PLAG ameliorates LPS-induced ALI by attenuation of neutrophil infiltration into alveolar via a prompt resolution of TLR4 signaling.

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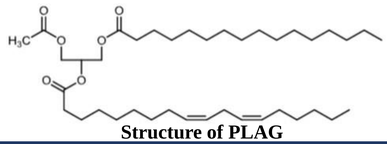
Abstract

Acute lung injury (ALI) is an acute respiratory failure that is associated with excessive neutrophil recruitment into the bronchoalveolar space and results in severe mortality. To evaluate 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) as a therapeutic agent for ALI, PLAG was administered orally to mice intranasally challenged with lipopolysaccharide (LPS). The excessive neutrophil infiltration in the bronchoalveolar lavage fluid (BALF) was detected in LPS-treated mice. Intranasally introduced LPS especially stimulates tissue-resident macrophages in the pulmonary tissue and induces neutrophil-attracting chemokine production such as MIP-2. Co-administrated PLAG dramatically ameliorated neutrophil infiltration into the bronchoalveolar region via modification of TLR4 signaling. We found that PLAG effectively accelerated endocytosis of LPS-TLR4 complex and promoted the NADPH oxidase activity through the formation of Rac, p47phox assembly into membrane for ROS production which results in elimination of endotoxin in Raw264.7 cells. PLAG also triggered a prompt TLR4 signals occurred in endosome mediated by TRIF and terminated signaling including MIP-2 expression when endocytosed endotoxin is cleared. Moreover, immunofluorescence microscopes of RAW264.7 cells showed that PLAG accelerated the endocytosis of the LPS-TLR4 complex and the clearance of the internalized LPS. Our results suggest that PLAG promotes the clearance of invaded endotoxin and eventually triggers an earlier termination of MIP-2 secretion in the endotoxin-cleared macrophages, and might be used as a potential therapeutic agent to prevent ALI through speedy resolution of endotoxin.

Introduction

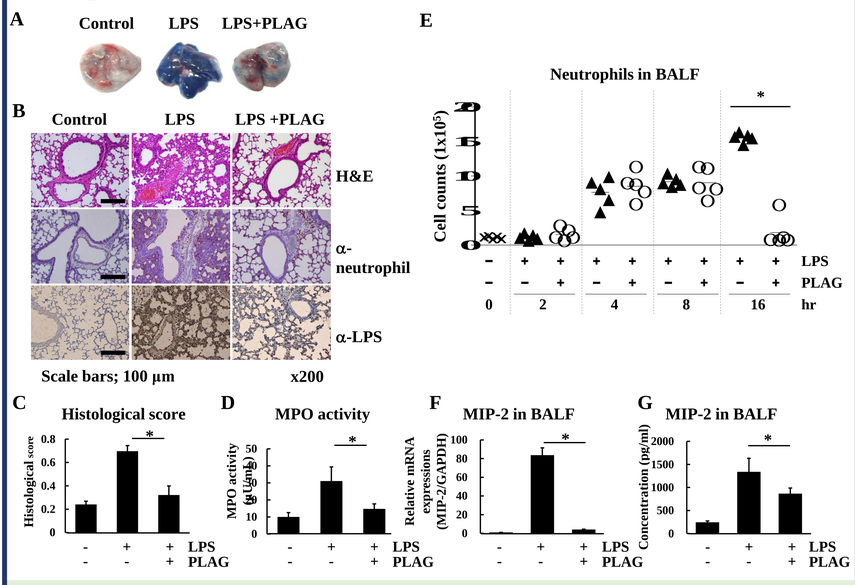
- Acute lung injury (ALI) are severe respiratory inflammatory lung diseases. ALI is characterized by the disruption of the lung alveolar–capillary membrane barrier, leading to a massive infiltration of neutrophils into the interstitium and the bronchoalveolar space, as well as an excessive inflammatory response. **Bernard GR, *et al.* Am J Respir Crit Care Med 149, 818-824 (1994).**
- The activated NOX produced reactive oxygen species (ROS) which is able to directly kill the pathogens and stimulate the increase of IL-8 and MIP-2. **Leverence JT, *et al.* Chem Biol Interact 189, 72-81 (2011).**
- TLR4 associated signal pathway is classified according to its use of two main adaptor proteins, referred to as myeloid differentiation primary response protein 88 (Myd88) and TIR domain-containing adaptor protein inducing IFN-β (TRIF). **Akira S, *et al. Nat Rev Immunol* 4, 499-511 (2004).**
- PLAG (1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol), which is an acetylated diacylglycerol (DAG), is a mono-acetyl-diglyceride that has been isolated from the antlers of sika deer and chemically synthesized from glycerol, palmitic acid, and linoleic acid. **Yang HO, *et al. Chemical & pharmaceutical bulletin* 52, 874-878 (2004).**
- PLAG was shown to exert a therapeutic effect with pegfilgrastim to treat chemotherapy-induced neutropenia by modulating neutrophil transmigration and PLAG administration significantly reduced 5-FU/scratching–induced oral mucositis and cachexia models. **Yoo N, *et al. Cancer Lett* 377, 25-31 (2016). Lee HR, *et al. Front Oncol* 6, 209 (2016).**



