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# PLAG enhances macrophage mobility for efferocytosis of apoptotic neutrophils via membrane redistribution of P2Y2

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Neutrophil activity, including trapping of damage-associated molecular patterns by neutrophil extracellular traps (NETs), is an important response to microbial infection. Most activated neutrophils commit to apoptosis and are removed by activated macrophages in the process of efferocytosis. Improper clearance of apoptotic neutrophils often causes an unnecessary and exaggerated immune response and subsequent chronic inflammation. Effective macrophage mobility toward activated neutrophils, which is triggered by binding of 'find-me' signals to receptors such as P2Y2, is a crucial step for the timely clearance of apoptotic neutrophils. In this paper, we investigated the effect of 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) on efferocytosis and the underlying molecular mechanisms. In a coculture of apoptotic neutrophils with macrophages, PLAG treatment increased levels of efferocytosis of apoptotic neutrophils. PLAG induced faster translocation of P2Y2 from lipid rafts to nonlipid raft plasma membrane domains in macrophages. This repositioning of P2Y2 enables the polarization of the cytoskeleton by association of the receptor with cytoskeletal proteins such as α-tubulin and actin to improve the mobility of macrophages. The formation of vesicular, chylomicron-like structures by PLAG was a prerequisite for the induction of this macrophage activity, as none of these effects was seen when the vesicle receptor GPIHBP1 was absent. Taken together, these data showed that PLAG is a powerful immune resolvent that triggers the prompt clearance of apoptotic neutrophils by enhanced efferocytosis activity. PLAG could therefore be an effective lipid-based efferocytosis enhancer for use as a therapeutic drug to prevent inflammatory disease caused by uncontrolled immune responses.

## Introduction

It is remarkable that our body maintains and recycles roughly a million cells every second, generating new cells and clearing damaged cells from sites of disease or infection [1,2]. If unnecessary cells cannot be cleared properly, they can undergo secondary apoptosis and release their intracellular contents, resulting in the induction of an unnecessary immune response, which can cause autoimmune or immunoregulation diseases such as asthma and chronic obstructive pulmonary disease (COPD) [3,4]. Therefore, proper and rapid

**Abbreviations**
PLAG, 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol; DAMP, damage-associated molecular pattern; NET, neutrophil extracellular trap; GPIHBP1, glycosylphosphatidylinositol anchored high-density lipoprotein binding protein 1; COPD, chronic obstructive pulmonary disease.

clearing of unwanted cells is necessary to create new spaces in which normal cells can grow and maintain tissue function and health. In general, the clearance of unwanted cells is mediated by specialized engulfer phagocytes, such as macrophages. This phagocytic action, called efferocytosis, plays a very important role in maintaining tissue homeostasis [1,5–7]. Macrophages detect the presence of unwanted cells by recognizing soluble 'find-me' molecules consisting of nucleotides or chemokines secreted from apoptotic cells [8,9]. Nucleotides secreted from dead cells are key factors for macrophage recruitment. Macrophages have receptors that recognize these nucleotides, among which P2Y2 plays a crucial role in the migration of macrophages toward target cells [9–11]. Recent studies have shown that P2Y2 induces polarization of actin proteins and is attached to the cytoskeleton, leading to the movement of the engulfer cells. P2Y2 is also known to be involved in stabilization of microtubules in colon cells which helps to relieve colitis. P2Y2 activated by nucleotides binds directly to integrin proteins by moving from the lipid raft to nonlipid raft domain within the cell membrane [12–15]. Therefore, if macrophages can be rapidly targeted to apoptotic cells through the control of P2Y2 activation, the macrophages could effectively eliminate the apoptotic cells and reduce unnecessary immune responses.

Neutrophils are one of the earliest immune cells recruited to infection sites. When an infection occurs, neutrophils release toxic substances at the site and capture microbes using neutrophil extracellular traps (NETs), a unique cell death structure formed by neutrophils. Therefore, neutrophils are very important immune cells in host defense against bacterial and fungal infection. Neutrophils form NETs by releasing components such as neutrophil elastase, myeloperoxidase, and nucleotides [16,17]. These components, called damage-associated molecular patterns (DAMPs), lead to an immune response to capture microbes and eventually recruit engulfer cells for the elimination of the damaged cells by efferocytosis [18,19]. However, when apoptotic neutrophils are not cleared optimally due to a lack of efficient efferocytosis at the infection site, an unnecessary immune response such as systemic lupus erythematosus and rheumatoid arthritis could occur in response to the secondary lysis and release of noxious neutrophil substances [20–23]. Thus, proper efferocytosis of apoptotic neutrophils is crucial for tissue homeostasis, because its dysregulation can lead to unwanted inflammation, autoimmunity, and an exacerbated immune response.

The natural compound 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) was discovered in the antlers of Sika deer. This compound can be synthesized from glycerol, palmitic acid, and linoleic acid. We have previously confirmed that our synthetic PLAG is chemically identical to the naturally isolated form [24,25]. We recently confirmed its pharmacological activity against hepatic injury and its ability to augment the effect of pegfilgrastim on gemcitabine-induced neutropenia and oral mucositis. PLAG also ameliorates arthritis in joints by reducing neutrophil infiltration [26–29]. However, the effect of PLAG on damaged cell clearance, including apoptotic neutrophil clearance, has not been carefully studied. Thus, we investigated the effect of PLAG on apoptotic neutrophil efferocytosis through activation of macrophages. The apoptotic neutrophil efferocytotic effect of macrophages was increased by PLAG treatment, which was confirmed to be due to the promotion of macrophage mobility. In addition, the regulation of macrophage mobility was confirmed to be due to faster polarization of the cytoskeleton induced by the acceleration of P2Y2 migration to the nonlipid raft domain. Taken together, these results suggest that PLAG may be a very effective lipid-based agent for the prevention and treatment of diseases caused by the unnecessary immune response.

## Result

### Effect of PLAG on the induction of apoptotic neutrophil efferocytosis

To evaluate the role of PLAG in efferocytosis, apoptotic neutrophils were cocultured with macrophages and the efferocytotic index was calculated by FACS. The efficacy of apoptotic neutrophil phagocytosis in the PLAG-treated group was increased compared with that of the control group in a dose-dependent manner (Fig. 1A,E, Video S1). In the control group, clearance of apoptotic neutrophils by macrophage phagocytosis was observed by confocal microscopy within 2 h, whereas in the PLAG-treated (100 $\mu g \cdot mL^{-1}$) group, this was seen within 30 min (Fig. 1A,D). In addition, the number of remaining apoptotic neutrophils significantly decreased in the PLAG-treated group (Fig. 1B). Collectively, the data from confocal microscopy and FACS showed that apoptotic neutrophils were more effectively cleared after PLAG treatment. The secretion of IL-10, a cytokine expressed at the resolving stage of phagocytosis, was also found to increase more rapidly in the PLAG treatment group (Fig. 1C). Together, these data suggest that PLAG is a powerful agent for the resolution of inflammation via effective clearance of activated apoptotic neutrophils by prompt engulfment.

