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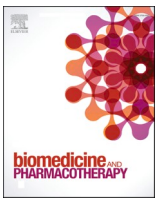

1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol treatment inhibits abnormal tumor growth by regulating neutrophil infiltration in a non-small cell lung carcinoma mouse model

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ABSTRACT

Excessive neutrophil infiltration into the tumor microenvironment (TME) is an important factor that contributes to tumor overgrowth and limited immunotherapy efficacy. Neutrophils activate various receptors involved in tumor progression, while suppressing the infiltration and activity of cytotoxic T cells and creating optimal conditions for tumor growth. Therefore, the appropriate control of neutrophil infiltration is an effective strategy for tumor treatment.

In the present study, 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) inhibited tumor overgrowth by suppressing excessive neutrophil infiltration, resulting in >74.97 % reduction in tumor size in a Lewis lung carcinoma (LLC-1) mouse model. All subjects in the positive control group died during the 90-day survival period, whereas only four subjects in the PLAG treatment group survived. PLAG had a significantly higher tumor growth inhibitory effect and survival rate than other neutrophil infiltration-targeting inhibitors (e.g., Navarixin, lymphocyte antigen 6 complex locus G6D antibody [aLy6G]). The ability of PLAG to regulate neutrophil infiltration and inhibit tumor growth depends on thioredoxin-interacting protein (TXNIP). In tumors lacking TXNIP expression, PLAG failed to control neutrophil infiltration and infiltration-related factor release, and the inhibitory effect of PLAG on tumor growth was reduced. PLAG-mediated inhibition of neutrophil infiltration enhances the efficacy of immune checkpoint inhibitors (ICIs), increasing the antitumor efficacy and survival rate by 30 %.

In conclusion, PLAG could be a novel alternative to anti-tumor drugs that effectively targets excessive neutrophil infiltration into cancer tissues.

1. Introduction

Neutrophils are vital immune cells that capture and eliminate exogenous and endogenous pathogens from the body. These cells are utilized in the human body to induce an appropriate immune response because of their ability to rapidly eliminate pathogens through a process known as NETosis [1,2]. Despite their beneficial role, excessive neutrophil activation leads to pathogenesis. Overactivation of neutrophils can cause tissue damage by infiltrating the target tissue and triggering an inflammatory response through the overactivation of the signaling pathway and the induction of a tumor-friendly immune response [3–10]. For example, neutrophils secrete factors such as

Abbreviation: **A2BR,**, Adenosine 2B Receptor; **aPD-L1,**, Programmed death-Ligand1 antibody; **CD,**, Cluster of differentiation; **FACS,**, Fluorescence-Activated Cell Sorter; **GPCR,**, G-protein coupled receptors; **IACUC,**, Institutional animal care and use committee; **ICI,**, Immune-checkpoint inhibitor; **IHC,**, Immunohistochemistry; **IVIS,**, *In vivo* image system; **LLC-1,**, Lewis lung carcinoma; **MAPK,**, Mitogen-activated protein kinase; **Mip-2,**, Macrophage inflammatory protein-2; **mpk,**, Milligram per kilogram body weight mg/kg BW; **MPO,**, Myeloperoxidase; **MPRO,**, Mouse promyelocyte; **NEDD-4,**, Neural precursor cell-expressed developmentally down-regulated protein 4; **NLR,**, Neutrophil-to-lymphocyte ratio; **NSCLC,**, Non-small cell lung carcinoma; **PD-L1,**, Programmed death-Ligand1; **PLAG,**, 1-Palmitoyl-2-limoleoyl-3-acetyl-roc-glycerol; **PP2A,**, Phosphatase 2 A; **TILs,**, Tumor-infiltrating lymphocytes; **TINs,**, Tumor-infiltrating neutrophils; **TME,**, Tumor microenvironment; **TXNIP,**, Thioredoxin interacting protein.