Structure of PLAG

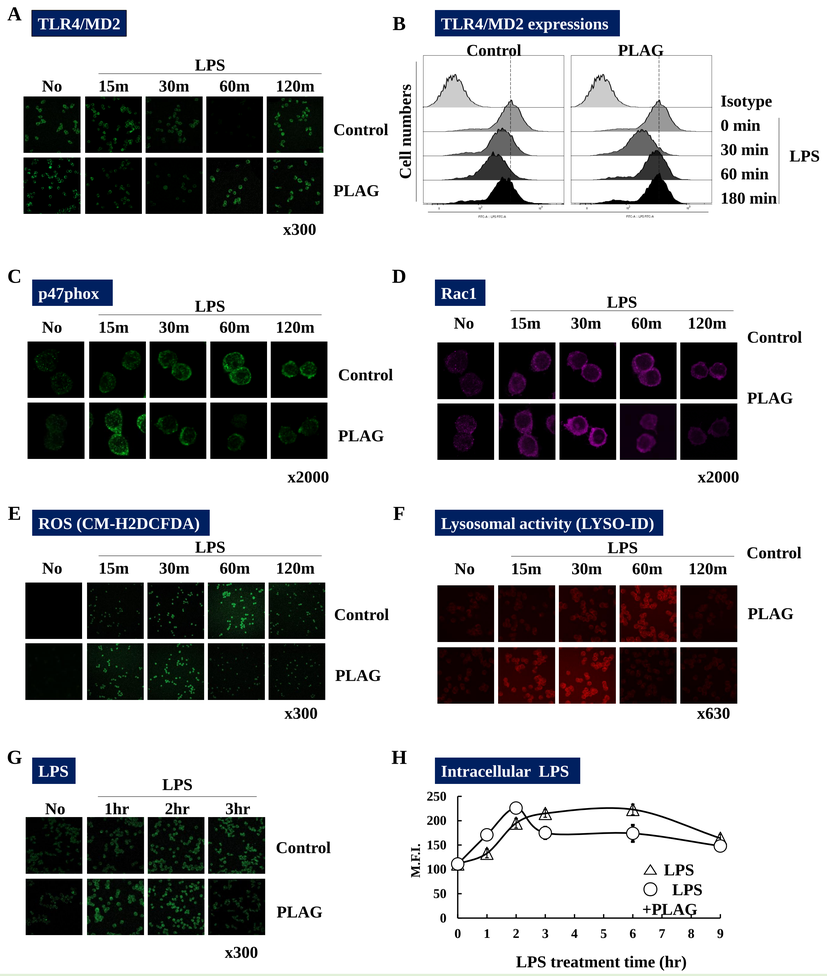
Result

1. PLAG resolved LPS-induced ALI through regulation of excessive neutrophil infiltration



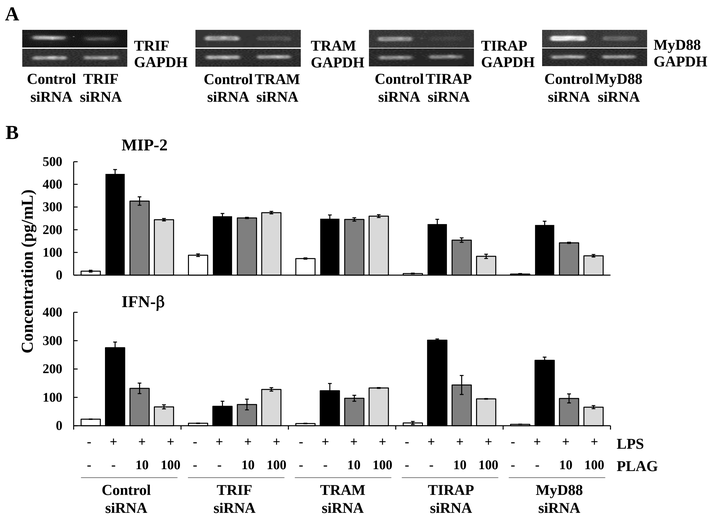
Mice were divided into four separate groups (n=5 per group): control, LPS-treated, and PLAG/LPS o-treated. LPS (25 mg/kg) was intranasal injected, and PLAG (250 mg/kg) was administered orally. Evans blue dye (50 mg/kg) was injected intravenously 30 min before sacrifice following PLAG/LPS treatment for 16 hr. In mice treated with PLAG/LPS, Evans blue stained albumins were decreased in the lungs compared to LPS only treated group **(Figure 1A)**. Intranasal LPS administration induced vast inflammatory cell infiltration into the lung tissue compared to control **(Figure 1B).** PLAG/LPS co-treated mice exhibited considerably reduced inflammatory cell infiltration into the alveolar and had normal alveolar morphology, and the histological score has also shown the same effect **(Figure 1C, 1D).** PLAG/LPS co-treated animals exhibited the faster returned homeostasis in the neutrophil number of BALF after 16 hr **(Figure 1E)**. To determine PLAG role in control of excessive and successive neutrophil infiltration on the lung tissue of ALI, the mRNA expressions of inflammation-related molecule were examined in BALF cells **(Figure 1F)**. Furthermore, the secreted MIP-2 level was also significantly increased in BALF following LPS administration, and markedly decreased in PLAG addition **(Figure 1G)**.