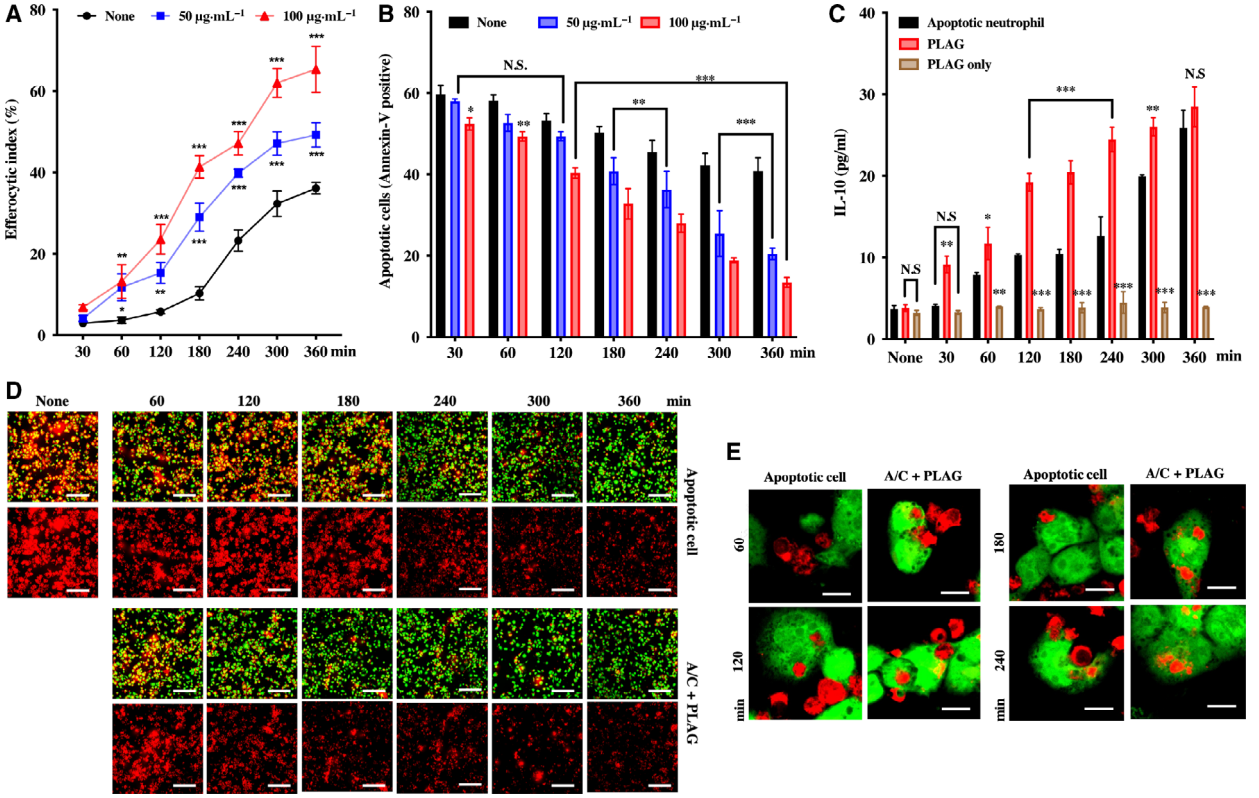


**Fig. 1.** Effect of PLAG on the induction of apoptotic neutrophil efferocytosis. Differentiated THP-1 cells were pretreated with PLAG for 1 h and then stimulated by apoptotic neutrophils. (A) Efferocytotic index was calculated by FACS. Compared to apoptotic neutrophil only group: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (each experiment $n = 6$). Mean ± SD. (B) Number of unengulfed apoptotic neutrophils was quantitated by FACS. Compared to control: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (each experiment $n = 6$). Mean ± SD. (C) Changes in IL-10 cytokine levels in the culture medium were determined by ELISA. Compared to control: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (each experiment $n = 6$). N.S., not significant. Mean ± SD. (D) The degree of clearance of apoptotic neutrophils was confirmed using confocal microscopy. Apoptotic neutrophils were tagged with red fluorescence and macrophages tagged in green fluorescence. Scale bar = 100 μm. (E) The engulfment of apoptotic neutrophils was confirmed using confocal microscopy. Apoptotic neutrophils were tagged with red fluorescence and macrophages tagged in green fluorescence. Scale bar = 20 μm.

## Increase of macrophage mobility in the PLAG-treated cells

Macrophage mobility for efferocytosis of apoptotic neutrophils was determined by a wound-healing assay as shown in Fig. 2A. Increased mobility of macrophages in the PLAG treatment group was observed as increased wound healing (Fig. 2B). DAMP molecules, including nucleotides excreted from apoptotic neutrophil, provide cytoskeletal modification in macrophages that promotes movement toward dead cells. A transwell assay examined macrophage mobility in response to DAMP from apoptotic neutrophils. Migration of macrophages was observed at 60 min in the PLAG treatment group, and this migration was more obvious at 120 min (Fig. 2C). Cytoskeletal modification was observed in macrophages that migrated to the bottom chamber containing apoptotic neutrophils. Polarization of the macrophage cytoskeleton was observed at 15 min in the PLAG group, whereas it began at 30 min in the control group (Fig. 2E). From these results, the promotion of apoptotic neutrophil clearance by efferocytosis in the PLAG-treated group might be due to increased macrophage mobility toward DAMPs secreted from apoptotic neutrophils.

## Enhanced movement of P2Y2 receptor from the lipid raft to nonlipid raft in the PLAG-treated cell

To analyze the cause of increased mobility of PLAG-treated macrophages, the distribution of P2Y2 on the plasma membrane was examined. The P2Y2 receptor is generally located in lipid rafts (Fig. 3A). In the apoptotic neutrophil-stimulated macrophage, translocation of the P2Y2 receptor toward nonlipid raft

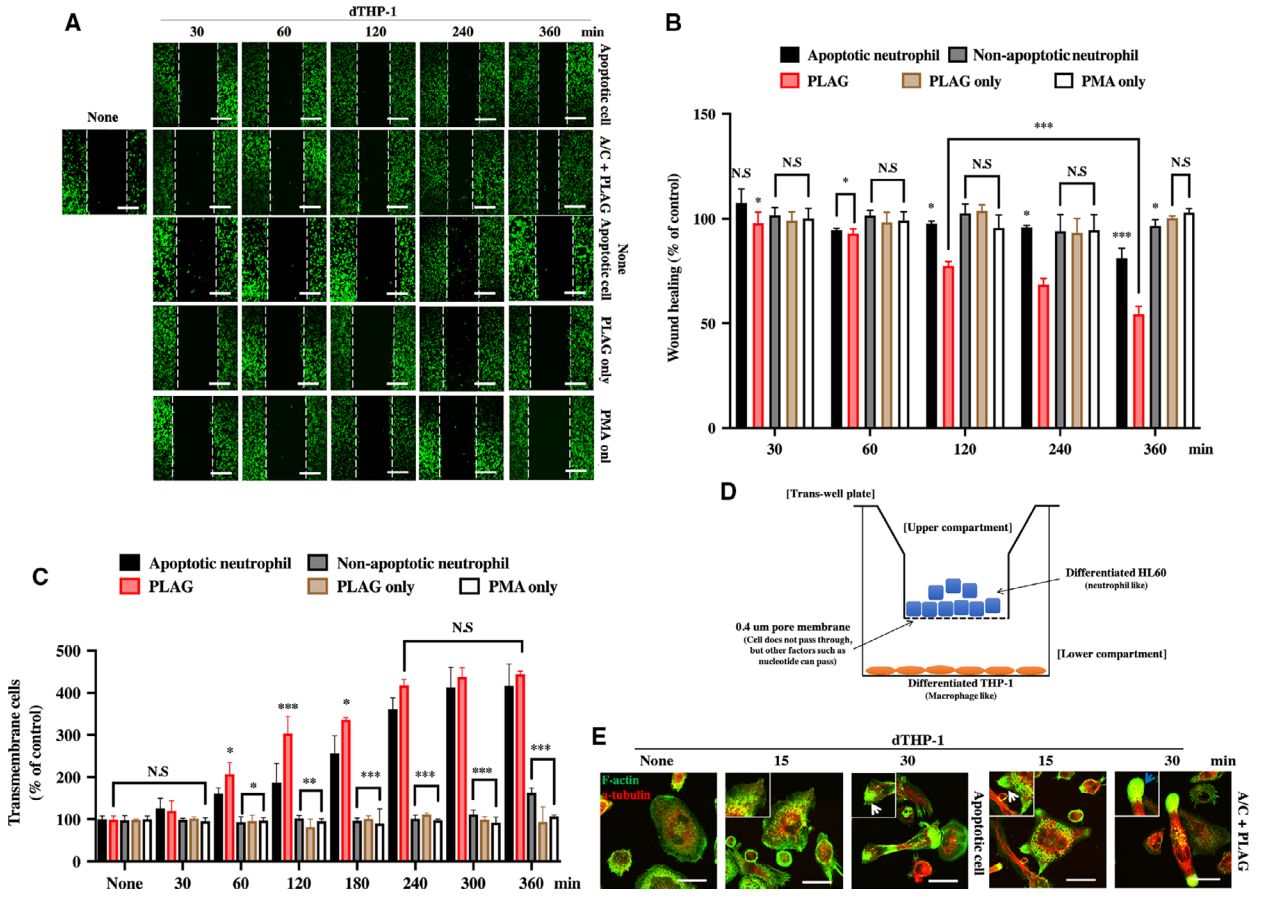


**Fig. 2.** Effect of PLAG on the increase of macrophage mobility. (A, B) The mobility of macrophages stimulated by apoptotic neutrophils was determined by wound healing assay. Degree of wound healing was quantified using IMAGE J. To assess the mobility of macrophages toward apoptotic neutrophils, nonapoptotic neutrophils were used to test the activity of subgroups. Compared to apoptotic neutrophils only: $*P < 0.05$, $***P < 0.001$ (each experiment $n = 6$). N.S., not significant. Scale bar = 100 μm. Mean ± SD. (C) The degree of chemoattraction of macrophages toward apoptotic neutrophils was determined. Compared to apoptotic neutrophils only group: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (each experiment $n = 6$). N.S., not significant. Mean ± SD. (D) Model for nondirect contact stimulation using transwell insert plate. (E) The degree of cytoskeletal polarization and colocalization with actin protein was determined by confocal microscopy. Scale bar = 20 μm.

domains of the plasma membrane was observed at 60 min. However, in the PLAG-treated macrophages cultured with apoptotic neutrophils, this translocation occurred within 30 min (Fig. 3A). Mobility of macrophages for efferocytosis in the presence of apoptotic neutrophils could be initiated by the assembly of cytoskeletal proteins around 'find-me' molecule receptors such as P2Y2. Association of actin protein with P2Y2 as shown by immunoprecipitation was obviously accelerated in PLAG-treated cells (Fig. 3C). In the protruded cytoskeleton of apoptotic neutrophil-stimulated macrophages, P2Y2 was colocalized with caveolin-1, a lipid raft marker, at 30 min (Fig. 4, left panel), but no colocalization of P2Y2 with caveolin-1 was observed at 30 min in the PLAG-treated group (Fig. 4, right panel). Together, these data show that PLAG promotes the association of P2Y2 with cytoskeleton proteins by promoting its translocation out of lipid rafts, and subsequently accelerates macrophage movement toward 'find-me' signals for efferocytosis.