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epidermal growth factor (EGF), high-mobility group box-1 (HMGB-1), myeloperoxidase (MPO), and neutrophil elastase to promote tumor progression through the activation of tumor-specific receptors such as growth factor receptors, proteinase-activated receptor 2 (PAR2), and receptors for advanced glycation end products (RAGE) [11–15]. Furthermore, neutrophils promote cancer metastasis by inducing the release of matrix metalloproteinases (MMPs) [16,17]. Neutrophils can also capture and induce the apoptosis of cytotoxic T cells via the surface expression of programmed death ligand 1 (PD-L1), inhibiting their infiltration and reducing the antitumor efficacy of immune checkpoint inhibitors (ICIs) [18–20]. Therefore, tumors consistently attract neutrophils to the tumor microenvironment by increasing the expression of factors that promote neutrophil maturation and migration, increase neutrophil survival, and inhibit the removal of activated neutrophils. Recent studies have shown that patients with cancer and above-average neutrophil counts have worse prognosis than those with average neutrophil counts. When accompanied by a high neutrophil-to-lymphocyte ratio (NLR), an increase in the number of neutrophils is an indicator of poor prognosis in patients with cancer [21–25]. Therefore, the effective control of neutrophil infiltration is an essential therapeutic strategy for suppressing tumor growth.

Our previous studies have demonstrated that 1-palmitoyl-2-linoleoyl-3-acetyl-rac-glycerol (PLAG) can alleviate various inflammatory diseases such as rheumatoid arthritis, acute lung injury, and oral mucositis by suppressing excessive neutrophil infiltration [26–29]. In addition, controlling neutrophil infiltration with PLAG inhibited non-small cell lung carcinoma (NSCLC) metastasis and significantly increased the antitumor effects of the adriamycin/cyclophosphamide (AC)regimen in a triple-negative breast cancer (TNBC) model. Furthermore, PLAG facilitates tumor suppression by increasing the efficacy of the PD-L1 antibody (aPD-L1) in urothelial cancer [30–32]. However, the exact mechanisms underlying the antitumor effects of PLAG have not yet been elucidated.

Therefore, in the present study, we suggested that the mechanism underlying the antitumor effect of PLAG involves the regulation of neutrophil infiltration. We demonstrated that PLAG not only exhibits improved efficacy as compared to conventional neutrophil infiltration inhibitors because of its unique mechanism of action, but also has the potential to achieve complete remission of NSCLC through combinational treatment with ICIs.

2. Materials and methods

2.1. PLAG synthesis and manufacture

PLAG was provided by the New Drug Production Headquarters, a GMP facility of Enzychem Lifesciences Corporation (Jecheon-si, South Korea), and used based on the information provided by the manufacturer.

2.2. Cell culture

Lewis lung carcinoma (LLC-1) NSCLC cells and mouse promyelocytic (MPRO) cells were obtained from the American Type Culture Collection (ATCC, VA, USA). 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; WelGENE, Seoul, Korea), and were incubated at 37°C with 5 % CO_2.

2.3. Compound preparation

Anti-lymphocyte antigen 6 complex locus G6D (aLy6G, clone 1A8) was purchased from BioXcell (BioXcell, MA, USA), and Navarixin (SCH527123, MK-7123) was purchased from Medchemexpress (Medchemexpress LLC, NJ, USA). Reagents were prepared according to the manufacturer's protocol and refrigerated until use. Administration of aLy6G and Navarixin was performed using intraperitoneal (IP) and oral (PO) injections, respectively. The mice were treated with 10 mg/kg aLy6G, which was administered each Tuesday, and Navarixin (30 mg/kg), which was administered daily.

2.4. Tumor inoculation in mice (syngeneic implantation)

Five-week-old male C57BL/6 mice were obtained from Daehan Bio-Link (Yong-in, South Korea) and housed in sterile filter-topped cages. Animals were anesthetized with isoflurane and placed on the right flank. A solution containing a total of 1×10^5 LLC-1 cells, 50 μL culture medium, and 50 μL Matrigel (BD Biosciences, NJ, USA) was subcutaneously injected into the right upper thigh using a 0.5 mL insulin syringe with a permanently attached 29 G needle (Becton Dickinson, NJ, USA). The mice were then allowed to rest on the heating pad until they fully recovered. After confirming that the tumor size of the implanted cells reached the long axis (0.5 cm) from day 4 post-implantation, the mice were separated into groups and the average tumor size of each group was calculated. Mice were orally administered with 25, 50, and 100 mg/kg of PLAG (n=6 mice per group) daily, with or without 5 mg/kg anti-PD-1 biweekly (BW). The negative control group (n=6) did not receive any treatment. The tumor burden was calculated every three days after treatment. The tumor volume was approximated using a simplified ellipsoidal formula: (short axis × short axis × long axis) × 2. The mice were sacrificed four weeks after implantation and perfused with PBS. The tumors were extracted and fixed in 10 % formaldehyde. Hematoxylin and eosin (H&E) staining was performed on tissue sections to examine their morphology. All the animal experiments were approved by the IACUC Korea Research Institute of Bioscience and Biotechnology (approval number: KRIBB-AEC-22018).

