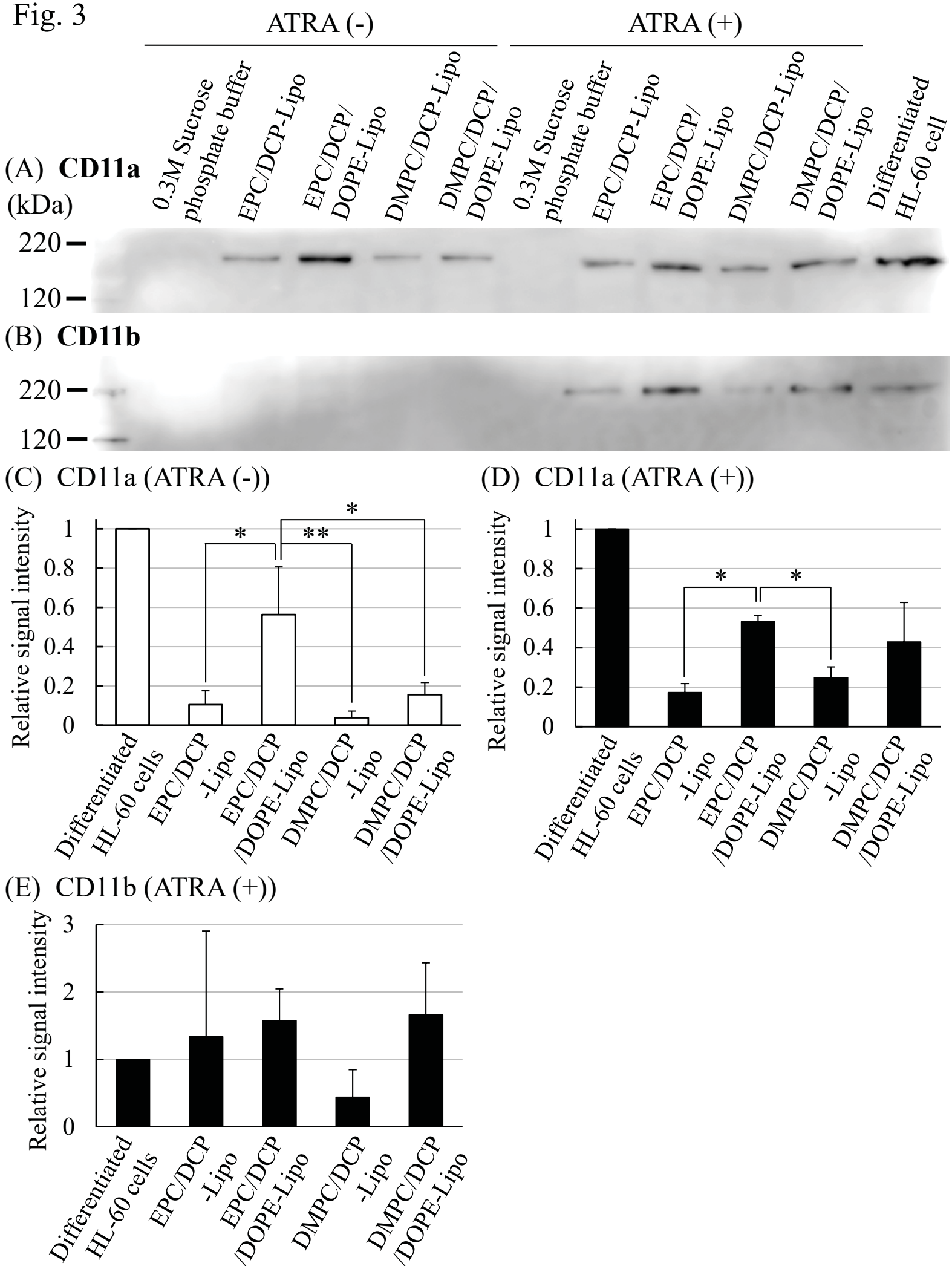


Fig. 4

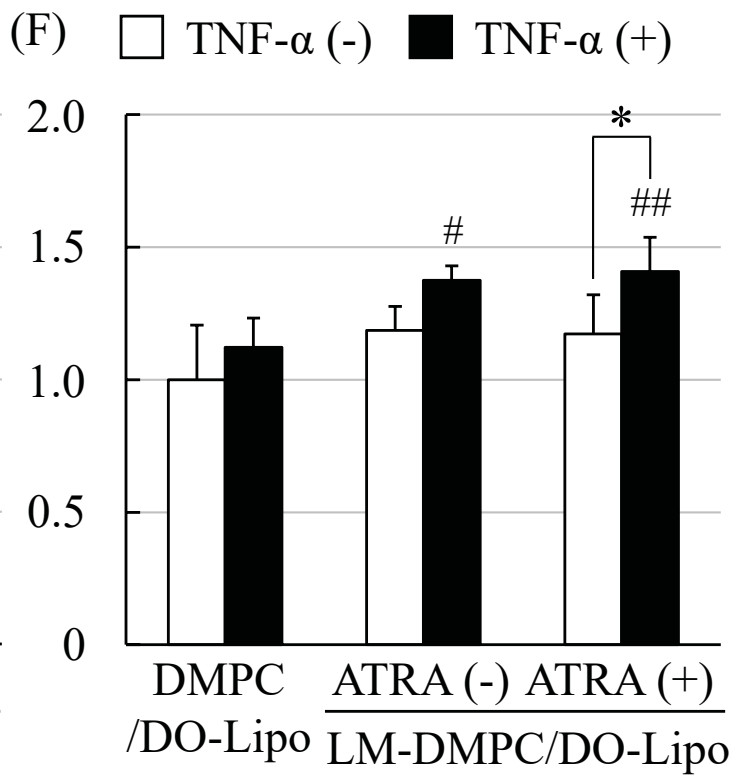
