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PLAG co-treatment increases the anticancer effect of Adriamycin and cyclophosphamide in a triple-negative breast cancer xenograft mouse model

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ABSTRACT

Chemotherapy induces tumor cell death and inhibits tumor progression, but the accompanying immune responses in the surrounding dying tissue cause significant inflammation. These responses, such as excessive neutrophil infiltration into tumor tissue, are the main causes of resistance to anticancer treatment. The development of drugs that reduce neutrophil infiltration into tumors is necessary to increase the anticancer effect of chemotherapy.

Here, we show that the antitumor effect of the chemotherapy AC regimen (Adriamycin and cyclophosphamide) was increased by 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) cotreatment in the MDA-MB-231 triple-negative breast cancer xenograft mouse model. Tumor growth was inhibited up to 56% in mice treated with AC and inhibited up to 94% in mice cotreated with AC and PLAG. Side effects of chemotherapy, such as a reduction in body weight, were alleviated in mice cotreated with AC and PLAG. Excessive neutrophil infiltration caused by the AC regimen was successfully cleared in mice cotreated with AC and PLAG.

We conclude that PLAG inhibits excessive neutrophil infiltration that aids tumor growth. Reduced neutrophils and increased lymphocytes in PLAG-treated mice can maximize the antitumor effect of the AC regimen and inhibit tumor growth.

1. Introduction

Chemotherapy agents, the first generation of anticancer drugs, remain the most widely used, reliable, and effective treatment for cancer by directly killing excessively proliferating tumors [1–4]. However, extensive tumor cell death causes the release of damage-associated molecular pattern (DAMP) molecules from dying tumor cells, which in turn induces an excessive immune response and recruit immune cells to tumor lesions. The immune cells recruited to tumors treated with chemotherapy determine chemotherapy resistance [5,6]. Recruited immune cells can transform the tumor microenvironment and subsequently affect tumor cell growth and metastasis, without causing further tumor cell death.

The release of substantial amounts of DAMPs, such as adenosine, from dying chemotherapy-treated tumor cells, stimulates the recruitment of neutrophils into tumor lesions. The DAMPs activate specific signaling pathways within tumors or induce the expression of neutrophil infiltration–related factors, such as C-X-C motif chemokine (CXCL) 1 and 8 [7–9]. These infiltrating neutrophils help establish tumor microenvironments that induce the release of tumor growth factors, such as neutrophil elastase and myeloperoxidase (MPO), rather than the release of factors that cause tumor destruction [10,11]. In addition, infiltrating neutrophils promotes

Abbreviations: PLAG: 1-Palmitoyl-2-limoleoyl-3-acetyl-roc-glycerol, TNBC: Triple negative breast cancer; TINs: Tumor infiltrating neutrophils, TME: Tumor microenvironment; BM: Bone marrow, WBC: White blood cells; DAMPs: Damage associated molecular patterns, AC-regimen: Adriamycin and Cyclophosphamide regimen; CD: Cluster of differentiation, MPO: Myeloperoxidase; mpk: Milligram per kilogram, Mip-2: Macrophage inflammatory protein-2; CXCL: C-X-C motif ligand, MMPs: Matrix metalloproteinases.

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tumor cell metastasis in chemotherapy-treated tumors [12–14]. Therefore, it is particularly important to use a combination of drugs and other treatments that reduce neutrophil infiltration into tumor tissue to increase the effectiveness of chemotherapy [15–17].

In this study, our results show that the antitumor effectiveness of the AC regimen (Adriamycin and cyclophosphamide) was significantly increased by 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) cotreatment in the MDA-MB-231 triple-negative breast cancer (TNBC) xenograft mouse model. Tumor growth was delayed by 45% after AC therapy and delayed by 95% after cotreatment with AC and PLAG compared with untreated tumor-bearing mice. We did not observe infiltration of active neutrophils into tumor tissue after AC and PLAG cotreatment, which is consistent with the inhibition of tumor growth observed. In other words, rapidly proliferating and damaged tumor cells release DAMPs that induce chemokines to recruit neutrophils into tumor tissues to support tumor growth. PLAG reduces the release of DAMPs and inhibits tumor growth.