2.5. Anti-PD-1 delivery

Anti-PD-1 (aPD-1, clone RMP1–14) was purchased from BioXcell (MA, USA) and prepared according to the manufacturer's instructions. The reagents were refrigerated until further use. aPD-1 (5 mg/kg) was delivered using biweekly IP injections.

2.6. Fluorescence-activated cell sorting (FACS) analysis

Single-cell suspensions of extracted tumors were prepared using a 40 μm mesh strainer. The cells were then incubated with specific fluorochrome-conjugated antibodies for 30 min at room temperature. After washing the samples twice using FACS buffer, 1x lysing solution (BD Biosciences, Franklin Lakes, NJ, USA) was added, and samples were incubated for 15 min with gentle agitation. The samples were then washed twice and resuspended in FACS buffer for further analysis. Single cells were sorted using FACSVerse™ and analyzed using the FlowJo software™ (FlowJo. LLC, OR, USA).

2.7. Complete blood count (CBC) analysis

Hematopoietic cell analysis was performed using a CBC analyzer (Mindray, Shenzhen, China). Whole blood samples were collected via cardiac puncture and stored in EDTA-coated tubes. For analysis, secreted serum was extracted by centrifuging at 6000 rpm for 10 minutes in a refrigerated centrifuge at 4°C.

2.8. ELISA

The levels of secreted proteins in mouse plasma were analyzed using factor-specific ELISA according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). The absorbance was measured at 450 nm using a Varioskan-LUX microplate reader (Thermo Fisher Scientific, MA, USA).

2.9. Gene silencing with small interfering RNA (siRNA) and small hairpin RNA (shRNA)

shRNA plasmids were purchased from OriGene Technologies (Rockville, MD, USA). For transient transfection, the cells were washed twice with PBS. Target RNA was resuspended in Opti-MEM (Thermo Fisher Scientific, MA, USA) containing Lipofectamine 2000 (Thermo Fisher Scientific, MA, USA). The target RNA mixture was added to the cell plate and cultured for 72 h to transfect cells with the target RNA. Next, the transfected cells were selected with 10 μg/mL puromycin for 4 days, and the cells transfected with shRNA were collected.

2.10. Immunohistochemistry (IHC) staining

Tumors collected from mice were fixed with 10 % formaldehyde, embedded in paraffin, and sectioned into 5 μm slices. To block endogenous peroxidase activity, sections were incubated with 3 % H_2O_2 for 10 min and then blocked with bovine serum albumin. Next, the sections were washed in PBS and incubated with a specific antibody overnight at 4 °C. The negative controls were incubated with normal primary serum IgG from the same target species as the primary antibody.

2.11. Tumor inoculation (orthotopic inoculation)

Five-week-old male C57BL/6 mice were obtained from Daehan Bio-Link (Yong-in, South Korea) and housed in sterile filter-topped cages. Mice were anesthetized using isoflurane and placed in the right lateral decubitus position. A solution containing a total of 0.5×10^5 LLC-1 (Luc-2 tagged) cells with 20 μL culture medium and 30 μL Matrigel (BD Biosciences, NJ, USA) was injected into the right lung using a 0.5 mL insulin syringe with a permanently attached 31 G needle (Becton Dickinson, NJ, USA). The mice were allowed to rest on the heating pad until they fully recovered. One week after implantation, the size of the implanted tumor was quantified using an IVIS (MA, USA). Mice were anesthetized using isoflurane and received an IP injection of D-luciferin. All images were obtained with a 30-second exposure time. The average luminescent intensity was measured in photons/second/cm^2/steradian and reported for each mouse chest. The mice were orally administered 100 mg/kg PLAG (n=5 rats per group) daily, with or without 5 mg/kg anti-PD-1, biweekly.

2.12. Overall survival analysis

Mantel-Cox survival curves were plotted 90 days after the initiation of compound treatment. Survival data, excluding deaths and moribund conditions, were collected until the last week of observation.

