PLAG reduces the infection risk via acceleration of bacteria clearance through NF-κB activation in *Pseudomonas aeruginosa* induced pneumonia model with chemotherapy-induced neutropenia

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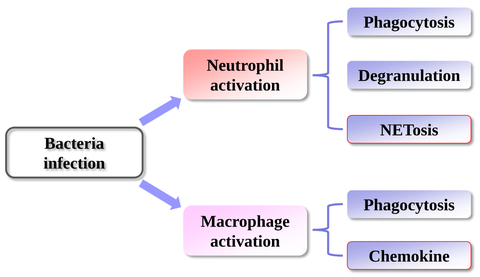
 # 1044

Abstract

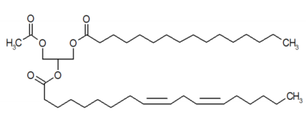
Pseudomonas aeruginosa K (PAK) is an opportunistic pathogen that cause an acute and chronic infectious disease in immunocompromised condition. Also, PAK is responsible for 90% of deaths in hospitalized cystic fibrosis patients. NF-κB and STAT3 are important signal molecules in inflammatory responses. Recently, researches on the interaction between NF-κB and STAT3 in inflammation have been actively conducted. Previously, we reported that PLAG (1-palmityoyl-2-linoleoyl-3-acetyl-rac-glycerol) has an effect on the regulation of STAT3 activation in diverse disease models. In this study, we investigated whether PLAG has effects on bacterial clearance in pneumonia mice with chemotherapy-induced neutropenia (CIN). To establish immunocompromised mice model, cyclophosphamide and doxorubicin (AC regimen) were injected to mice. And PAK was administrated by intranasal injection to the AC regimen-treated mice and followed by oral administration of PLAG. We observed that PLAG increased survival rate, and bacteria clearance of PAK-infected neutropenic mice was also enhanced by PLAG administration at early time. To reveal the mechanism of PLAG on increase of bacteria elimination, we examined the effect of PLAG on neutrophil recruitment and NETosis. We found that PLAG promoted NETosis of neutrophils than only PAK-treated cells through NF-κB activation via down-regulation of STAT3. In conclusion, we suggest that role of PLAG on bacterial clearance in pneumonia model with CIN is to accelerate PAK clearance by enhancing NETosis and regulating interaction between NF-κB and STAT3 activation. Therefore, PLAG can help immunocompromised patients at high risk of infection.

Introduction

- *P. aeruginosa*, a gram negative and opportunistic bacterium, can cause acute or chronic diseases, cystic fibrosis, sepsis and pneumonia. *P. aeruginosa* is one of the main causes of pneumonia, which degrades lung function and increases mortality of infected people. **Fujita T, *et al*,. Kansenshogaku Zasshi 2010;84(5):588-91.**
- Neutrophils play a key function in the innate immunity against bacteria infection; Phagocytosis, degranulation and NETosis. Among them, NETosis is an effectively mechanism in eliminating infecting pathogen. When infected with pathogens, neutrophils release DNA web-like structure and eliminate captured pathogen. **Ranzk N, *et al*,. Semin Immunopathol 2013;35(4):513-30.**
- NF-κB and STAT3 are important signal molecule in innate immune responses. NF-κB is a major signal pathway against pathogen infection, but STAT3 induces immunosuppressive gene, IL-10. **McFarland BC, *et al*,. Mol Cancer Res 2013;11(5):494-505.**
- PLAG, 1-palmitoryl-2-linoleoyl-3-acetyl-rac-glycerol, is a synthetic DAG derivative. We confirm that PLAG has an effect on diverse disease models such as LPS induced lung inflammation, rheumatoid arthritis, and so on. **Yoon SY, *et al*,. Immune Netw 2015;15(2):100-9.**



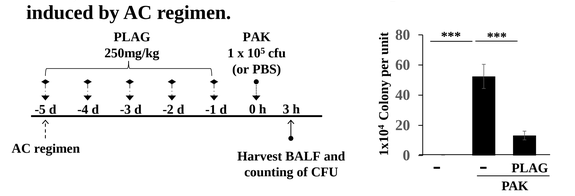
Elimination of pathogen by neutrophil and macrophage

