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1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) attenuates gemcitabine-induced neutrophil extravasation

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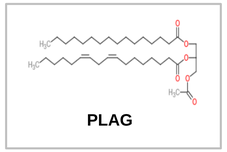
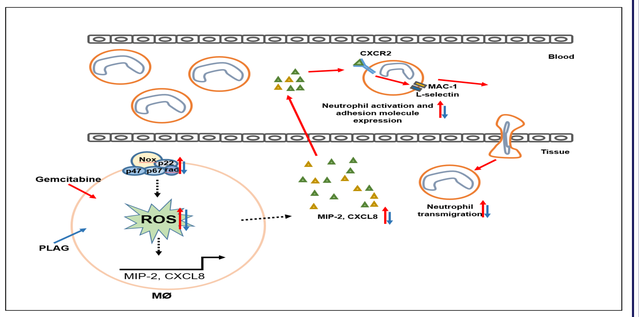
Abstract

Neutrophils are the most abundant leukocyte in circulation and are the first responder to foreign particles and/or injury. Some chemotherapeutics are known to induce depletion of blood neutrophils as its high cytotoxicity. However, it is also possible that circulating neutrophils undergo extravasation in response to chemotactic gradients induced by anti-cancer agents. Here, we found that 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) attenuates gemcitabine-induced neutrophil extravasation via the inhibition of neutrophil-attracting chemokine expression in macrophages using *in vivo* and *in vitro* approaches. First, we observed that there were increased neutrophils infiltrated into the peritoneal cavity following gemcitabine treatment in 4T1 tumor-bearing mice. In addition, gemcitabine increased MIP-2 mRNA expression in the peritoneal cells as well as in other tissues of the mice. Likewise, gemcitabine induced the migration of circulating neutrophils into the peritoneal cavity in normal mice, and PLAG effectively decreased neutrophil migration by inhibiting the expression of adhesion molecules. Inhibition of CXCR2 by its antagonist, reparixin, abrogated gemcitabine-induced neutrophil migration, indicating that chemokines produced by gemcitabine mainly support neutrophil activation. *In vitro* experiments demonstrated that PLAG inhibited NADPH oxidase (NOX)-mediated reactive oxygen species production induced by gemcitabine, which is the upstream of MIP-2 and/or CXCL8. Importantly, PLAG down-regulated gemcitabine-induced membrane translocation of the cytosolic NOX subunit, Rac1, and phosphorylation of p47phox. Altogether, this study suggests the potential of PLAG as a therapeutic strategy to modulate chemotherapy-induced neutrophil activation for cancer patients undergoing chemotherapeutic treatment.

Introduction

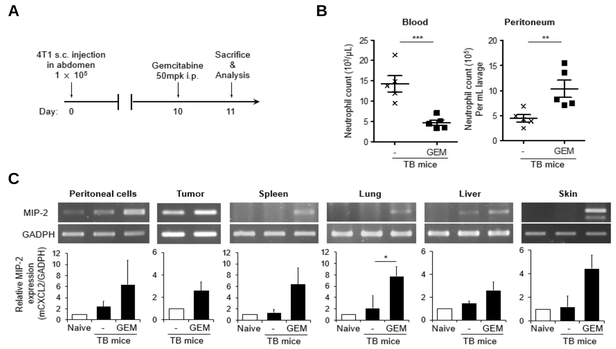
- Cancer patients undergoing treatment with chemotherapeutics often suffer from a rapid decline of circulating neutrophils. This complication is called chemotherapy-induced neutropenia (CIN). **Crawford J., *et al* (2004). Cancer 100(2), 228–237.**
- Neutropenic patients are susceptible to infection, which necessitates dose reduction or cessation of chemotherapy. **Caggiano V., *et al* (2005). Cancer 105(9), 1916–1924.**
- Chemotherapeutics often activates NADPH oxidase and generates reactive oxygen species (ROS) production, which leads to cancer progressions and/or complications. Fiorini, C., et al. (2015). **Biochimica et Biophysica Acta (BBA)-Molecular Cell Research**, *1853*(3), 549-560.
- PLAG, synthetic monoacetyl-diacylglyceride, is an effective regulator of inflammation in diverse disease model. **Yoon, S. Y., et al. (2015). Immune Netw 15(2): 100-109.**