2.13. Statistics

Data were analyzed using ANOVA (Prism 9, GraphPad Software, CA, USA), and $P<0.05$ was considered statistically significant.

3. Results

3.1. PLAG treatment inhibits the overgrowth of NSCLC in LLC-1 implanted mice

Tumor burden was measured in syngeneic LLC1 cell-implanted mice, and the antitumor effect of PLAG was investigated, as shown in Fig. 1**A**. Briefly, mice were orally administered PLAG (25/50/100 mg/kg) daily for 4 weeks. In the control group, the tumor size increased rapidly 12 days after inoculation. However, in the PLAG-treated group, tumor growth and weight decreased by an average of 74.97 % in a dose-dependent manner (25 mg/kg, 57.39 % / 50 mg/kg, 78.49 % / 100 mg/kg, and 89.01 %, respectively) (Fig. 1**B, C**). The half-maximal inhibitory dose (ED50) for tumor size and weight reduction was 14.19 and 13.82 mpk (mg/kg), respectively. (Fig. 1**D, E**). In the 90-day survival test, all subjects in the positive control group died by day 64, whereas four subjects in the PLAG treatment group (50 mg/kg) survived until 90 days (medium survival, 39.5 day vs. undefined) (Fig. 1**F**).

3.2. PLAG suppresses the infiltration of neutrophils in tumors

Excessive infiltration of tumor-friendly neutrophils is an important factor that promotes tumor overgrowth. Quantitative analysis of neutrophil infiltration into tumors was conducted according to the gating strategy shown in Fig. 2**A**. Neutrophil infiltration was higher in the positive control group than in the PLAG (50 mg/kg) treated group (average 62.5 % vs. 29.5 %) (Fig. 2**B, C**). As the number of infiltrating neutrophils decreased, the number of neutrophils in the blood returned to the normal range following PLAG treatment. (Fig. 2**D**). Quantitative analysis of the proportion of active infiltrating neutrophils showed that the Ly6G/MPO double-positive area was significantly reduced following