2. PLAG induced the faster endocytosis and recovery of TLR4 and promoted the engulfed LPS clearance than LPS alone



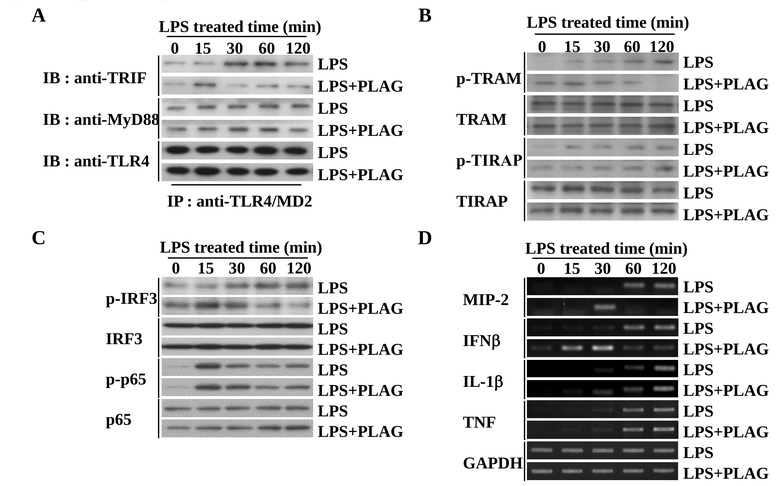
PLAG role for ALI protection were investigated *in vitro* system as well as LPS-mediated ALI pathogenesis. LPS(100ng/mL) stimulates its cognate receptor TLR4 and subsequently induces LPS engulfment with aid of TLR4 in the RAW264.7 cells. The internalized LPS/TLR4 complex was evaluated by analysis of surface spanning TLR4 using anti-TLR4/MD2 antibody. PLAG(100μg/mL) 1h pre-treated Raw 264.7 cells showed the faster endocytosis of LPS/TLR4 complex and the earlier recovery of TLR4 on surface membrane than LPS alone analyzed confocal and FACS **(Figure 2A, 2B)**. PLAG accelerated the internalization of TLR4 receptor and promoted its returns to surface membrane. ROS generation is closely regulated by nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase system. LPS-stimulated Raw 264.7 cells showed the recruitment of p47phox and Rac1 from cytosol to membrane was observed at 30 min and sustained until 120 min after treatment. In PLAG/LPS co-treated cells, the recruitment and return to homeostasis of p47phox and Rac1 were observed at 15 and 60 min, respectively **(Figure 2C, 2D)**. ROS production was detected at the earlier time, 15 min, in the PLAG added group than only LPS treated group **(Figure 2E, 2F).