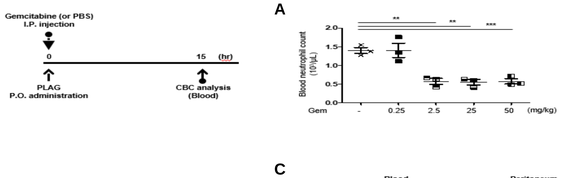
Results

1. Gemcitabine induces neutrophil extravasation and infiltration into the peritoneum by producing chemokine MIP-2 in vivo in 4T1 tumor-bearing mice.



Male Balb/c mice of 8 to 10 weeks of age were subcutaneously injected with 4T1 mammary carcinoma at day 0, and the mice were intraperitoneally injected with saline (control) or 50mg/kg gemcitabine for 24h at day 10. Blood, peritoneal fluids and tissue samples were collected for further analysis. **A,** Schematic of the experimental design. **B,** Cell counts and cell differentiation were determined by complete blood count (CBC) analysis, and neutrophils were counted. The number of neutrophils from the blood and the peritoneal cavity was compared. Each group contains five mice, and bars represent the mean ± SD.

2. PLAG attenuates gemcitabine-induced neutropenia by repressing neutrophil extravasation from blood into the peritoneum.

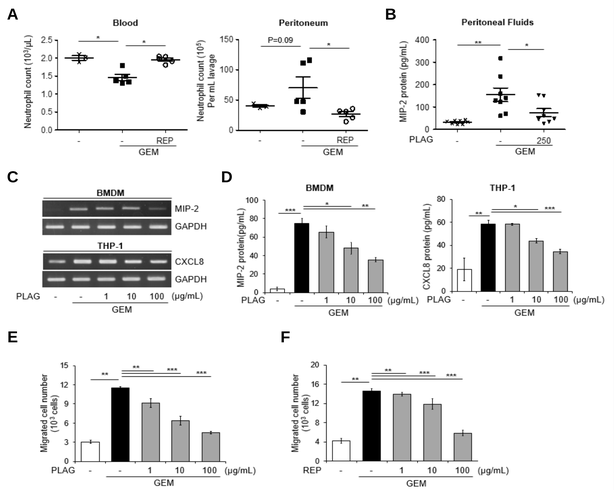


	Control	Gem	Gem + PLAG 50	Gem + PLAG 250
Neut (1x10^3/μL)	1.37±0.22	0.62±0.16	0.92±0.18	1.09±0.14

	Blood			Peritoneum		
	Control	Gem	Gem +PLAG	Control	Gem	Gem +PLAG
Neut (1x10^3/μL)	2.00±0.11	1.46±0.20	1.96±0.10	2.00±0.11	1.46±0.20	1.96±0.10

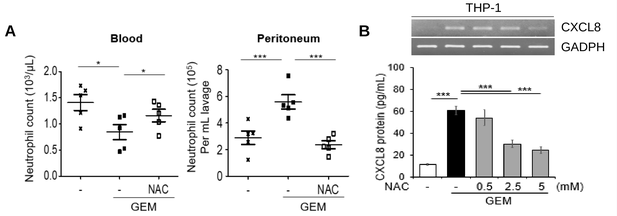
A, Male balb/c mice of 8-10 weeks of age were intraperitoneally injected with different concentrations of gemcitabine for 24h and then the number of blood neutrophils was determined by CBC analysis. **B,** Male balb/c mice of 8-10 weeks of age were orally administrated with various concentrations of PLAG, and then intraperitoneally injected with 50mg/kg gemcitabine for 24h. The number of blood neutrophils were determined by CBC analysis. Each group contains seven mice, and bars represent the mean ± SD. **C,** The number of neutrophils from the blood and the peritoneal cavity was compared. Each group contains five mice.