PLAG successfully reduces excessive neutrophil infiltration to suppress chemoresistance and prevent tumor growth. Excessive infiltration of active neutrophils into tumor tissue induces chemoresistance and excessive tumor growth. Therefore, reduction of neutrophil infiltration is an important developmental strategy to maximize the antitumor effect of chemotherapy.

2. Materials and methods

2.1. PLAG synthesis

PLAG was synthesized by the New Drug Production Headquarters, a GMP facility of Enzychem Lifesciences Corporation (Jecheon-si, South Korea) and used in accordance with the manufacturer's instructions.

2.2. Cell culture

MDA-MB-231 cells, a human breast cancer cell line, were obtained from the ATCC (American Type Culture Collection, MD, USA). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM; WelGENE, Seoul, Korea) containing 10% fetal bovine serum (HyClone, MA, USA) and 1% antibiotics (100 mg/L streptomycin, 100 U/mL penicillin) at 37 °C in a 5% CO_2 atmosphere.

2.3. Syngeneic tumor implantation and processing

Five-week-old female Balb/c nu/nu mice were obtained from Nara Biotech (Yong-in, South Korea) and housed in sterile filter-topped cages. The animals (n = 6 for each treatment group) were anesthetized using isoflurane and placed in the right lateral decubitus position. A total of 1×10^6 MDA-MB-231 cells in a solution containing 50-μL culture medium and 50-μL Matrigel (BD Biosciences, NJ, USA) was subcutaneously injected into the right thigh using a 29-G needle attached to a 0.5-mL insulin syringe (Becton, Dickinson and Company, NJ, USA). The mice were then allowed to rest on a heating pad until fully recovered. Starting 3 weeks after cell implantation, the mice were given daily oral doses of 25-, 50-, or 100 mg/kg PLAG (n = 6 mice per group) with or without the AC regimen given once per week. A negative control group (n = 6 tumor-bearing mice) was left untreated. Tumor burden was calculated every three days after implantation. Tumor volume was approximated by a simplified ellipsoidal formula: (short axis) x (short axis) x (long axis)/2. The animals were humanely sacrificed 9 weeks after implantation and perfused with phosphate-buffered saline. The tumors were extracted and fixed with 10% formaldehyde. Hematoxylin and eosin staining and immunohistochemical staining were performed on the tissue sections to analyze tissue morphology. All animal experiments were approved by the Institutional Animal Care and Use Committee, Korea Research Institute of Bioscience and Biotechnology (approval number: KRIBB-AEC-18241).

2.4. AC regimen delivery

Adriamycin and cyclophosphamide (Sigma-Aldrich, MA, USA) were prepared in accordance with the manufacturer's protocol and refrigerated until use. Adriamycin (2 mg/kg) and cyclophosphamide (20 mg/kg) were injected intraperitoneally at 17:30 every Thursday.

2.5. Complete blood count (CBC) analysis

After the mice were humanely killed, whole-body mouse blood was obtained by cardiac puncture and stored in an EDTA-coated tube until analysis. Hematopoietic analysis was performed using a complete blood count analyzer (Mindray, Shenzhen, China). The serum was separated by centrifugation at 6000 rpm for 10 min at 4 °C to measure levels of secreted proteins.

2.6. Enzyme-linked immunosorbent (ELISA) assay

The levels of secreted proteins in the mouse plasma were analyzed by factor-specific enzyme-linked immunosorbent assay in accordance with the manufacturer's protocol (R&D Systems, MN, USA). The absorbance was measured at 450 nm using an EMax Endpoint ELISA microplate reader (Molecular Devices Corporation, CA, USA).