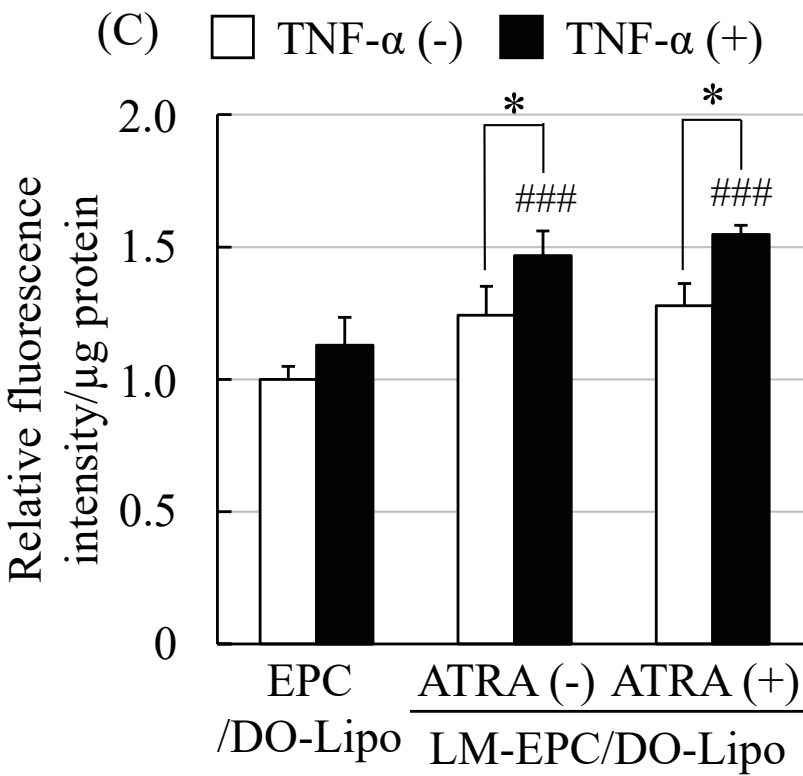