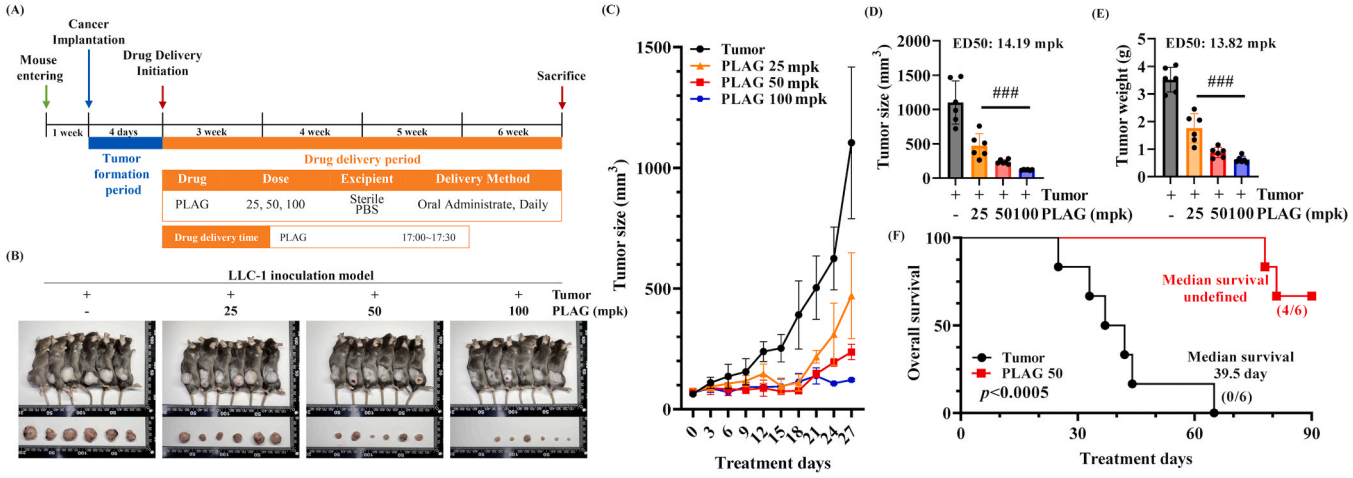


Fig. 1. PLAG inhibits tumor progression in a dose-dependent manner in the LLC-1 syngeneic inoculation model. (A) Schematic diagram of experimental design to evaluate the tumor progression inhibitory effect of PLAG in LLC-1 implanted mice. (B) Tumor burden and size were measured on the day of sacrifice in control mice and PLAG-treated groups. (C) Changes in tumor size of mice from each treatment group measured at 3-day intervals. (D, E) Quantitative comparison of tumor size (D) and weight (E) according to each treatment group. (F) Comparison of 90-day survival between the control group and the PLAG treatment group. Statistical significance was compared with tumor control: ###$P<0.001$. Results are shown as the mean ± standard deviation (SD).