2.7. Measurement of extracellular adenosine (eAdo)

Mice with MDA-MB-231 xenografts were treated concurrently with AC and PLAG. Mouse serum and extracellular adenosines were perfused using tissue storage solution (Miltenyi Biotec, Bergisch Gladbach, Germany) for 12 h. The perfused solution and serum were collected and centrifuged at 8800 rpm for 5 min to remove insoluble particles. Extracellular adenosines were analyzed using an adenosine assay kit (Cell Biolabs Inc., San Diego, CA, USA). The fluorescence was measured at 560 nm excitation and 590 nm emission using a Varioskan LUX microplate reader (Thermo Fisher Scientific, MA, USA).

2.8. Immunohistochemical (IHC) staining

Tumor tissue from the mice were fixed in 10% formaldehyde, embedded in paraffin, and sectioned into 5-μm slices. The sections were treated with 3% H_2O_2 for 10 min to block endogenous peroxidase activity and then blocked with bovine serum albumin. Then, the sections were washed in phosphate-buffered saline and incubated with an antibody overnight at 4 °C. Negative controls were incubated with normal serum IgG for the species from which the primary antibody was obtained.

2.9. Overall survival analysis

We plotted Mantel–Cox survival curves to 120 days after compound treatment initiation. Survival data were collected until the final observation week, excluding death or moribundity.

2.10. Statistics

The data were analyzed using one-way analysis of variance (Prism 9, GraphPad Software, CA, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cotreatment with AC and PLAG enhances antitumor effects in the MDA-MB-231 xenograft model

Chemotherapy is the gold standard for TNBC tumors because they are highly resistant to targeted therapies. However, dose optimization to achieve complete tumor death is difficult owing to side effects caused by chemotherapy, such as rapid weight loss. Therefore, combination therapy with an agent that lowers side effects and that can also increase the effect of the AC regimen is recommended. In this study, MDA-MB-231 cells were implanted in mice and allowed to grow for 3 weeks before drug delivery. Mice were treated with the AC regimen and/or 50 mg/kg PLAG for 6 weeks (Fig. 1A). Tumor size was evaluated every 3 days for 39 days, and tumor weight was assessed on the day of death. We observed significant antitumor efficacy of AC and PLAG cotreatment in the TNBC xenograft implantation model. The AC regimen alone decreased tumor growth by 56% compared with the negative control group, and AC and PLAG cotreatment decreased tumor growth by 94% compared with the negative control group (Fig. 1B,C,D). Mice treated with AC alone had a body weight reduction of approximately 28% compared with normal mice, whereas weight loss was minimal in the group treated with PLAG alone (Fig. 1E).

3.2. Cotreatment with AC and PLAG reduced tumor-infiltrating neutrophils in tumor tissue

Tumor-infiltrating neutrophils (TINs) in the tumor microenvironment promote tumor growth and reduce antitumor activity of the AC regimen. We found that the number of TINs stained with Ly6G antibody, neutrophil elastase antibody, and MPO antibody by immunohistochemical staining was reduced in the tumor tissue of mice treated with PLAG alone. However, increased numbers of TINs in tumor tissue were maintained in AC-treated mice (Fig. 2A and B). These data provide evidence that PLAG reduces infiltration of neutrophils into tumor tissue.

TINs in tumor-bearing mice migrate to the tumor tissue in response to chemokines such as hCXCL1 and hCXCL8 and macrophage inflammatory protein 2 (mMip-2). Implanted human cancer cells (MDA-MB-231) express CXCL1 and CXCL8, and surrounding cells secrete mMip-2. In addition, host-tissue damage due to the AC regimen induces mMip-2 expression. PLAG reduced excessive neutrophil infiltration into tumor tissue by reducing chemokines to their normal levels (Fig. 2C). Tumor cell death by treatment with the AC regimen is mediated by apoptosis. We found that PLAG did not interfere with the antitumor effectiveness of the AC regimen (Fig. 2D) and was highly effective at reducing expression of chemokines that attract excessive neutrophils.