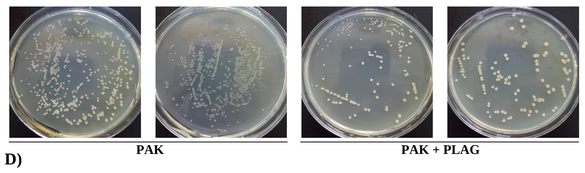


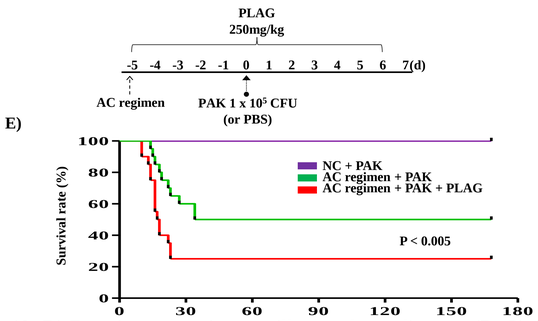
PLAG

Result

1. PLAG improves clearance and viability of *P. aeruginosa* K (PAK) -infected mice under immunocompromised condition induced by AC regimen.

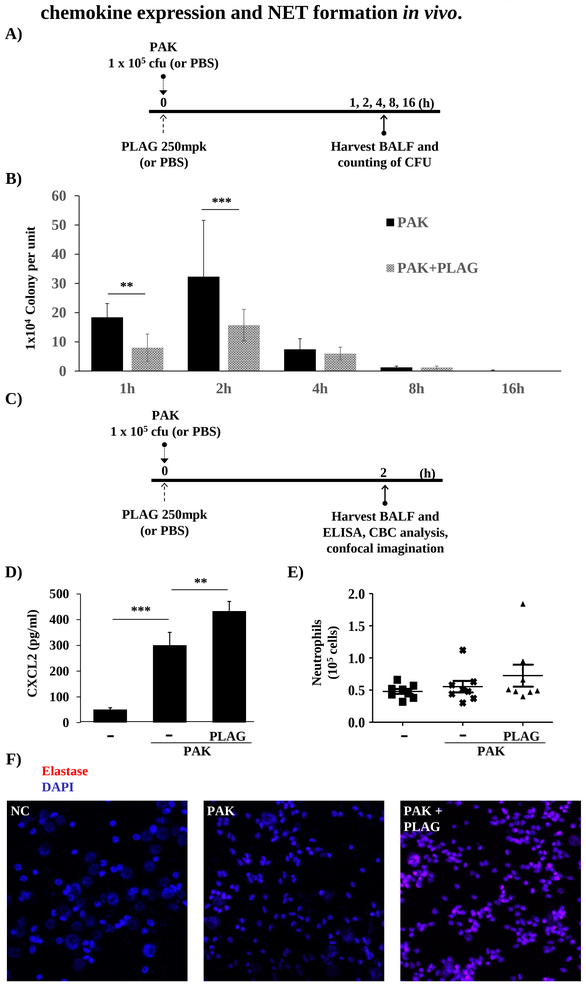






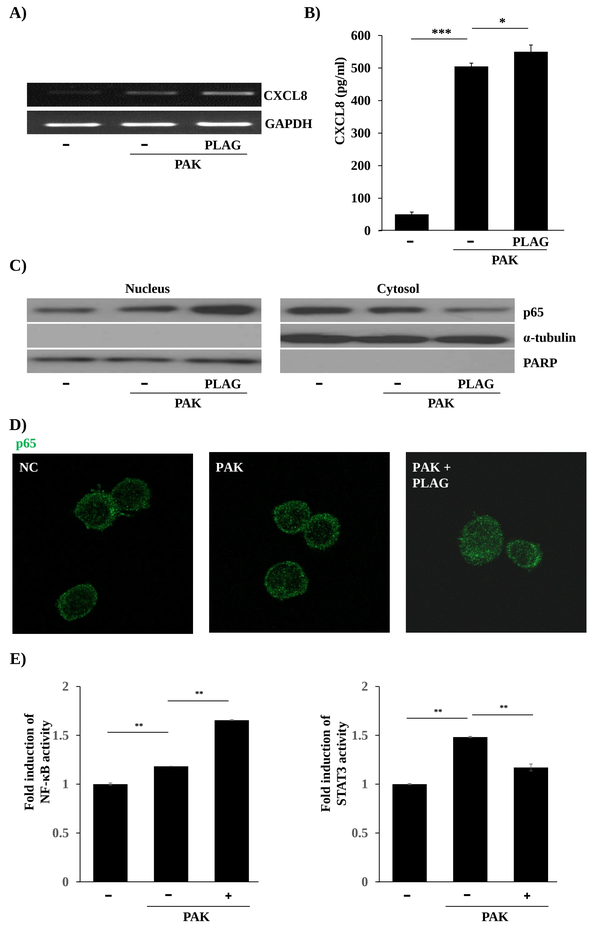
(A, D) Experimental design was illustrated as a diagram. **(B, C)** After PAK infection 3h, bacterial counts in lung and BALF are expressed as CFU in AC regimen induced neutropenic mice. Student's t-test was performed to determine the p values, and p values less than 0.05 were considered statistically significant. *p<0.05, **p<0.01, ***p<0.05 **(E)** Survival rate of infected mice under neutropenic condition. AC regimen treatment started 5 days after, PAK was inoculated into lung of mice by intranasal injection. Difference in survival between the 3 experimental groups of mice was compared by using the Mantel-Cox-log-rank test.