** PLAG facilitates LPS-induced TLR4 endocytosis in short time and accelerates LPS-induced ROS production and return to homeostasis via earlier clearance of invading pathogen, LPS **(Figure 2G, 2H)**.

3. PLAG affected TRIF-dependent endosomal signaling rather than MyD88 pathway under LPS stimulation



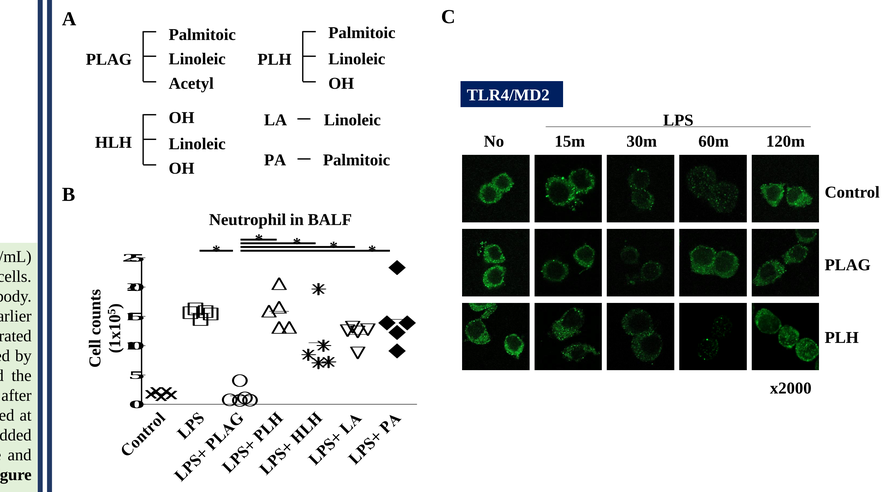
To determine whether PLAG modulates the major pathway of LPS-induced MIP-2 production, Raw264.7 cells were transfected with the specific siRNAs of Myd88 and TIRAP for Myd88-dependent signal and siRNAs of TRIF and TRAM for TRIF-dependent signal pathway associated molecules. The targeted all mRNAs were effectively downregulated **(Figure 3A)**. After 24hr, cells were incubated with 10 or 100 μg/mL of PLAG or DMSO (as solvent control) for 1 hr. LPS was treated with 100 ng/mL for 16 hr, culture supernatants were assayed by ELISA to check the secretion levels of MIP-2 and IFN-β respectively In TRIF or TRAM silenced group, activity of PLAG for modulation of MIP-2 and IFN-β productions was completely abolished. But, in TIRAP or Myd88 silenced group, PLAG effect was still observed with dose dependent manner. Which apparently suggests that PLAG has activity for MIP-2 modulation is mainly dependent on TRIF, endosomal associated signal pathway **(Figure 3B)**.