3.3. Inhibition of tumor growth by PLAG is concentration dependent

We tested whether PLAG alone can inhibit tumor growth in the TNBC xenograft model. We administered 25-, 50-, or 100-mg/kg PLAG to TNBC xenograft models for 6 weeks, and tumor size and tumor weight were evaluated on the same schedule as for cotreatment of AC and PLAG (Fig. 3A). Tumor size and tumor weight were suppressed by PLAG in a concentration-dependent manner. Tumor size was minimal in mice treated with 100-mg/kg PLAG during the 6-week treatment period (Fig. 3B and C). On the day of death, the tumor weight was reduced by approximately 89% in the mice treated with 100-mg/kg PLAG compared with the mice in the negative control group (Fig. 3D). Importantly, the AC regimen reduced tumor size but also rapidly reduced mouse body weight. PLAG alone inhibited excessive tumor growth and caused no side effects, such as weight loss (Fig. 3E).

3.4. Levels of tumor-infiltrating neutrophils reduce in PLAG-treated mice

PLAG treatment inhibited tumor growth and excessive infiltration of neutrophils into the tumor microenvironment. The uncontrolled infiltration of neutrophils into the tumor is important for tumor growth. We stained TINs in the tumor tissue by immunohistochemistry using Ly6G antibody, neutrophil elastase antibody, and MPO

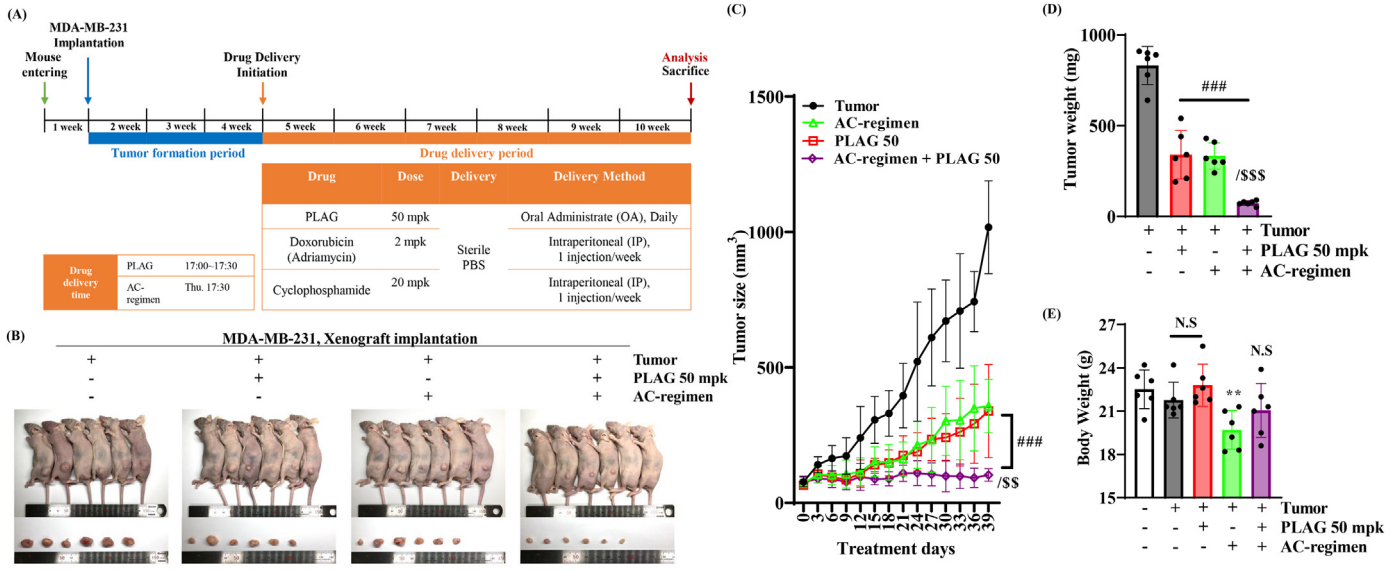

Fig. 1. Inhibition of cancer progression by AC and PLAG cotreatment in the MDA-MB-231 triple-negative breast cancer xenograft model.
(A) Experimental design to evaluate prevention of tumor progression by cotreatment with AC and PLAG. (B) Tumor burden and tumor size were measured in mice cotreated with AC and PLAG on the day of death. (C) Analysis of increased tumor size in each group measured at 3-day intervals for 39 days. (D) Tumor weight was measured in mice cotreated with AC and PLAG on the day of death. (E) Body weight was measured in mice cotreated with AC and PLAG on the day of death.
Data are presented as mean ± SD. Compared with the negative control group: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; compared with the tumor-bearing untreated group: #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$; compared with the AC-treated group: \$$P < 0.05$, \$\$$P < 0.01$, \$\$\$$P < 0.001$ (n = 6 for each experiment); N.S., not significant.