## Promoted movement of P2Y2 receptor to nonlipid raft by PLAG was dependent on GPIHBP1, vesicle recognizing receptor

GPIHBP1 is a chylomicron receptor on the plasma membrane that recognizes vesicles. To determine whether PLAG acts as a vesicle, we silenced GPIHBP1 in the macrophage cells. In the GPIHBP1-silenced cells, the increase of efferocytosis and the acceleration of phagocytosis induced by PLAG were not observed

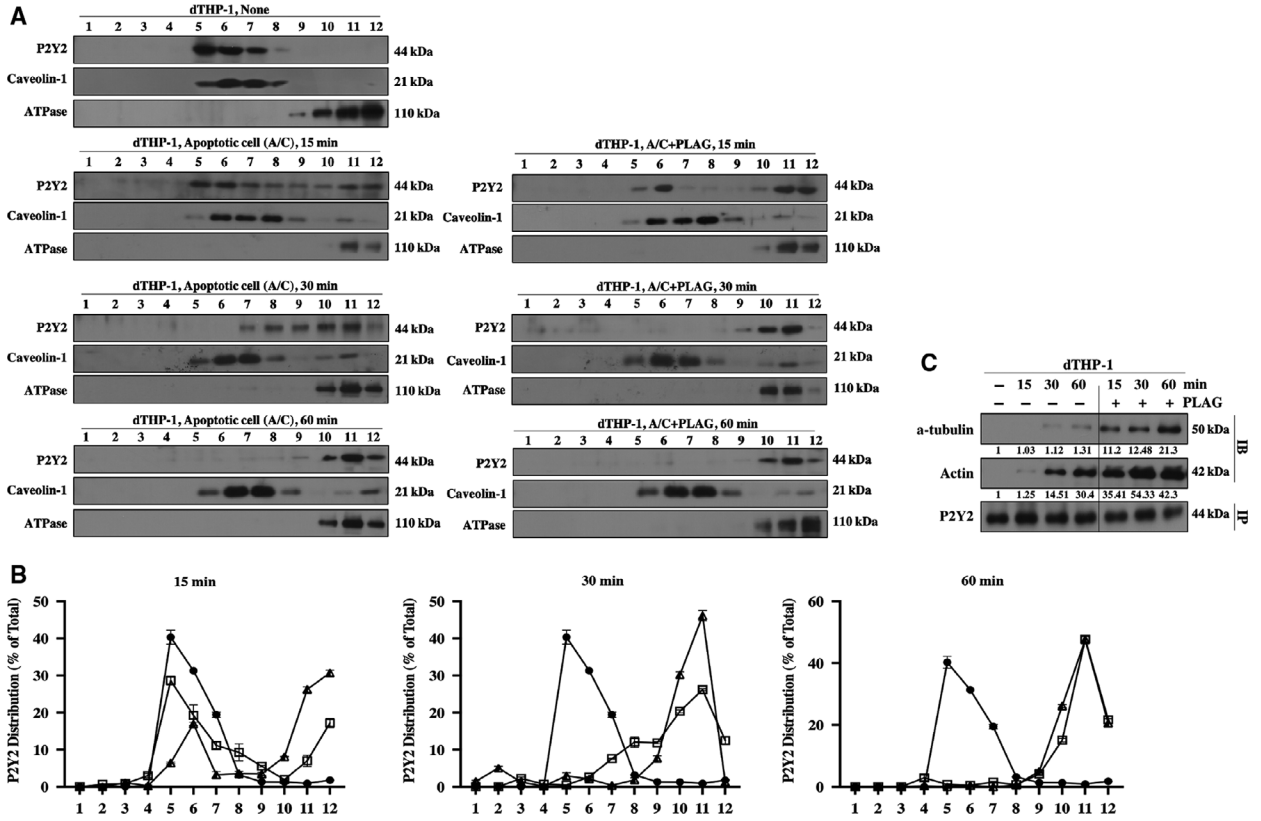


**Fig. 3.** PLAG enhanced P2Y2 redistribution to nonlipid raft domain and colocalization with cytoskeleton rearrangement proteins. (A) The membrane distribution change of P2Y2 was determined by the lipid raft fractionation method. Caveolin-1 was used as a lipid raft marker. (B) The distribution of P2Y2 in each band was quantified and plotted. •, None; □, Apoptotic neutrophil; △, PLAG. (C) The binding of P2Y2 with proteins related to polarization of the cytoskeleton was detected by immunoprecipitation (each experiment $n$ = 3). Mean ± SD.

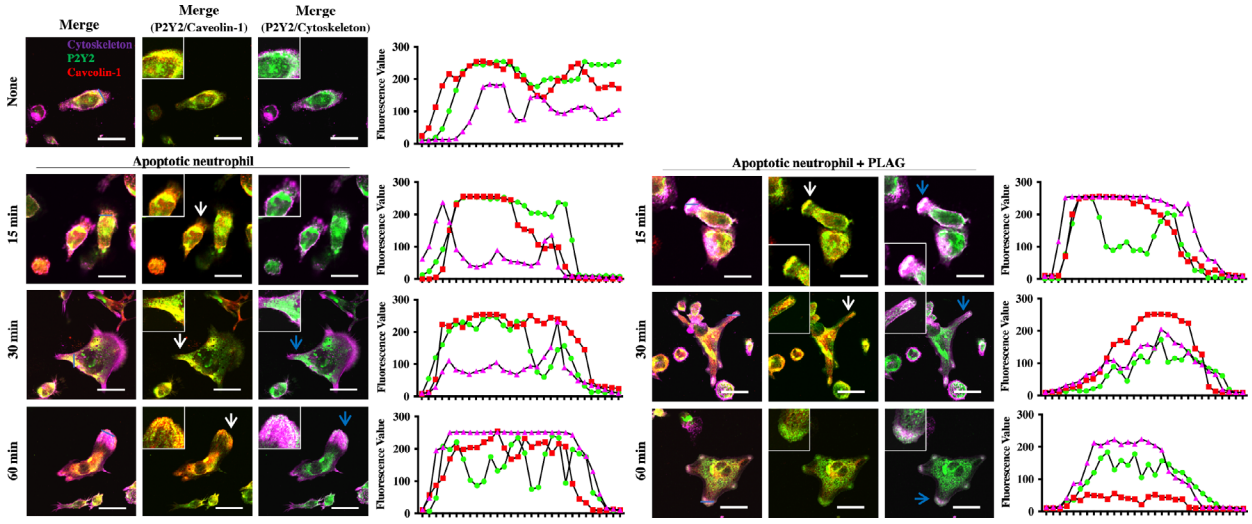


**Fig. 4.** Induction of P2Y2 activity by PLAG through changing the lipid raft. Colocalization shift of P2Y2 and cobinding with cytoskeleton by membrane distribution change were confirmed by confocal microscopy. White arrow indicates that P2Y2 is colocalized with caveolin-1, and the blue arrow indicates that P2Y2 is colocalized with the cytoskeleton. Scale bar = 20 μm. The degree of colocalization on the blue line between P2Y2, caveolin-1, and cytoskeleton was quantified using the IMAGE J (Fiji) (Bethesda, MD, USA).

(Fig. 5A) and apoptotic neutrophil clearance was not promoted (Fig. 5B). Furthermore, neither the enhanced macrophage mobility in the transwell assay (Fig. 5E) nor the promotion of IL-10 secretion by macrophages was observed in the GPIHBP1-silenced cells (Fig. 5F). P2Y2 colocalization with cytoskeletal proteins through accelerated redistribution was also not observed in the GPIHBP1-silenced and PLAG-treated cells. PLAG effects on efferocytosis, rapid movement to apoptotic neutrophils, and return to homeostasis were not observed in the GPIHBP1 silenced cells, as verified in coimmunoprecipitation assay with actin and α-tubulin (Fig. 5D). In addition, P2Y2 redistribution speed was not increased by PLAG treatment in the GPIHBP1-silenced cells (Fig. 6). These results indicated that the biological activity of PLAG on the membrane distribution of P2Y2 form lipid rafts to nonlipid raft domains might be dependent on the micellar structure of PLAG, rather than properties of the molecule itself.

