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PLAG enhances macrophage mobility for efferocytosis of active neutrophils via membrane re-distribution of P2Y2

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Abstract

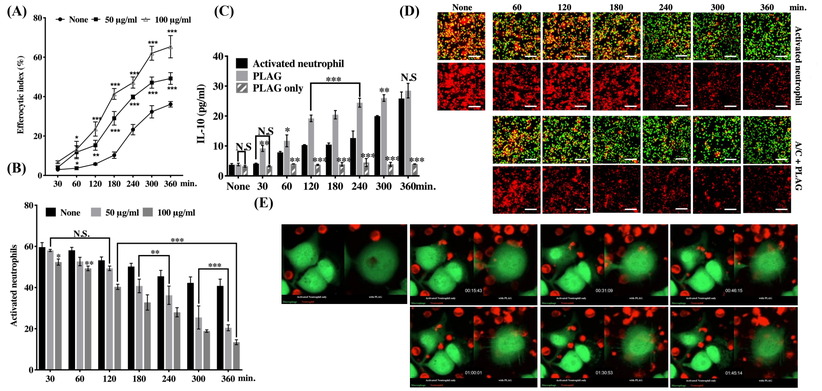
Neutrophil activity is prerequisite during chemotherapy. The DAMP (Damage Associated Molecular Pattern) molecules generated by chemotherapy could be effectively trapped by activated neutrophil called 'NETosis'. Efferocytosis of macrophages should remove most activated neutrophils including NETosis A timely removal of activated neutrophils is essential for the prevention of abnormal activation of immune response and metastatic activity of cancer cells induced by tumor microenvironment (TME). Particularly, appropriate clearance of the activated neutrophils by efferocytosis should be carried out because activated neutrophils have a detrimental effect on TME.

In this research, we investigated the effect of 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) on efferocytosis and its underlying molecular mechanisms. In a co-culture of activated neutrophils with macrophages, PLAG increased the activity of efferocytosis for elimination of activated neutrophils. PLAG accelerated translocation of P2Y2 from lipid rafts to non-lipid-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. Through these protein assemble, PLAG encouraged macrophage mobility toward the activated neutrophils. Formation of micelle including PLAG, chylomicron-like structures, was a prerequisite for induction of this macrophage activity. PLAG effect on this activity was not observed in the absence of GPIHBP1,micelle receptor.

Taken together, these data showed that PLAG triggered a prompt clearance of activated neutrophils through enhancement of efferocytosis activity. Subsequently, PLAG could have effects on modulation of TME. PLAG could be utilized as an effective lipid-based TME modulator via the prevention of abnormal activation induced by uncontrolled immune response during chemotherapy.