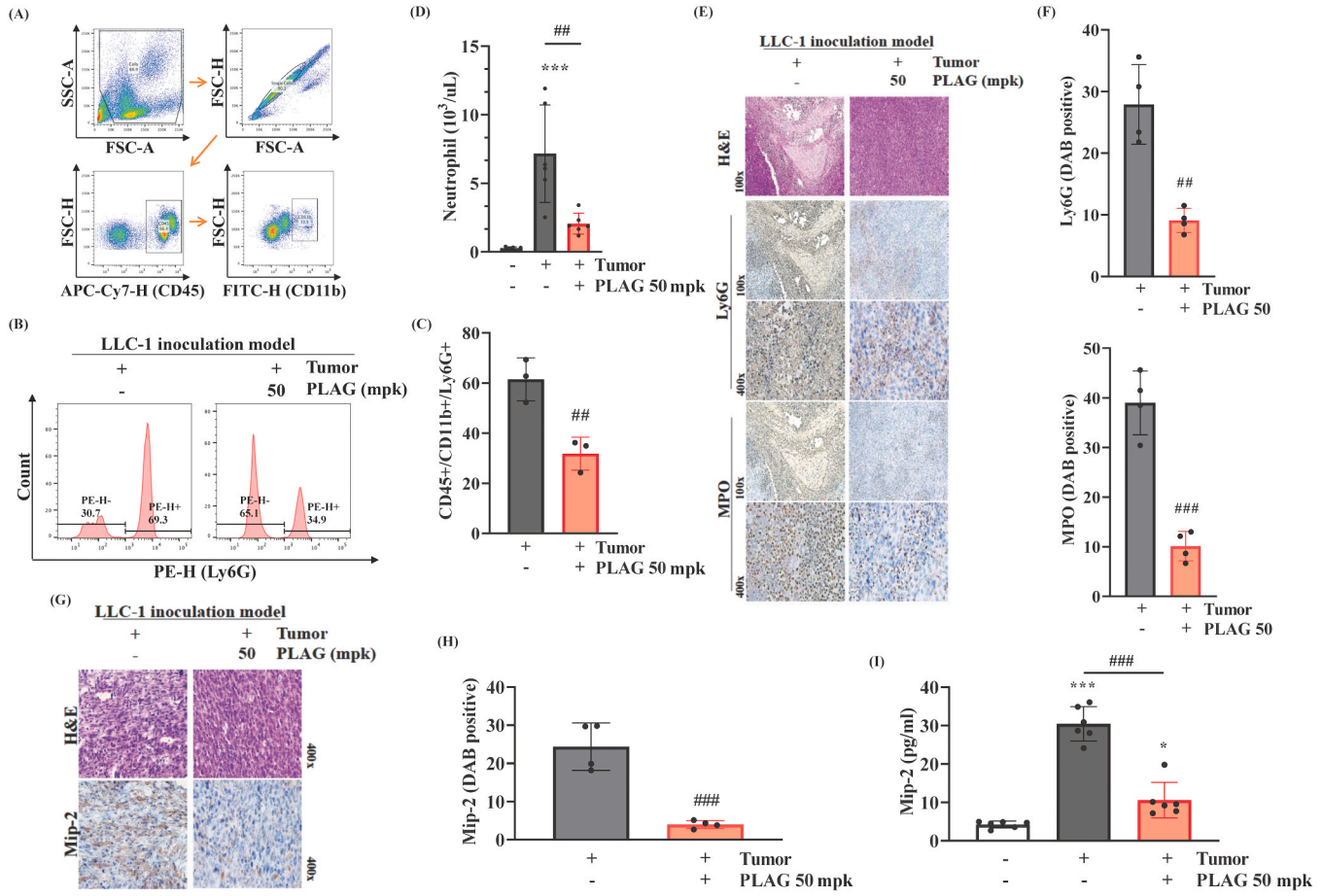


Fig. 2. Controlling abnormal tumor infiltration of neutrophils by PLAG treatment in LLC-1 inoculated mouse model. (A) FACS gating strategy for tumor-infiltrating neutrophil analysis. (B, C) Quantitative analysis of tumor-infiltrating neutrophil changes following PLAG treatment using FACS. (D) Analysis of changes in blood neutrophil count according to PLAG treatment. (E, F, G, H) Immunohistochemistry (IHC) analysis of tumor-infiltrating neutrophil changes and activity following PLAG treatment with anti-Ly6G/anti-MPO antibodies (E and F) and anti-Mip-2 (G and H). (I) Quantitative analysis of changes in Mip-2 concentration in blood following PLAG treatment using ELISA. Significance compared with negative control $^{*}P<0.033$, $^{***}P<0.001$, with tumor control $\#\#P<0.002$, $\#\#\#P<0.001$ ($n=3$ in FACS analysis, $n=4$ in IHC, $n=6$ in CBC and ELISA). Results are shown as the mean ± SD.

PLAG treatment compared to a positive control group (mean, 39 % vs. 10.1 %) (Fig. 2**E, F**). This was due to decreased expression and release of macrophage inflammatory protein-2 (MIP-2), a chemokine that induces neutrophil migration (Fig. 2**G, H, I**).

3.3. PLAG effectively inhibits the excessive infiltration of neutrophils into tumors

Neutrophil infiltration into tumors is an important factor contributing to excessive tumor growth; thus, effective control of neutrophil infiltration is a vital strategy for suppressing excessive tumor growth. Quantitative analysis of tumor size and neutrophil infiltration after LLC-1 inoculation showed that tumor size in the positive control group gradually increased from week 2, but did not change significantly in the PLAG 50 mg/kg treatment group (Fig. 3**A, B**). The number of active infiltrating neutrophils was significantly increased with tumor size in the positive control group and MIP-2 expression also significantly increased. In contrast, neutrophil infiltration was effectively regulated in the PLAG-treated group (Fig. 3**C-F**), accompanied by a decrease in the expression and release of MIP-2 (Fig. 3**G-I**).

3.4. PLAG has excellent efficacy in suppressing tumor growth as compared to comparators targeting neutrophil infiltration inhibition

To objectively verify the role of neutrophil infiltration control in the tumor growth inhibitory effect of PLAG, the extent of tumor growth inhibition was compared between the group treated with PLAG and the group treated drugs that inhibit neutrophil infiltration. Compared with the group treated with Navarixin, a CXCR2 antagonist that inhibits neutrophil migration, and Ly6G antibody that suppresses neutrophil activity, the tumor weight in the PLAG-treated group was significantly lower at 3 weeks after inoculation (average tumor suppressive efficacy, PLAG: 81.41 %, Navarixin: 31.63 %, aLy6G: 51.61 %) (Fig. 4**A, B**). In particular, PLAG increased the 90-day survival rate as compared to the comparative test drugs without side effects, such as weight loss, observed with Navarixin treatment (Fig. 4**C, G**).

Navarixin did not affect the total number of neutrophils in the blood; thus, tumor neutrophil infiltration gradually increased from week 3 after inoculation. However, PLAG effectively controlled the total number of neutrophils and significantly inhibited neutrophil infiltration (Fig. 4**D-F,** Supplement Fig.4).