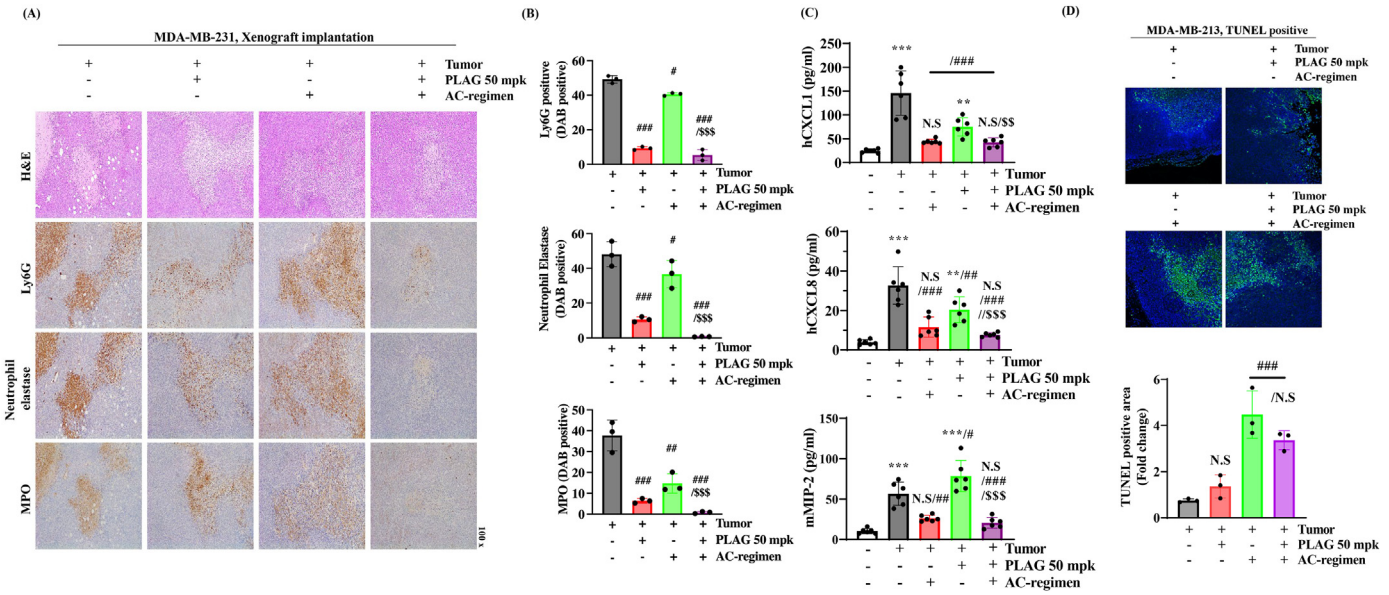

Fig. 2. Reduced neutrophil infiltration after cotreatment with AC and PLAG in the MDA-MB-231 triple-negative breast cancer xenograft model.
(A) Tumor-infiltrating neutrophils in PLAG-treated tumor-bearing mice were identified by immunohistochemical staining using anti-Ly6G, anti-neutrophil elastase, and anti-myeloperoxidase antibodies. (B) We analyzed 3,3′-diaminobenzidine-positive cells in tumor tissues stained with anti-Ly6G, anti-neutrophil elastase, and anti-myeloperoxidase antibodies. (C) Enzyme-linked immunosorbent assay was used to evaluate expressed chemokines associated with neutrophil infiltration in serum of mice cotreated with AC and PLAG. (D) Validation of apoptosis activity in tumor tissue of mice cotreated with AC and PLAG by TUNEL assay.
Data are presented as mean ± SD. Compared with the negative control group: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; compared with the tumor-bearing untreated group: #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$; compared with the AC-treated group: $$P < 0.05$, $$$P < 0.01$, $$$$P < 0.001$ (n = 6 for each experiment); N.S., not significant.