### Induction of P2Y2 trafficking activity through caveolae formation of Structural PLAG

To determine the mechanism of GPIHBP1-dependent PLAG action, we examined the caveolae formation of the lipid raft domain and the trafficking activity of P2Y2. As a result, treatment of PLAG accelerated P2Y2 endocytosis and recycling time to the plasma membrane (Fig. 7A). In addition, we confirmed that the formation of caveolae, which plays a crucial role in the endocytosis of the receptors present in the lipid raft domain, had already occurred at 5 min following PLAG treatment (Fig. 7B). This action of PLAG did not occur in the GPIHBP1-silenced group (Fig. 7E,F). The colocalization of GPIHBP1 and P2Y2 was examined to confirm the activation of caveolae formation and P2Y2 trafficking in the processing of structural PLAG. As a result, the two proteins showed polarity at the same position according to PLAG treatment (Fig. 7C). However, there was no difference in the lipid raft distribution of P2Y2 in the group treated with PLAG alone (Fig. 7D). These results confirm that the treatment of PLAG leads to a 'ready to activation' state before ligand binding rather than to direct activation of P2Y2, allowing the ligand binding to react more rapidly (Fig. 8).

## Discussion

The immune function of active neutrophils plays a significant role in the defense of the human body. Especially important is the function of trapping of microorganisms and molecules such as DNA derived from tissue damage. NETosis, an active neutrophil, is a very fast and inevitable process for the elimination of invading microbe and release of DAMP in a wide area of our body [16,17]. However, if activated neutrophils are not cleared quickly or excessive NETosis occurs, inappropriate immune responses will be sustained and eventually cause undesirable inflammatory disease. Therefore, activated neutrophils and excessively expended NET should be removed via efferocytosis on time [21–23,30].

In this paper, we have studied about an efficient substance that has a capability for suppressing the exaggerated immune response by prompt clearance of apoptotic neutrophils through enhanced efferocytosis. We found that it accelerated phagocytosis for clearance of apoptotic neutrophils (Fig. 1A,D,E) and promoted IL-10 expression for return to homeostasis after efferocytosis in the macrophage was also observed in the PLAG-treated cells (Fig. 1C). For rapid efferocytosis, macrophage mobility must increase so that they can access apoptotic neutrophils. In the present study, PLAG rapidly increased the migration of macrophages toward apoptotic neutrophils (Fig. 2). This movement of macrophages for phagocytosis is generally triggered by the rearrangement of the cytoskeleton [9,31]. In our in vitro cell culture system, macrophage movement and rearrangement of the cytoskeleton was triggered by apoptotic neutrophils within 30 min, whereas in the PLAG-treated cells, the accelerated cytoskeletal rearrangement was observed within 15 min.

Generally, the onset of efferocytosis by macrophage was triggered by increased mobility through the recognition of 'find-me' signals. Especially, recognition of nucleotide by P2Y2 receptor, which was G-protein-coupled receptor (GPCR), is an important part of macrophage activity [32–34]. Recent studies have shown that GPCR, such as P2Y2, induces polarization of actin proteins and is attached to the cytoskeletons through endocytosis and recycling to the plasma membrane [35–37]. Also, it leads to leading-edge formation for the movement of cells. In particular, the change of receptor localization from lipid rafts to other membrane domains is an important step in the induction of its activity [9,15]. Our results showed that translocation of P2Y2 to nonlipid raft domains was observed at 60 min in macrophages stimulated by apoptotic neutrophils, whereas in the PLAG-treated group translocation was initiated at 15 min and cells were already moving at 30 min. Association of the P2Y2 receptor with cytoskeletal proteins, actin and α-tubulin, and actin polarization were also observed at 15 min (Figs 2D and 3D).

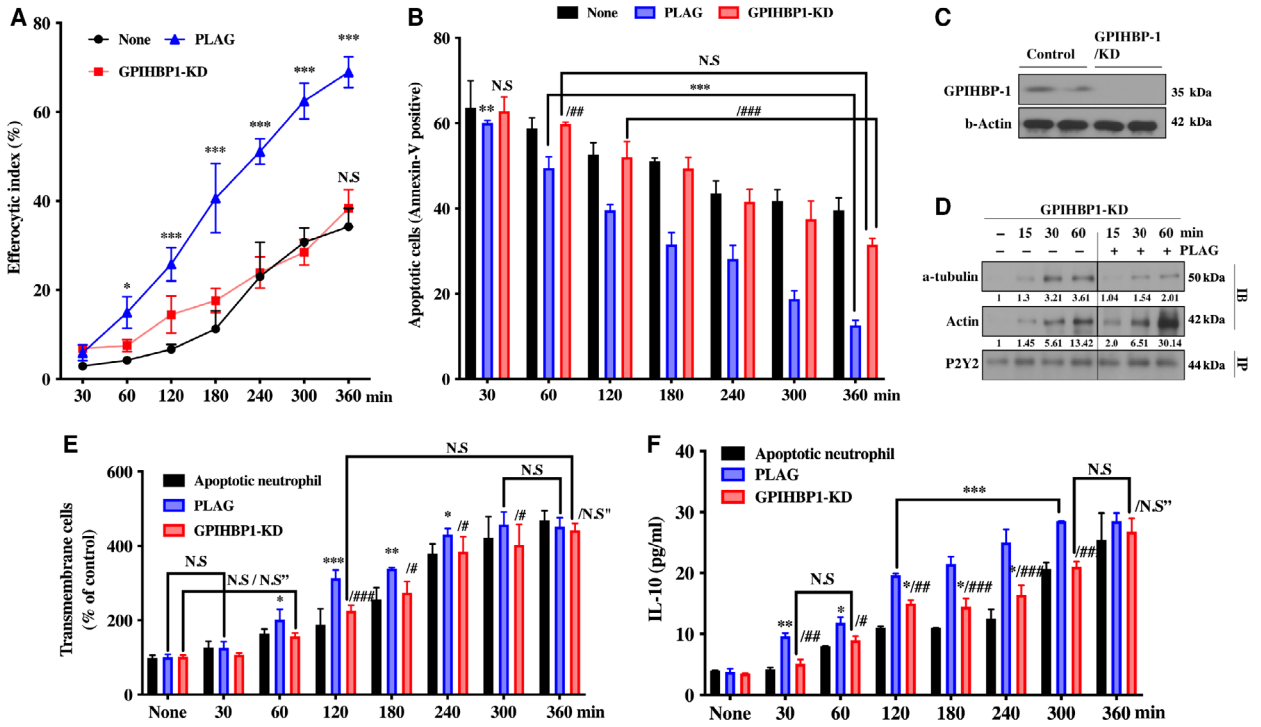


**Fig. 5.** PLAG-promoted movement of the P2Y2 receptor out of lipid rafts is dependent on GPIHBP1. The effect of PLAG was examined in macrophages with GPIHBP-1 knockdown via siRNA transfection. (A) Efferocytotic index was calculated by FACS. Compared to apoptotic neutrophil only group: $^{*}P < 0.05$, $^{***}P < 0.001$ (each experiment $n = 6$). N.S., not significant. Mean ± SD. (B) Number of unengulfed apoptotic neutrophils was quantitated by FACS. Compared to apoptotic neutrophil only group: $^{**}P < 0.01$, $^{***}P < 0.001$ (each experiment $n = 6$). Compared to PLAG group: $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ (each experiment $n = 6$). N.S., not significant (compared to apoptotic neutrophil only group). Mean ± SD. (C) Knockdown of GPIHBP-1 via siRNA transfection was confirmed. (D) Coimmunoprecipitation of P2Y2 with proteins related to polarization of the cytoskeleton. (E) The degree of chemoattraction of macrophages toward apoptotic neutrophils was determined. Compared to apoptotic neutrophil only group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (each experiment $n = 6$). Compared to PLAG group: $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ (each experiment $n = 6$) N.S., not significant (compared to apoptotic neutrophil only group). N.S.", not significant (Compared to PLAG group). Mean ± SD. (F) Changes of IL-10 cytokine levels in the culture medium were measured by ELISA. Compared to apoptotic neutrophil only group: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (each experiment $n = 6$). Compared to PLAG group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ (each experiment $n = 6$) N.S., not significant (compared to apoptotic neutrophil only group). N.S.", not significant (compared to PLAG group). Mean ± SD.