2. PLAG enhances clearance of PAK via up-regulation chemokine expression and NET formation *in vivo*.



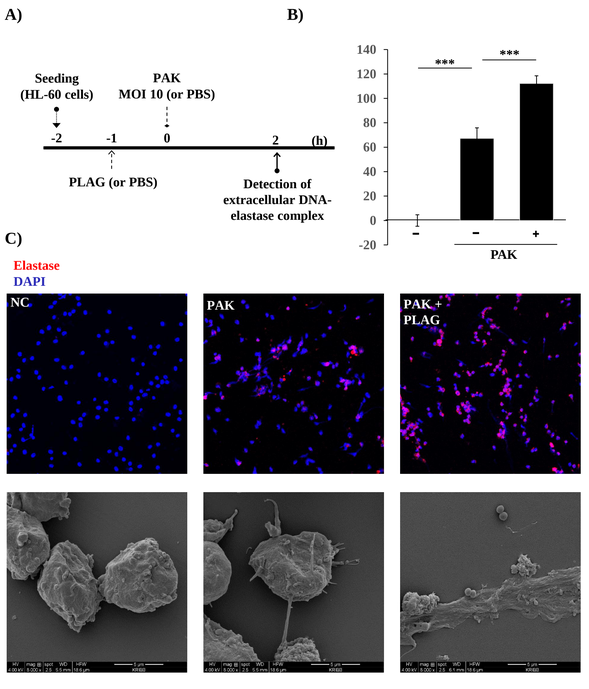
(A, C) Experimental design was illustrated as a diagram. **(B)** Pulmonary bacterial clearance. After PAK infection, bacterial counts in lung and BALF are expressed as CFU in mice. **(D, E, F)** After PAK infection for 2h, we harvested BALF and measured CXCL2 level **(D)** and counted neutrophils **(E)** in BALF. And we visualized NET formation by using confocal microscopy **(F)**. The bars represent the mean ± SD. Student's t-test was performed to determine the p values, and p values less than 0.05 were considered statistically significant. **p<0.001, ***p<0.005.