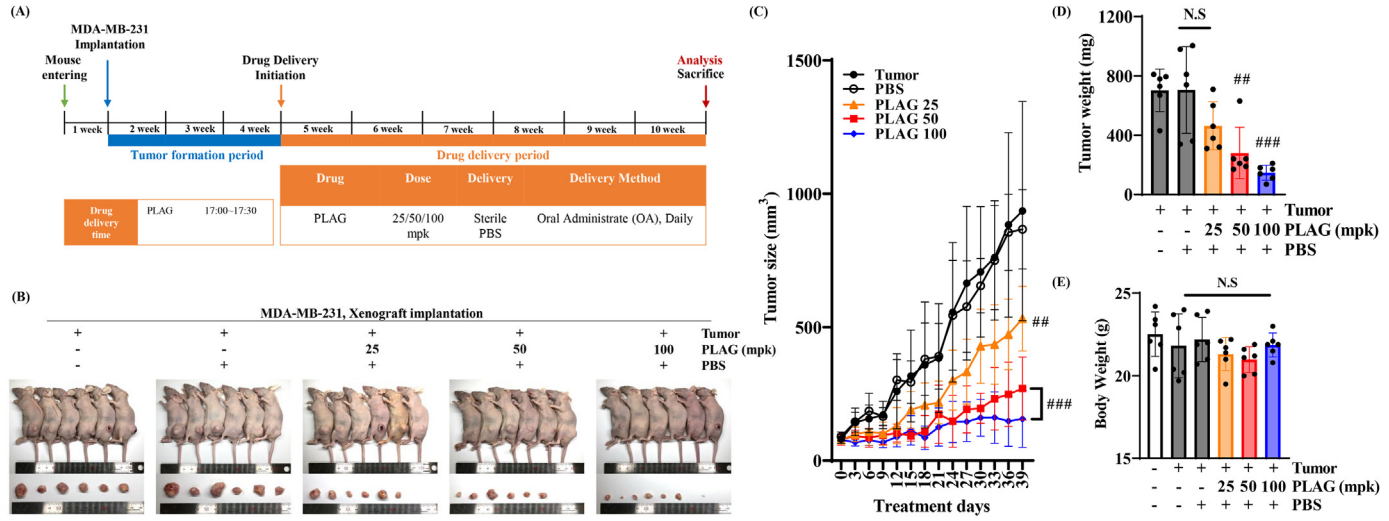

Fig. 3. Concentration-dependent inhibition of cancer progression by PLAG treatment in the MDA-MB-231 triple-negative breast cancer xenograft model.
(A) Experimental design to evaluate prevention of tumor progression with the treatment of 25-, 50-, or 100-mg/kg PLAG in the mouse model. (B) On the day of death, tumor burden and tumor size were measured in mice treated with 25-, 50-, or 100-mg/kg PLAG. (C) The tumor size of each group was measured every 3 days for 39 days. (D) Tumor weight of mice in each group was measured on the day of death. (E) Body weight of mice in each group was measured on the day of death.
Data are presented as mean ± SD. Compared with the negative control group: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; compared with the tumor-bearing untreated group: #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$; N.S., not significant.

antibody and found that TINs were reduced in a dose-dependent manner in the PLAG treatment group (Fig. 4A and B). The TINs are mediated by tumor-derived neutrophil infiltration–related factors (hCXCL1 and hCXCL8) and surrounding cells that produce neutrophil infiltration-inducing factors (mMip-2). Induced hCXCL8 and mMip-2 levels in tumor-bearing mice were reduced by PLAG treatment in a dose-dependent manner (Fig. 4C). These data suggest that PLAG reduces chemokines in tumor tissue, which leads to a reduction in neutrophil infiltration into tumor lesions.