To determine whether PLAG acts as a vesicle in this context, the biological activity of PLAG was examined using the same experiment in GPIHBP1-silenced cells. GPIHBP1 (glycosylphosphatidylinositol anchored high-density lipoprotein binding protein 1) protein is expressed as membrane protein for recognizing vesicles like chylomicron [38]. As a result, the effect of PLAG promoted efferocytosis, and macrophage mobility was not observed in the GPIHBP1-silenced cells. In addition, accelerated translocation of P2Y2 to the nonlipid raft was also not detected (Figs 5 and 6). Recent studies have shown that the recruitment of GPI-anchored protein (AP) in lipid rafts induces the formation of caveolae and the activation of specific receptors [39–41]. It is also known to induce rapid repositioning into the plasma membrane through the formation of the recycling endosome of the receptor [42]. Our study shows that the action of structural PLAG induces recruitment of GPIHBP1, which is GPI-AP, and caveolae that formed through it rapidly accelerated the trafficking activity of P2Y2 for cytoskeleton rearrangement (Fig. 7).

The promotion of P2Y2 receptor translocation by PLAG provides more rapid movement of macrophages toward nucleotides generated by apoptotic neutrophils, allowing the macrophages to eliminate the apoptotic neutrophils rapidly. Eliminating apoptotic neutrophils in a timely manner is very important for the prevention of exaggerated immune responses and maintenance of homeostasis. Our results showed that the vesicle structure of PLAG induced rapid macrophage mobility and more effective phagocytosis of apoptotic neutrophils and that these biological activities were rapidly initiated as a result of translocation of the

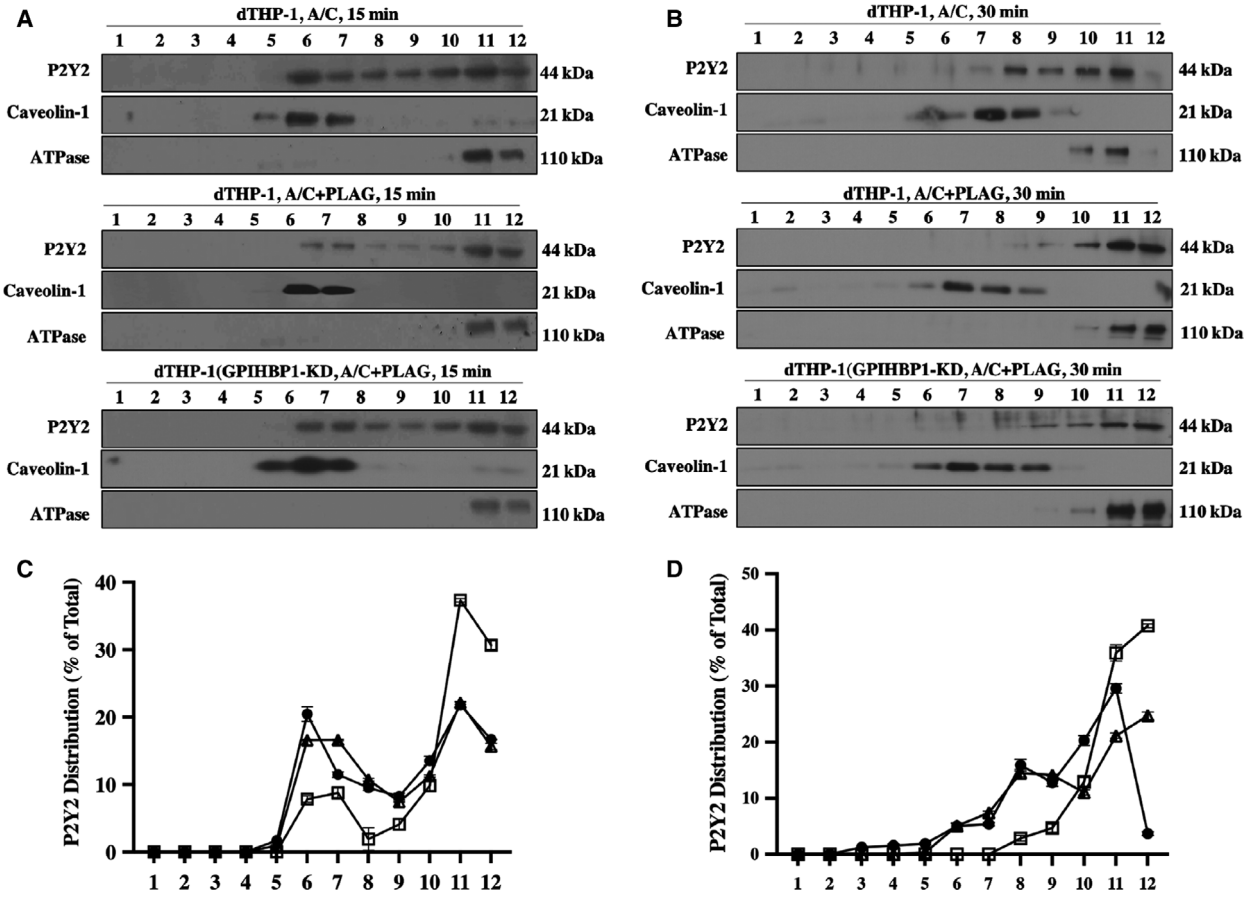


**Fig. 6.** PLAG regulated macrophage lipid raft via GPIHBP1. (A, B) The membrane distribution change of P2Y2 in GPIHBP-1 knockdown cells was confirmed by the lipid raft fraction method. Change of P2Y2 distribution by PLAG treatment was quantified at the same time. Caveolin-1 was used as a lipid raft domain marker. (C, D) The distribution of P2Y2 in each band was quantified and plotted. ●, None; □, apoptotic neutrophil with PLAG; △, GPIHBP1-KD group (each experiment $n = 3$). Mean ± SD.

P2Y2 receptor to nonlipid raft membrane domains for earlier rearrangement of the cytoskeleton in macrophages (Fig. 8).

Taken together, we suggest that PLAG is a very effective lipid-based drug for prevention and treatment of inflammatory diseases occurring because of sustained immune responses caused by delayed elimination of apoptotic neutrophils.

## Methods

### Cell culture

THP-1 and HL60 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). THP-1 cells were grown in RPMI1640 medium (WelGENE, Seoul, Korea) containing 10% fetal bovine serum (HyClone, Waltham, MA, USA), 1% antibiotics (100 $mg{\cdot}L^{-1}$ streptomycin, 100 $U{\cdot}mL^{-1}$ penicillin), and 0.4% 2-Mercaptoethanol (Sigma Aldrich, St. Louis, MO, USA). HL60 cells were grown in RPMI1640 medium containing 20% fetal bovine serum, and 1% antibiotic solution (100 $mg{\cdot}L^{-1}$ streptomycin, 100 $U{\cdot}mL^{-1}$ penicillin). Cells were grown at 37 °C in 5% $CO_2$ atmosphere. To differentiate THP-1 cells into macrophage-like cells, cells were grown in medium with 1% phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich) for 72 h. To differentiate HL60 cells into neutrophil-like cells, cells were grown in a medium with 10% DMSO (Sigma Aldrich) for 5 days.

### Determination of efferocytosis index and apoptotic cell clearance

For determining apoptotic differentiation, HL60 were stained with Annexin-V PE (Molecular probes, Eugene, OG, USA) for 30 min in binding buffer. Differentiated THP-1 cells were stained with 10 mM CellTracker Green CMFDA (Molecular probes) for 30 min in PBS. Apoptotic