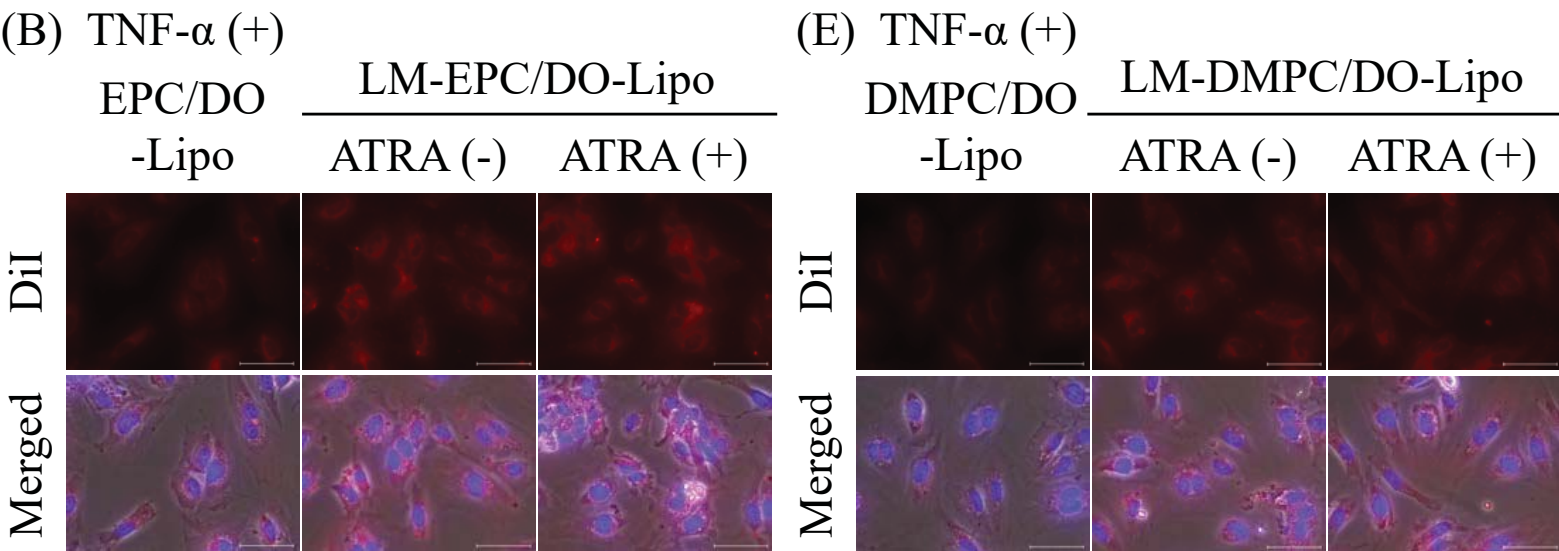
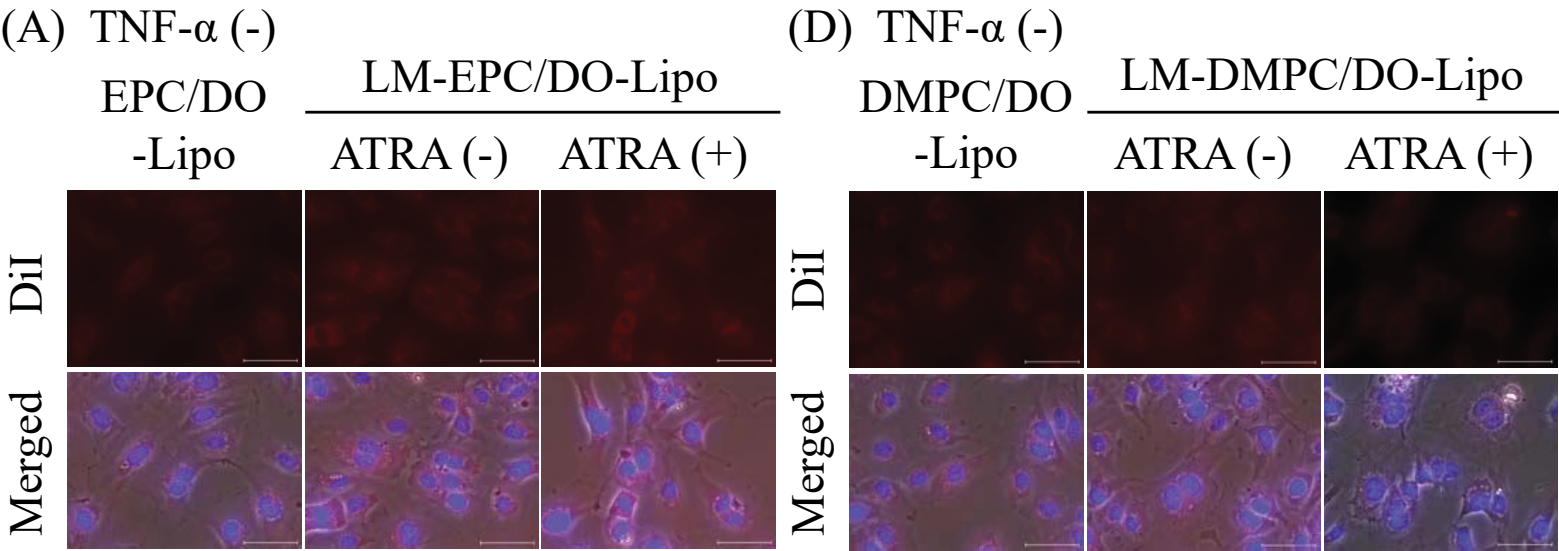