4. Discussion

The goal for patients receiving cancer treatment is complete destruction of tumors. Chemotherapy is easily administered and can quickly reduce tumor size. However, chemotherapy causes non-specific tissue damage, including damage of tumor cells, and it activates the host's immune system [18,19]. High doses or frequent treatments of chemotherapy induce uncontrolled immune responses and cause serious side effects, such as symptoms of

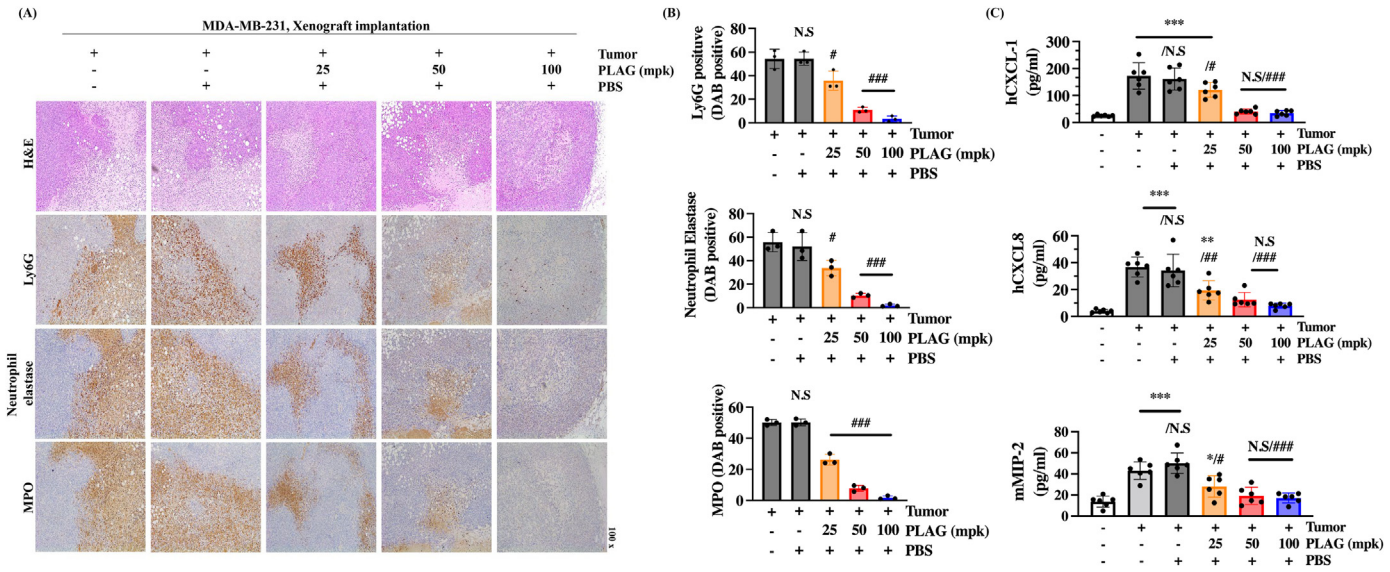

Fig. 4. Neutrophil infiltration into tumors in PLAG-treated mice.
(A) Tumor-infiltrating neutrophils in mice treated with 25-, 50-, or 100-mg/kg PLAG were measured by immunohistochemical staining using anti-Ly6G, anti-neutrophil elastase, and anti-myeloperoxidase antibodies. (B) We analyzed 3,3′-diaminobenzidine-positive cells in tumor tissues stained with anti-Ly6G, anti-neutrophil elastase, and anti-myeloperoxidase antibodies. (C) Enzyme-linked immunosorbent assay was used to evaluate expressed chemokines associated with neutrophil infiltration in serum of mice treated with PLAG.
Data are presented as mean ± SD. Compared with the negative control group: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; compared with the tumor-bearing untreated group: $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$; N.S., not significant.

inflammation, which limit the use of chemotherapy [20–22]. The symptoms of inflammation induced by chemotherapy are accompanied by excessive infiltration of neutrophils, which is the most important cause of chemotherapy resistance [13,15]. Therefore, excessive neutrophil infiltration should be appropriately controlled to maximize the antitumor effect of chemotherapy.