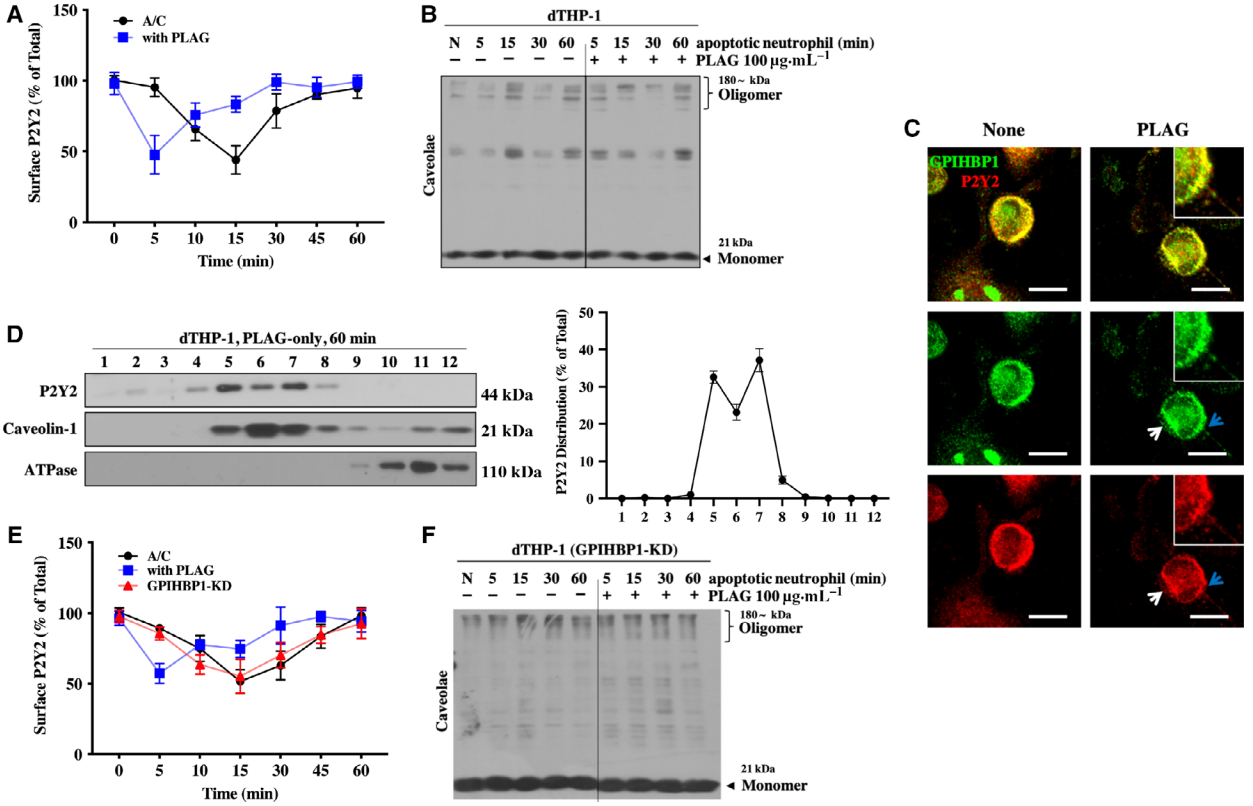


**Fig. 7.** Mechanism of P2Y2 redistribution of structural PLAG. (A) The surface membrane expression of P2Y2 (Trafficking) over time was quantitated using FACS (each experiment $n = 6$). Mean ± SD. (B) The changes of caveolae formation in lipid raft over time were confirmed by western blotting. (C) The colocalization and polarization changes of GPIHBP1 and P2Y2 by structural PLAG treatment were confirmed by confocal microscopy. The white arrows indicate the polarization regions of the two proteins by structural PLAG treatment, and the blue arrows indicate the magnified region. Scale bar = 20 μm. (D) Distribution change of P2Y2 during PLAG alone treatment. (E) The changes of P2Y2 surface membrane expression in GPIHBP1 knockdown cells were quantitated by FACS (each experiment $n = 6$). Mean ± SD. (F) The changes of caveolae formation by PLAG treatment in GPIHBP1 knockdown cells were confirmed by western blotting over time.

neutrophils were cocultured with macrophages for a period of time and then the supernatant (nonefferocytosis neutrophil) was transferred to a new tube. A cell scraper was used to collect the efferocytotic macrophages, which were combined with the collected supernatant and centrifuged at 260 ***g*** for 10 min. Cells were washed twice using PBS and then resuspended in PBS containing 0.2% BSA. The efferocytotic index was analyzed by FACS (BD Bioscience, Franklin Lakes, NJ, USA). Briefly, the fluorescence intensities of apoptotic neutrophils stained with Annexin-V PE in FACS gates of CMFDA-stained macrophages were measured. Nonphagocytosis apoptotic neutrophils measure the PE fluorescence index in CMFDA negative gates.

## Live cell image

Differentiated HL60 cells were stained with 5 uM CellTracker Red CMTPX (Molecular probes) for 30 min in PBS and lead to apoptosis by PMA treatment. Differentiation THP-1 were stained with 5 μM CellTracker Green CMFDA (Molecular probes) for 30 min in PBS. The coculture plate was put on the stage of LSM800 (Carl Zeiss, Thornwood, NY, USA) for 120 min. Fluorescence overlay videos were recorded using ZEN program (Carl Zeiss).

## Indirect contact macrophage stimulation (indirect coculture)

Differentiated THP-1 cells were seeded in 24-well plates, and apoptosis was induced in the differentiated HL-60 cells by PMA. For coculture, the media was removed from precultured differentiated THP-1 cells, and 0.5 mL of fresh media was added to the plate. Insert modified Boyden chamber (SPL lifescience, Seoul, Korea) with 0.4-μm pore polycarbonate membrane inserts into each well, and add 0.5 mL of media suspended in apoptotic HL-60 to the upper chamber for stimulation. PLAG is placed in the lower chamber 1 h before stimulation with apoptotic HL-60.

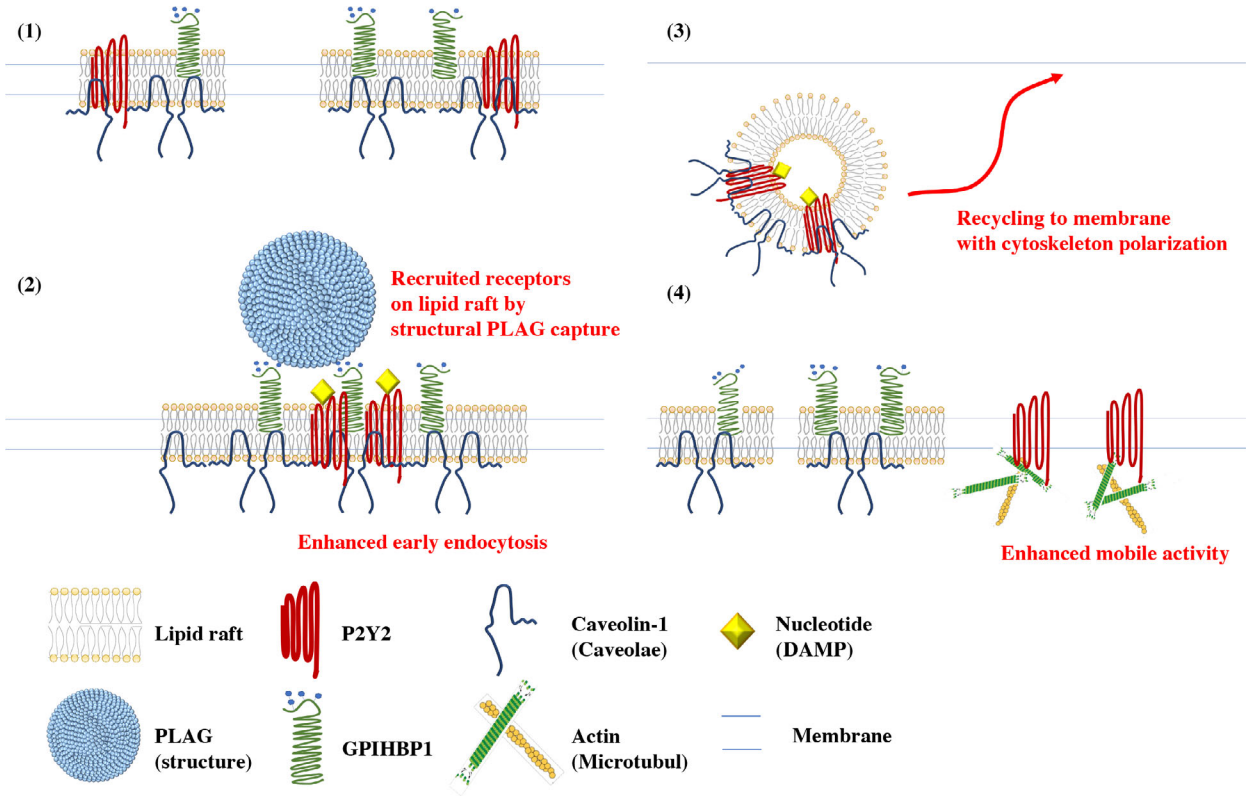


**Fig. 8.** Mechanism of induction of macrophage mobility through membrane redistribution induction of structural PLAG. (1) P2Y2, caveolin-1, and GPIHBP1 are distributed on lipid raft. (2) AS structural PLAG treatment, GPIHBP1 gathers at a specific position to catch it. P2Y2 and caveolin-1 on the lipid raft move together, and caveolae formation then takes place rapidly for endocytosis. (3) Rapidly, endocytosis of P2Y2 is quickly recycled to the membrane. (4) When P2Y2 is recycled, the actin component of cytoskeleton moved together and polarized rapidly. This reaction leads to an increase in the mobility of macrophages.

## Macrophage cytoskeleton polarization

Cells were seeded in 12-well plates with cover glass and incubated until 100 % confluence was reached in each well. After incubation, wound cell monolayer is made on the center of the well and stimulated for an indicated period of time. After stimulation for the indicated time and dose at 37 °C in a 5% $CO_2$ atmosphere, the cells were fixed with 3.7 % formaldehyde for 20 min and permeabilized with 0.2 % Triton X-100 for 20 min. For staining the specific proteins, cells were washed with PBS twice and reacted with specific antibody (α-tubulin; Cell Signaling Technology, Danvers, MA, USA, #2125, 1 : 1000/ Actin; Santa Cruz, Dallas, TX, USA, sc-70319, 1 : 1000) for overnight at 4 °C. Cells were washed with PBS twice and reacted with secondary antibody (Alexa 488-conjugated antibody for actin/ Alexa 546-conjugated antibody for α-tubulin, 1 : 5000). Fluorescence was detected by confocal microscopy (Carl Zeiss).