Fig. 5

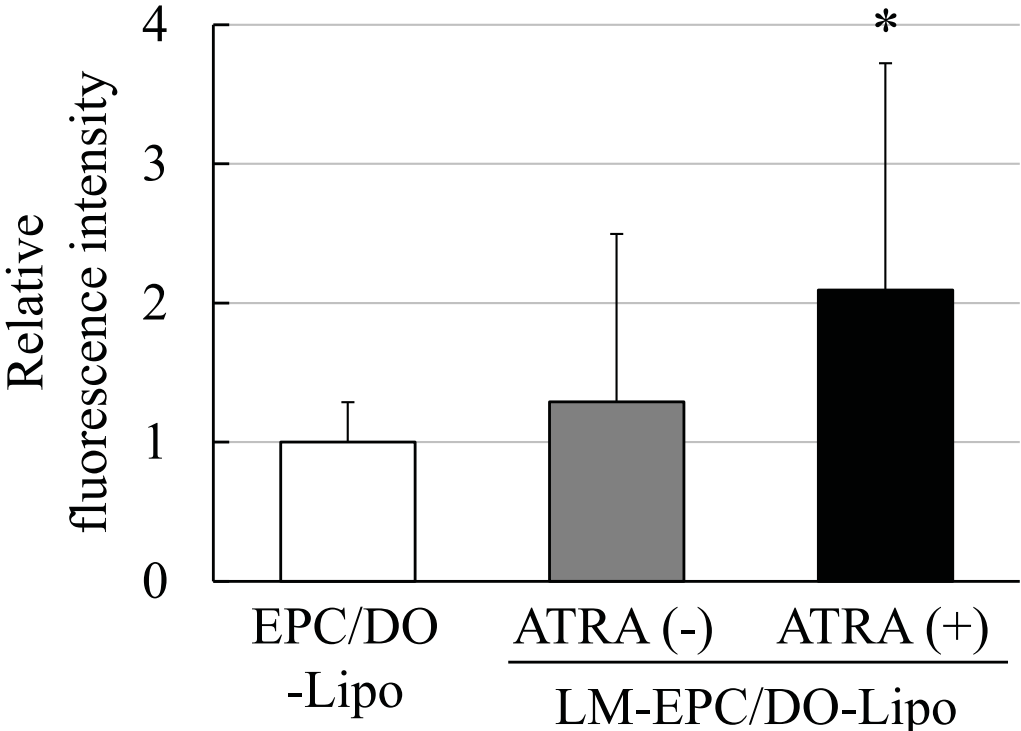
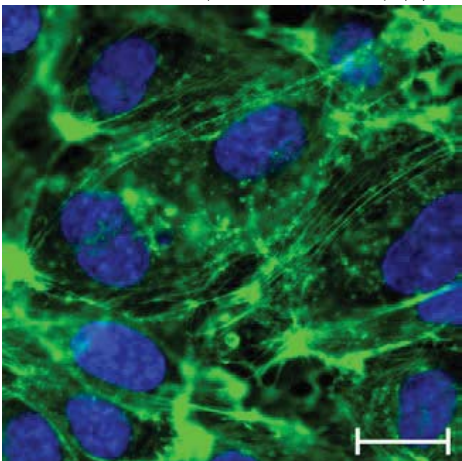
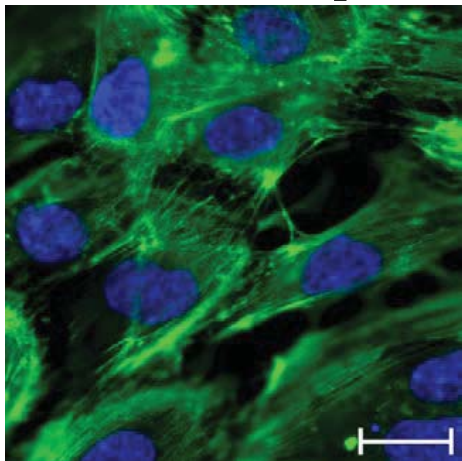


Fig. 6

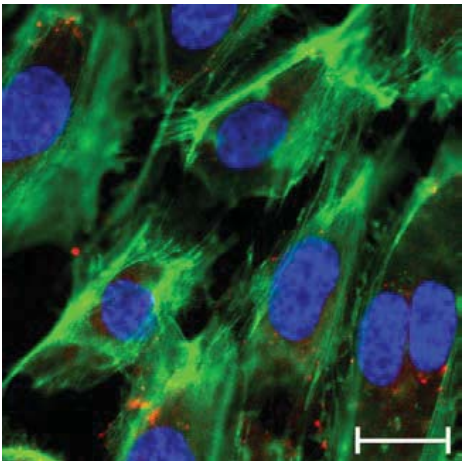
(A) Control (TNF- α (-))



(B) EPC/DO-Lipo



(C) LM-EPC/DO-Lipo
ATRA (-)



(D) LM-EPC/DO-Lipo
ATRA (+)

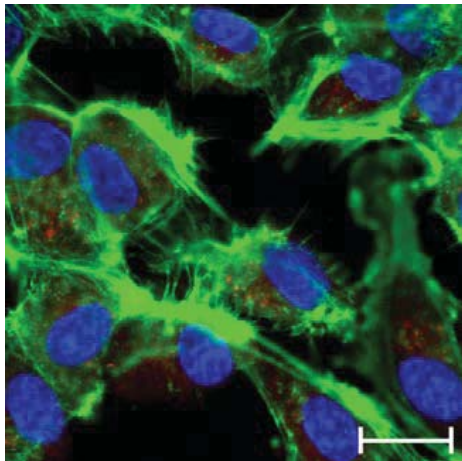


Fig. 7