This study demonstrates the role of PLAG in improving the antitumor effectiveness of the AC regimen in the MDA-MB-231 TNBC xenograft model. We found that tumor size decreased by 56% in tumor-bearing mice treated with AC alone compared with that in the negative control group, but the tumors from mice cotreated with AC and PLAG decreased in size by about 94% (Fig. 1). Four out of six tumor-bearing mice that received no AC or PLAG treatment died within 120 days. However, there were no deaths within 120 days in tumor-bearing mice treated with PLAG alone or cotreated with AC and PLAG. In the AC regimen alone, two out of six mice died within 120 days (Suppl. Fig. 1). Many neutrophils were recruited into the tumor tissue during MDA-MB-231 tumorigenesis. These recruited neutrophils support tumor growth and progression. PLAG treatment decreased the number of TINs (Fig. 2A–C). PLAG, in combination with AC, suppressed excessive infiltration of neutrophils in tumor tissue by regulating neutrophil-attractant factors that originated from the implanted tumor cells (hCXCL1 and hCXCL8) and from the surrounding non-tumor cells (mMip-2) that were generated by the AC regimen (Fig. 2D).

Chemokine induction by chemotherapy is mediated by the release of DAMPs. Elevated levels of DAMPs released in response to excessive tumor growth and non-specific tissue damage by the regimen contribute to the establishment of a tumor-friendly microenvironment [23–26]. Elevated levels of extracellular adenosine aid the rapid recruitment of neutrophils via specific receptor activation [27–29]. We found that excess extracellular adenosine in serum from untreated tumor-bearing mice was reduced compared to PLAG-treated mice, and the excess adenosine present in the tumor microenvironment also returned to normal levels after PLAG treatment (Suppl. Fig. 2). Thus, PLAG inhibits tumor growth by decreasing neutrophil infiltration in a concentration-dependent manner by regulating the expression of factors associated with neutrophil movement (Fig. 4).

According to a recent report, the neutrophil-lymphocyte ratio (NLR) is one of the key factors that determines the efficacy of the regimen [30–32]. High NLR values are frequently observed in AC-treated because many neutrophils are produced with excessive tumor growth and are recruited into tumor lesions. Also, the number of lymphocytes rapidly decreases in AC-treated [33,34]. High NLR values indicate tumor-friendly microenvironments. Cotreatment with AC and PLAG lowered the NLR values and restored the NLR values of normal mice (Suppl. Fig. 3).

We have previously reported that PLAG alleviates the symptoms of some inflammatory diseases by controlling excessive neutrophil infiltration [35–38]. It has also been suggested that PLAG controls tumor metastasis by downregulation of neutrophils [39,40]. Here, we demonstrated that reduction of neutrophil infiltration by PLAG treatment increases the antitumor effectiveness of the AC regimen in mice. Our data suggest that PLAG enhances the therapeutic potential of chemotherapy in cancer patients by mitigating excessive neutrophil infiltration and therefore reducing chemotherapy resistance.

Ethics approval

a. All animal care and experimental protocols were carried out according to the Korea Research Institute of Bioscience & Biotechnology (KRIBB) regulations and animal care and experimental protocols were approved by the Institutional animal care and use committee (IACUC), Korea Research Institute of Bioscience & Biotechnology (approval number: KRIBB-AEC-18241).
b. The authors confirmed that all methods were performed following the relevant guidelines and regulations.
c. The study was carried out in compliance with the ARRIVE guidelines.

Author contribution

Guen Tae Kim: Writing – Original draft, Methodology, Validation, Investigation, Formal analysis, Data curation Su-Hyun Shin: Investigation Eun Young Kim: Investigation, Formal analysis Hyowon Lee: Investigation Se Hee Lee: Investigation Ki-Young Sohn: Funding acquisition Jae Wha Kim: Project administration, Supervision, Conceptualization, Writing – Review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Guen Tae Kim reports financial support was provided by Enzychem Lifesciences Corporation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.06.051.

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