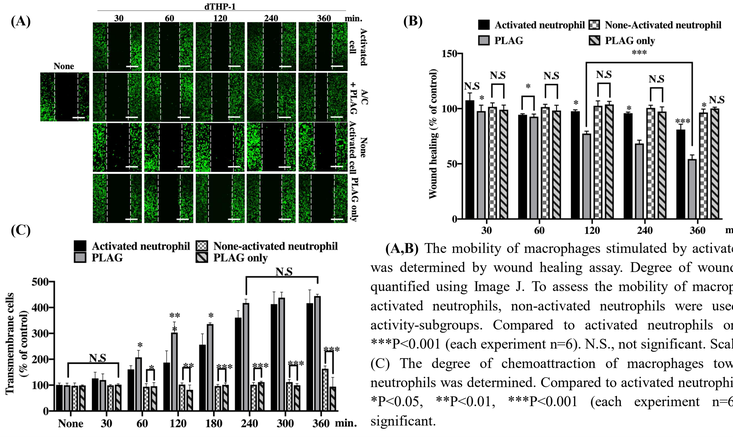
Result

Effect of PLAG on the induction of activated neutrophil efferocytosis



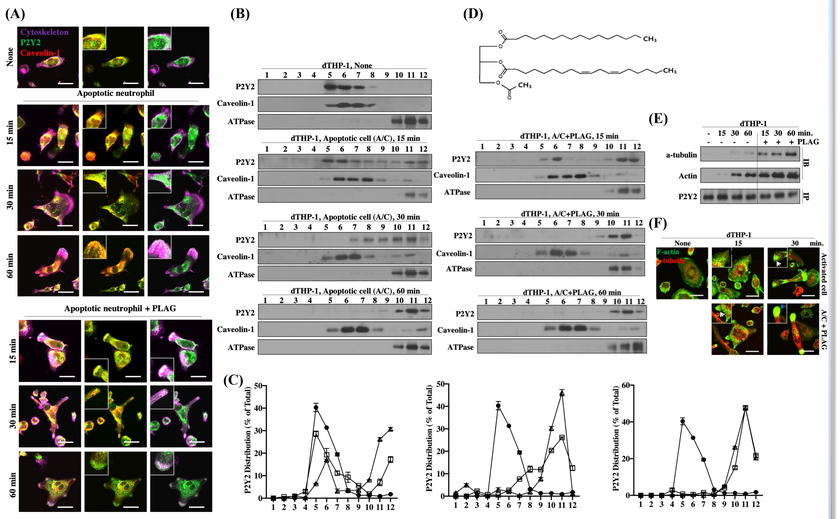
Differentiated THP-1 cells were pre-treated with PLAG for 1 h and then stimulated by activated neutrophils. **(A)** Efferocytotic index was calculated by FACS. Compared to activated neutrophil only group: *P<0.05, **P<0.01, ***P<0.001 (each experiment n=6). **(B)** Number of un-engulfed activated neutrophils was quantitated by FACS. Compared to control: *P<0.05, **P<0.01, ***P<0.001 (each experiment n=6). **(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. **(D)** The degree of clearance of apoptotic neutrophils was confirmed using confocal microscopy. Activated neutrophils were tagged with red fluorescence and macrophages tagged in green fluorescence. Scale bar; 100 um. **(E)** Efferocytosis of macrophages was visualized in real time.

Increase of macrophage mobility on the PLAG treated cells



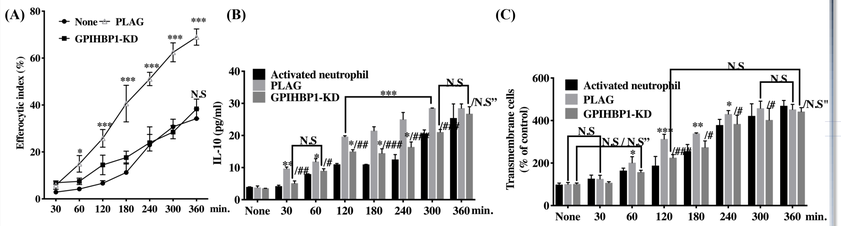
(A,B) The mobility of macrophages stimulated by activated neutrophils was determined by wound healing assay. Degree of wound healing was quantified using Image J. To assess the mobility of macrophages toward activated neutrophils, non-activated neutrophils were used to test the activity-subgroups. Compared to activated neutrophils only: *P<0.05, ***P<0.001 (each experiment n=6). N.S., not significant. Scale bar; 100 um. (C) The degree of chemoattraction of macrophages toward activated neutrophils was determined. Compared to activated neutrophils only group: *P<0.05, **P<0.01, ***P<0.001 (each experiment n=6). N.S., not significant.