## Macrophage movement (wound healing assay)

dTHP-1 cells were seeded in 12-well plates with cover glass and incubated to 100% confluence in each well. After incubation, a wound cell monolayer was made in the center of well, and treated with PLAG and apoptotic neutrophil stimulation for an indicated period of time. After treatment for the indicated time and dose at 37 °C in a 5% $CO_2$ atmosphere, the cells were fixed with 3.7% formaldehyde for 20 min and permeabilized with 0.2% Triton X-100 for 20 min and stained with 0.1% Phalloidin-FITC for 40 min. Fluorescence was detected by confocal microscopy (Carl Zeiss). The degree of wound healing was quantified using IMAGE J [National Institutes of Health (NIH), Bethesda, MD, USA].

## Transwell-Chemoattraction assay

Quantitative macrophage chemoattraction assays were performed using a modified Boyden chamber (SPL lifescience) with Matrigel-coated 8.0-μm pore polycarbonate membrane inserts in 24-well plates. The lower chamber was filled with apoptotic neutrophils for chemoattraction. The dTHP-1 cells ($5 \times 10^4$ cells·mL$^{-1}$) in serum-free medium were added into the upper chamber and treated with PLAG. The cells were allowed for chemoattraction for an indicated period of time at 37 °C in a 5% $CO_2$ atmosphere. The nonchemoattracted

cells were removed from the upper surface of the membrane by scraping with a cotton swab, and the number of chemoattracted cells was calculated by MTT assay.

## Confocal microscopy

To confirm the colocalization of proteins involved in the macrophage migration, dTHP-1 cells were seeded in 12-well plates with cover glass, and incubated to 60 % confluence in each well. After incubation, treated with PLAG and induced apoptotic neutrophil stimulation for an indicated period of time. After treatment for an indicated time at 37 °C in a 5% $CO_2$ atmosphere, the cells were fixed by treating with 3.7% formaldehyde for 20 min and permeabilized with 0.2% Triton X-100 for 20 min for staining purpose. Cells were washed with PBST twice and incubated with specific antibody (P2Y2; Santa Cruz, sc-518019, 1 : 1000/ Caveolin-1; Thermoscientific, Waltham, MA, USA, MA3-600, 1 : 1000) for overnight at 4 °C. Cells were washed with PBST twice and reacted with secondary antibody. For cytoskeleton detection, cells were stained with 0.1% Phalloidin-Cy5.5 for 40 min. Fluorescence was detected by confocal microscopy (Carl Zeiss).

In order to confirm the changes in the localization of cell membrane proteins following PLAG treatment, dTHP-1 cells were seeded in 12-well plates with cover glass, and incubated to 60 % confluence in each well. After incubation, treated with PLAG to induce stimulation for an indicated time period. After treatment for the indicated time at 37 °C in a 5% $CO_2$ atmosphere, the cells were fixed with 3.7 % formaldehyde for 20 min. Cells were washed with PBST twice and reacted with specific antibody (P2Y2; Santa Cruz, sc-518019, 1 : 1000/ GPIHBP1; Novus Biological, Littleton, CO, USA, NB110-41537 1 : 1000) for overnight at 4 °C. Cells were washed twice with PBST and reacted with secondary antibody. Fluorescence was detected by confocal microscopy (Carl Zeiss).

## ELISA

The levels of cytokine secretion in the cell supernatants or plasma were analyzed using an enzyme-linked immunosorbent assay (ELISA) (specific for cytokines obtained from BD Bioscience) according to the manufacturer's protocol. The absorbance was measured at 450 nm using an EMax Endpoint ELISA microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA) and the concentration ($pg{\cdot}mL^{-1}$) was calculated using a standard curve.

## 10. Immunoprecipitation (IP)

dTHP-1 cells treated with PLAG and stimulated to induce neutrophil apoptosis for various time periods at 37 °C in a 5% $CO_2$ atmosphere were lysed using ice-cold IP lysis buffer (25 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol). Extracted proteins were incubated with SureBeads Protein G specific antibody (P2Y2; Santa Cruz, sc-518019, 1:250) bound magnetic beads (Bio-Rad, Hercules, CA, USA). Beads were washed with PBS containing Tween 20 (PBST) and target proteins were eluted in 1× sample buffer and analyzed by western blotting.

## Lipid raft fractionation

Cells were centrifuged and resuspended in ice-cold lysis buffer (25 mM MES pH 6.5, 150 mM NaCl, 1% Triton X-100) for 30 min. High-density insoluble debris was removed by centrifugation. The supernatant was added to 2 volumes of OptiPrep (Sigma Aldrich) in MES buffer (25 mM MES pH 6.5, 150 mM NaCl), gently mixed by vortexing, and then centrifuged through a 10–40% continuous gradient using an SW60 rotor for 18 h at 168 661 ***g*** and 4 °C. After centrifugation, 12 fractions were harvested from the top to bottom. Protein concentration in each fraction was determined by Bradford protein assay and protein content was analyzed by western blotting.

## GPIHBP1 Knockdown (KD)—siRNA transfection

Small interfering RNA (siRNA) was purchased from Santa Cruz Biotechnology. For transient transfection, cells were washed twice with PBS and resuspended in transfection buffer (Lonza, Basel, Switzerland) containing the siRNA. Cell and siRNA mixtures were placed in a Nucleocuvette and electroporated using a 4D-nucleofector (Lonza). After transfection, cells were incubated in a differentiation medium for 72 h and treated with PLAG for apoptotic neutrophil stimulation for various time periods.

## Measurement of P2Y2 trafficking

dTHP-1 cells were resuspended with FACS buffer (1% FBS in PBS). Cells were treated with specific antibody (P2Y2; Santa Cruz, sc-518019, 1:20) for 30 min at 4 °C and reacted with PE-conjugated secondary antibody for 30 min at 4 °C. Then the cells were washed with FACS buffer twice and analyzed by flow cytometry.

## Caveolae formation assay

The dTHP-1 cells were centrifuged and resuspended in ice-cold lysis buffer (25 mM HEPES, pH 7.2, 150 mM NaCl, 1% Triton X-100) for 30 min. High-density insoluble debris was removed by centrifugation. Extracted proteins were incubated with 25 mM BMH (Thermoscientific) with gentle rotation for 1 h, then add 1 mM glycine and keep for 15 min, and finally add 4× nonreducing sample buffer and quantify by western blotting.

### Measurement of apoptotic neutrophils

The dHL60 cells were seeded in 60-mm plates and incubated for 24 h. Following incubation, the cells were stimulated using 100 nM PMA for 24 h at 37 °C in a 5% $CO_2$ atmosphere. Total cells were harvested by trypsinization, collected by centrifugation, washed with PBS, and resuspended in binding buffer. Cells were stained with Annexin-V for 15 min. Fluorescence intensity was analyzed using a flow cytometer (Fig. S1).

### Neutrophil Extracellular Trap (NET) formation

PMA-treated dHL60 cells were made to react with a plasma membrane-impermeable DNA-binding dye SYTOX Red (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction. The cells were mixed with 1 μM SYTOX dye in the culture medium and treated with PMA for a certain time period. dHL60 cells with NETosis were collected after centrifugation, washed with PBS, resuspended in DEPC-DW, and fluorescence intensity analyzed using a flow cytometer (Fig. S1).

### Statistics

The data were statistically analyzed using a one-way ANOVA (Prism 7, GraphPad Software, La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

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## Conflict of interest

The authors declare no conflict of interest.

## Author contributions

This study was conceived and designed by JW and KW; GT, SY, and JW performed the experiments; GT and JW tested for efferocytosis effect; and SY and KY synthesized and provided PLAG for testing purpose.