4. PLAG affected TRIF-dependent endosomal signaling rather than MyD88 pathway under LPS stimulation.



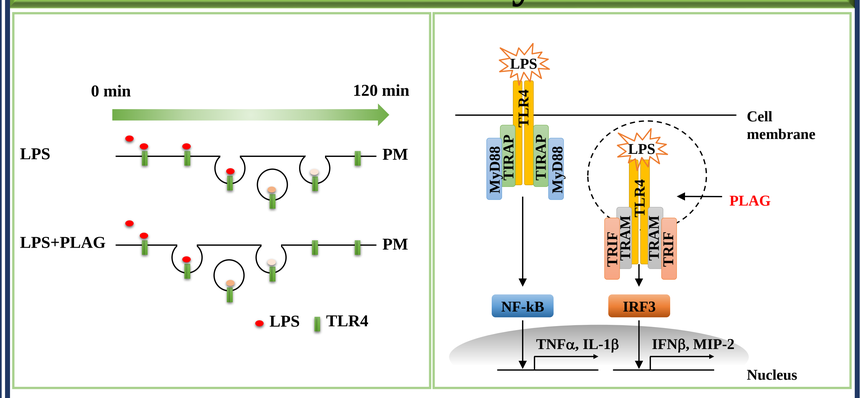
Assembled adaptor proteins to TLR4 were precipitated using anti-TLR4/MD2 in LPS (100 ng/mL) stimulated cells with or without PLAG (100μg/mL). Assemble of TRIF to TLR4/MD2 was detected at 30 min and sustained for 120 min in LPS treated cells. In PLAG/LPS co-stimulated cells, TRIF and TLR4 assembling was initiated at 15 min and dissembling was found at 60 min following LPS treatment. Meanwhile, MyD88 molecule assemble to TLR4 was not changed in PLAG/LPS stimulated cells. These data showed that PLAG accelerated assembling and dissembling of TRIF to TLR4 **(Figure 4A)**. PLAG has a preferential activity on the modulation of MIP-2 and IFN-β expression rather than that of IL-1β and TNF expression **(Figure 4D)** by TRAM, IRF specifically phosphorylation **(Figure 4B, 4C)**.

5. Specificity of PLAG in therapeutic effects of ALI was determined by comparisons with derivatives



To determine specificity of PLAG, acetylated diacylglycerol, PLAG derivatives were used to compare the biological efficacy related to therapeutic effect of ALI. PLH is a diacylglycerol (DAG) that consists of two fatty acid chains, palmitic acid, and linoleic acid. HLH is composed of linoleic acid and glycerol backbone. Linoleic acid (LA) or palmitic acid (PA) was also used **(Figure 5A)**. In the ALI animal model, LPS administration via intranasal induces massive neutrophil extravasation into alveolar which is easily detected in BALF. Neutrophils in the BALF in the PLAG (250 mg/kg) co-treated mice was dramatically reduced and returned to normal status. Whereas PLH, HLH, LA, and PA (250 mg/kg) had no effect in the reducing neutrophils in the BALF. These data indicated that PLAG has specific role in the blocking the excessive and successive neutrophil infiltration during LPS-induced ALI progression **(Figure 5B)**. In the LPS stimulated Raw264.7 cells, TLR4/MD2 internalization was observed at 30 min and prolonged for 120 min. PLAG (100μg/mL) co-treated cells showed the increased TLR4/MD2 internalization at 15 min and return to surface at 60 min. But PLH (100μg/mL) co-treated cell didn't show the accelerated internalization and early return to surface of TLR4/MD2 **(Figure 5C)**. These findings suggest that the acetylation of diacylglycerol is critical factor in blocking excessive neutrophil infiltration in the ALI animal model.

Summary



- **PLAG accelerates LPS-induced TLR4 endocytosis/exocytosis cycle.**
- **PLAG regulates TLR4 endocytosis related signaling pathway.**
- **PLAG stimulated the faster resolution of lung inflammation and may be suggested as a therapeutic agent on the various inflammatory diseases.**