Enhanced movement of P2Y2 receptor from the lipid raft to non-lipid raft in the PLAG treated cells

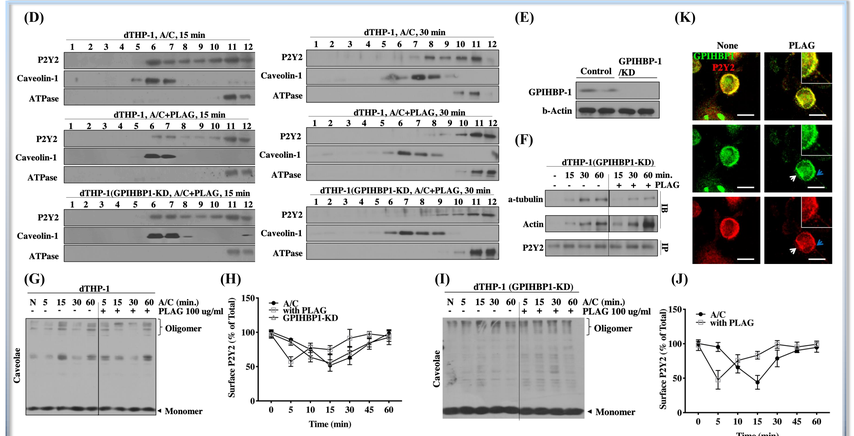


(A) Co-localization shift of P2Y2 and co-binding with cytoskeleton by membrane distribution change were confirmed by confocal. White arrow indicated that P2Y2 were co-localization with caveolin-1 and blue arrow indicate that P2Y2 were co-localization with cytoskeleton. Scale bar; 20 um. **(B)** The membrane distribution change of P2Y2 was determined by the lipid raft fractionation method. Caveolin-1 was used as a lipid raft marker. **(C)** The distribution of P2Y2 in each band was quantified and plotted. ●; None, □Apoptotic neutrophil, △; PLAG. **(D)** The simple structure of PLAG. **(E)** The binding of P2Y2 with proteins related to polarization of the cytoskeleton was detected by immunoprecipitation. **(F)** The degree of cytoskeletal polarization and colocalization with actin protein was determined by confocal microscopy. Scale bar; 20 um

Promoted movement of P2Y2 receptor to non-lipid raft by structural PLAG was dependent on GPIHBP1, vesicle recognizing receptor

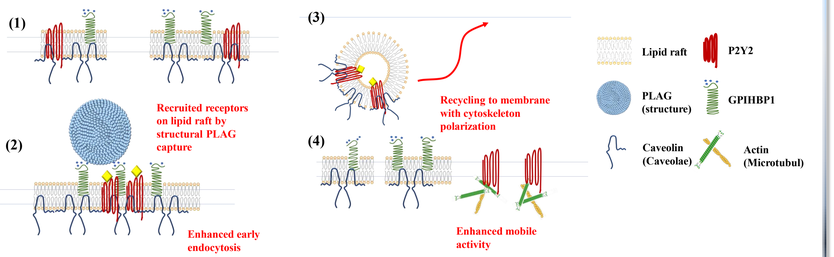


The effect of PLAG was examined in macrophages with GPIHBP-1 knock-down via siRNA transfection. **(A)** Efferocytotic index was calculated by FACS. Compared to activated neutrophil only group: *P<0.05, ***P<0.001 (each experiment n=6). N.S., not significant.



(B) 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). **(C)** 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<0.01 (each experiment n=6) N.S., not significant (Compared to apoptotic neutrophil only group). N.S.", not significant (Compared to PLAG group). **(D)** The membrane distribution change of P2Y2 in GPIHBP-1 knock-down cells was confirmed by the lipid raft fraction method. Change of P2Y2 distribution by PLAG treatment was quantified at the same time. **(E)** Knock-down of GPIHBP-1 via siRNA transfection was confirmed. **(F)** Co-immunoprecipitation of P2Y2 with proteins related to polarization of the cytoskeleton. **(G,I)** The changes of caveolae formation in Lipid raft over time were confirmed by Western blotting. **(H,J)** The surface membrane expression of P2Y2 (Trafficking) over time was quantitated using FACS. **(K)** The co-localization and polarization changes of GPIHBP1 and P2Y2 by structural PLAG treatment were confirmed by Confocal Scale bar; 20 um.

Conclusion



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. The promotion of P2Y2 receptor translocation by PLAG provides more rapid movement of macrophages toward nucleotides generated by activated neutrophils, allowing the macrophages to rapidly eliminate the activated neutrophils.