## Reference

1 Henson PM & Hume DA (2006) Apoptotic cell removal in development and tissue homeostasis. *Trends Immunol* **27**, 244–250.
2 Karaji N & Sattentau QJ (2017) Efferocytosis of pathogen-infected cells. *Front Immunol* **8**, 1863.
3 Nagata S, Hanayama R & Kawane K (2010) Autoimmunity and the clearance of dead cells. *Cell* **140**, 619–630.
4 Belchamber KBR & Donnelly LE (2017) Macrophages: macrophage dysfunction in respitory disease. *Results Probl Cell Differ* **62**, 299–313.
5 Trahtemberg U & Mevorach D (2017) Apoptotic cells induced signaling for immune-homeostasis in macrophages and dendritic cells. *Front Immunol* **8**, 1356.
6 Lauber K, Blumenthal SG, Waibel M & Wesselborg S (2004) Clearance of apoptotic cells getting rid of the corpses. *Mol Cell* **14**, 277–287.
7 Ravichandran KS & Lorenz U (2007) Engulfment of apoptotic cells: signals for a good meal. *Nat Rev Immunol* **7**, 964–974.
8 Ravichandran KS (2010) Find-me and eat-me signals in apoptotic cell clearance: progress and conundrums. *J Exp Med* **207**, 1807–1817.
9 Elliott MR, Chekeni FB, Trampont PC, Lazarowski ER, Kadl A, Walk SF, Park D, Woodson RI, Ostankovich M, Sharma P *et al.* (2009) Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature* **461**, 282–286.
10 Chen Y, Corriden R, Inoue Y, Yip L, Hashiguchi N, Zinkernagel A, Nizet V, Insel PA & Junger WG (2006) ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. *Science* **314**, 1792–1795.
11 Kronlage M, Song J, Sorokin L, Isfort K, Schwerdtle T, Leipziger J, Robaye B, Conley PB, Kim HC, Sargin S *et al.* (2010) Autocrine purinergic receptor signaling is essential for macrophage chemotaxis. *Sci Signal* **3**, ra55.
12 Sathanoori R, Bryl-Gorecka P, Müller CE, Erb L, Weisman GA, Olde B & Erlinge D (2017) P2Y2 receptor modulates shear stress-induced cell alignment and actin stress fibers in human umbilical vein endothelial cells. *Cell Mol Life Sci* **74**, 731–746.
13 Degagné É, Degrandmaison J, Grbic DM, Vinette V, Arguin G & Gendron FP (2013) P2Y2 receptor promotes intestinal microtubule stabilization and mucosal re-epithelization in experimental colitis. *J Cell Physiol* **228**, 99–109.
14 Liao Z, Seye CI, Weisman GA & Erb L (2007) The P2Y2 nucleotide receptor requires interaction with αv integrins to access and activate G12. *J Cell Sci* **120**, 1654–1662.
15 Martinez NA, Ayala AM, Martinez M, Martinez-Rivera FJ, Miranda JD & Silva WI (1321N) Caveolin-1 regulates the P2Y2 receptor signaling in human 1321N1 astrocytoma cells. *J Biol Chem* **291**, 12208–12222.
16 Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y & Zychlinsky A

(2004) Neutrophil extracellular traps kill bacteria. *Science* **303**, 1532–1535.
17 Cooper PR, Palmer LJ & Chapple ILC (2013) Neutrophil extracellular traps as a new paradigm in innate immunity: friend or foe? *Periodontol 2000* **63**, 165–197.
18 Nakazawa D, Shida H, Kusunoki Y, Miyoshi A, Nishio S, Tomaru U, Atsumi T & Ishizu A (2016) The responses of macrophages in interaction with neutrophils that undergo NETosis. *J Autoimmun* **67**, 19–28.
19 Farrera C & Fadeel B (2013) Macrophage clearance of neutrophil extracellular traps is a silent process. *J Immunol* **191**, 2647–2656.
20 Lee KH, Kronbichler A, Park DDY, Park Y, Moon H, Kim H, Choi JH, Choi Y, Shim S, Lyu IS *et al.* (2017) Neutrophil extracellular traps (NETs) in autoimmune diseases: a comprehensive review. *Autoimmun Rev* **16**, 1160–1173.
21 Muñoz LE, Lauber K, Schiller M, Manfredi AA & Herrmann M (2010) The role of defective clearance of apoptotic cells in systemic autoimmunity. *Nat Rev Rheumatol* **6**, 280.
22 Wang W, Peng W & Ning X (2018) Increased levels of neutrophil extracellular trap remnants in the serum of patients with rheumatoid arthritis. *Int J Rheum Dis* **21**, 415–421.
23 Khandpur R, Carmona-Rivera C, Vivekanandan-Giri A, Gizinski A, Yalavarthi S, Knight JS, Friday S, Li S, Patel RM, Subramanian V *et al.* (2013) NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. *Sci Transl Med* **5**, 178ra40.
24 Yang H, Kim S, Cho SH, Kim MG, Seo JY, Park JS, Jhon GJ & Han SY (2004) Purification and structural determination of hematopoietic stem cell-stimulating monoacetyldiglycerides from *Cervus nippon* (Deer Antler). *Chem Pharm Bull* **52**, 874–878.
25 Yang H, Park JS, Cho SH, Yoon JY, Kim MG, Jhon GJ, Han SY & Kim S (2004) Stimulatory effects of monoacetyldiglycerides on hematopoiesis. *Biol Pharm Bull* **27**, 1121–1125.
26 Ko YE, Yoon SY, Ly SY, Kim JH, Sohn KY & Kim JW (2018) 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) reduces hepatic injury in concanavalin A-treated mice. *J Cell Biochem* **119**, 1392–1405.
27 Yoo N, Lee HR, Shin SH, Sohn KY, Kim HJ, Han YH, Chong S, Kim MH, Yoon S & Kim J (2016) PLAG (1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol) augments the therapeutic effect of pegfilgrastim on gemcitabine-induced neutropenia. *Cancer Lett* **377**, 25–31.
28 Lee HR, Yoo N, Kim J, Sohn KY, Kim HJ, Kim MH, Han M, Yoon S & Kim J (2016) The therapeutic effect of PLAG against oral mucositis in hamster and mouse model. *Front Oncol* **6**, 209.
29 Kim YJ, Shin J, Shin SH, Kim J, Sohn KY, Kim HJ, Kang JK, Yoon S & Kim J (2014) 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol ameliorates arthritic joints through reducing neutrophil infiltration mediated by IL-6/STAT3 and MIP-2 activation. *Oncotarget* **8**, 96636–96648.
30 Bukong T, Cho Y, Iracheta-Vellve A, Saha B, Lowe P, Adejumo A, Furi I, Ambade A, Gyongyosi B, Catalano D *et al.* (2018) Abnormal neutrophil traps and impaired efferocytosis contribute to liver injury and sepsis severity after binge alcohol use. *J Hepatol* **69**, 1145–1154.
31 Freeman SA & Grinstein S (2014) Phagocytosis: receptors, signal integration, and the cytoskeleton. *Immunol Rev* **262**, 193–215.
32 Elliott MR, Koster KM & Murphy PS (2017) Efferocytosis signaling in the regulation of macrophage inflammatory responses. *J Immunol* **198**, 1387–1394.
33 Medina CB & Ravichandran KS (2016) Do not let death do us part: 'find-me' signals in communication between dying cells and the phagocytes. *Cell Death Differ* **23**, 979–989.
34 Beigneux AP, Davies B, Gin P, Weinstein MM, Farber E, Qiao X, Peale F, Bunting S, Walzem RL, Wong JS *et al.* (2007) Glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 plays a critical role in the lipolytic processing of chylomicrons. *Cell Metab* **5**, 279–291.
35 Eun S, Seo J, Park S, Lee J, Chang K & Kim H (2014) LPS potentiates nucleotide-induced inflammatory gene expression in macrophages via the upregulation of P2Y2 receptor. *Int Immunopharmacol* **18**, 270–276.
36 Saha S, Anilkumar A & Mayor S (2016) GPI-anchored protein organization and dynamics at the cell surface. *J Lipid Res* **57**, 159–175.
37 Cendrowski J, Mamińska A & Miaczynska M (2016) Endocytic regulation of cytokine receptor signaling. *Cytokine Growth Factor Rev* **32**, 63–73.
38 DeFea KA (2007) Stop that cell! β-arrestin-dependent chemotaxis: a tale of localized actin assembly and receptor desensitization. *Ann Rev Physiol* **69**, 535–560.
39 Min J & DeFea K (2011) β-arrestin-dependent actin reorganization: bringing the right players together at the leading edge. *Mol Pharmacol* **80**, 760–768.
40 Paulick MG & Bertozzi CR (2008) The glycosylphosphatidylinositol anchor: a complex membrane-anchoring structure for proteins. *Biochemistry* **47**, 6991–7000.

41 Chaudhary N, Gomez GA, Howes MT, Lo HP, McMahon KA, Rae JA, Schieber NL, Hill MM, Gaus K, Yap AS *et al.* (2014) Endocytic crosstalk: cavins, caveolins, and caveolae regulate clathrin-independent endocytosis. *PLoS Biol* **12**, e1001832.
42 Sabharanjak S, Sharma P, Parton RG & Mayor S (2002) GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. *Dev Cell* **2**, 411–423.

## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1**. Verification of NETosis formation and apoptosis of differentiated HL60 by PMA.

**Video S1**. Effect of PLAG on the induction of apoptotic neutrophil efferocytosis.