3. PLAG inhibits gemcitabine-induced MIP-2(CXCL8)-CXCR2-mediated neutrophil chemotaxis.

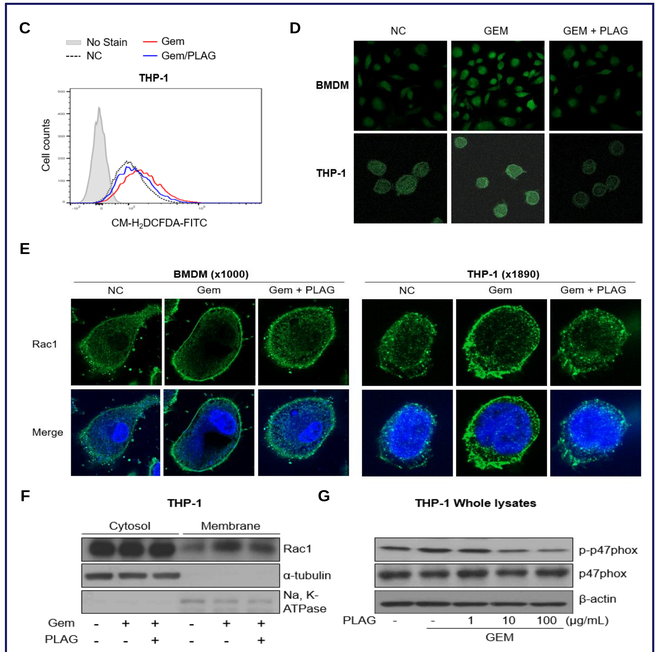


A, Reparixin was diluted in mineral oil and administered intraperitoneally at a dose of 50 mg/kg for 1 h before gemcitabine administration. Neutrophils in the blood or in the peritoneal cavity were counted using CBC analysis. Each group contains five mice, and bars represent the mean ± SD. **B,** Peritoneal fluids were harvested, and the protein level of MIP-2 was evaluated by ELISA. BMDMs and THP-1 cells were treated with various doses of PLAG and stimulated with gemcitabine (10μg/mL). **C,** The mRNA level of MIP-2 or CXCL8 was analyzed by RT-PCR, and **D,** the protein level of MIP-2 or CXCL8 in the conditioned medium was determined by ELISA. The schematic illustrates the protocol of in vitro migration assays, and transmigration of dHL-60 cells was decreased by **(E)** PLAG and **(F)** reparixin treatment. * p<0.05, ** p<0.01, *** p<0.001.

4. PLAG decreases gemcitabine-induced activation of NADPH oxidase and ROS production that mediates neutrophil migration.



A, NAC (50mg/kg) was administered intraperitoneally at a dose of 50 mg/kg for 1 h before gemcitabine administration. Neutrophils in the blood and the peritoneal cavity were counted using CBC analysis. Each group contains five mice, and the bars represent the mean ± SD. **B,** THP-1 cells were treated with various doses of NAC and stimulated with gemcitabine (10μg/mL), and the mRNA and protein level of CXCL8 were determined by RT-PCR and ELISA, respectively.



C, The level of intracellular ROS in BMDMs and THP-1 cells were analyzed by flow cytometry. **D,** CM-H_2DCFDA fluorescence imaging of ROS in BMDMs and THP-1 cells using a confocal laser scanning microscope. **E,** BMDMs and THP-1 cells were treated with gemcitabine and/or PLAG, and processed for immunofluorescence with an anti-Rac1 antibody. The same cells were also stained with DAPI to visualize nuclei. **F,** the cells were pretreated with PLAG for 1 h and stimulated with gemcitabine for 3 h, and cytosolic and membrane proteins were fractioned. The separated proteins were subject to western blot analysis and blotted with antibodies against: Rac1, Na/K-ATPase, and α-tubulin. **G,** Whole lysates of THP-1 cells were separated by SDS-PAGE and subject to western blot. The blot was probed with an anti-phospho-p47^{phox} antibody.

Summary

- Chemotherapy-Induced Neutropenia (CIN) is the consequence of excessive circulating neutrophil transmigration in response to chemotactic gradients produced by resident macrophages.
- PLAG attenuates gemcitabine-induced neutrophil extravasation by regulating neutrophil-attracting chemokine expression and gemcitabine-induced activation of NADPH oxidase and ROS generation.
- We expect that PLAG can be developed as a new potential therapeutic strategy for CIN.