3. PLAG increases PAK-induced NF-κB signaling pathway and CXCL8 production by inhibiting STAT3 activation in THP-1.



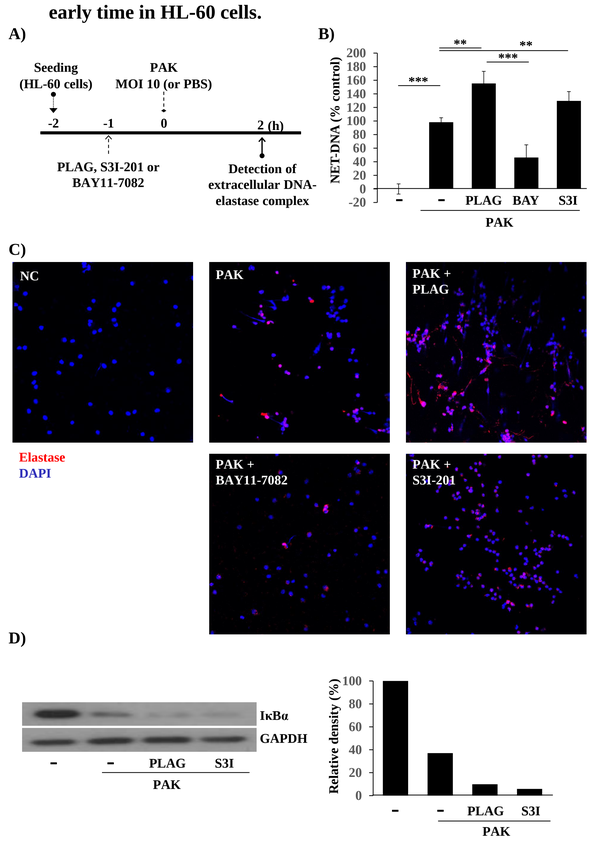
THP-1 cells were pretreated with PLAG and treated with PAK. And CXCL8 was analyzed by conventional RT-PCR **(A)** and ELISA **(B)**. **(C)** Western blot analyses of nuclear and cytosolic fractions from THP-1 cells. And detection of p65/RelA in the nuclear or cytosol fraction. To determine even protein loading and purity of nuclear and cytosolic fraction, probed with PARP and α-tubulin antibody. **(D)** Localization of p65 was visualized by using ZEN imaging software. **(E)** Reporter construct containing luciferase gene regulated by NF-κB or STAT3 activity was transfected to RAW264.7 cells and the effect of PLAG on expression of the luciferase gene was analyzed by reporter assay. The bars represent the mean ± SD. Student's t-test was performed to determine the p values, and p values less than 0.05 were considered statistically significant. *p<0.05, **p<0.01, ***p<0.05

4. PLAG enhances NETosis in HL-60 cells



(A) Experimental design was illustrated as a diagram. **(B, C)** After PAK infection 2h, we harvested HL-60 cells and detected extracellular DNA-elastase complex by using ELISA **(B)** and visualized by using confocal microscopy (x 400) or scanning electron microscope (SEM) (x 8000) **(C)**. The bars represent the mean ± SD. Student's t-test was performed to determine the p values, and p values less than 0.05 were considered statistically significant. ***p<0.005.

5. PLAG and STAT3 inhibitor enhance PAK-induced NETosis at early time in HL-60 cells.



(A) Experimental design was illustrated as a diagram. **(B, C)** After PAK treatment 2 h, we harvested BALF and detected extracellular DNA-elastase complex by using ELISA **(B)** and visualized by using confocal microscopy **(C)**. **(D)** After PAK treatment 30 min, the HL-60 cells were harvested, extracted total proteins, and degradation of IκBα detected by western blot. The bars represent the mean ± SD. Student's t-test was performed to determine the p values, and p values less than 0.05 were considered statistically significant. *p<0.05, **p<0.01, ***p<0.05

Pneumonia model

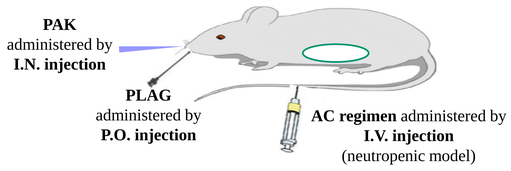


Diagram of neutropenic mice model

Conclusion

- PLAG effectively increases bacterial clearance and survival rate under immunocompromised condition.
- PLAG improves bacterial clearance via up-regulation of inflammatory responses, chemokine expression and NETosis.
- PLAG enhances NF-κB mediated anti-bacteria responses, chemokine expression and NETosis by down-regulating STAT3 activation.
- In this study, we suggest that PLAG can help cancer patients with neutropenic condition to decrease infection risk by enhancing innate immunity and anti-bacterial responses.