3.5. PLAG regulates tumor growth and neutrophil infiltration in a thioredoxin-interacting protein (TXNIP) dependent manner

In a previous study, we proposed that PLAG inhibited tumor growth by inducing receptor degradation in a TXNIP-dependent manner [33]. Based on these results, a quantitative comparison was performed to verify the role of TXNIP in the inhibitory effects of PLAG on tumor growth and neutrophil infiltration. The inhibitory effect of PLAG on

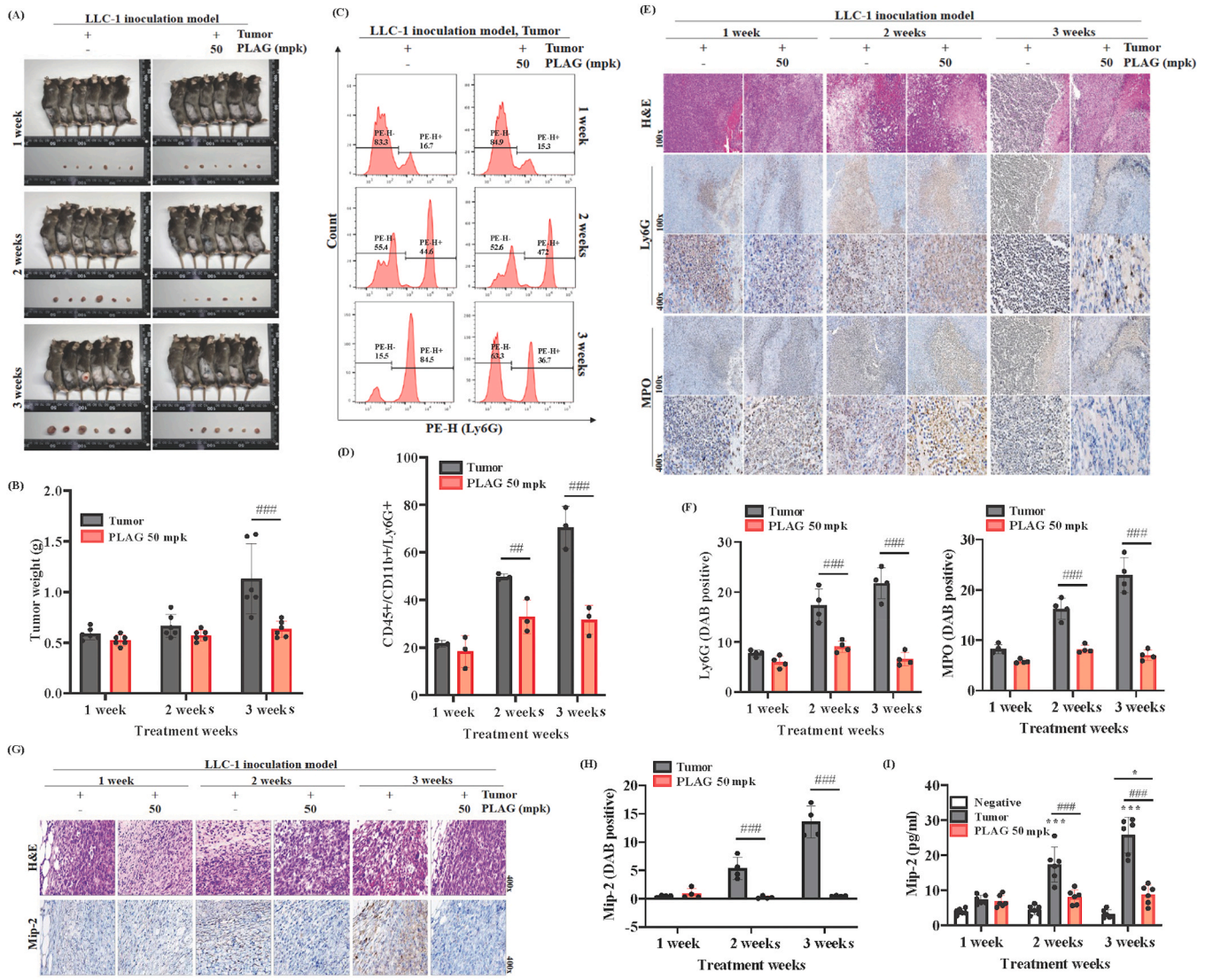


Fig. 3. PLAG regulates excessive infiltration of neutrophils accompanied by tumor growth. (A) Tumor burden and tumor size were measured in control mice and the PLAG-treated group for each week. (B) Changes in tumor weight following weekly. (C, D) Quantitative analysis of tumor-infiltrating neutrophil changes following PLAG treatment using FACS for each week. (E, F, G, H) IHC analysis of tumor-infiltrating neutrophil changes and activity following PLAG treatment with anti-Ly6G/anti-MPO antibodies (E and F) and anti-Mip-2 (G and H) for each week. (I) Quantitative analysis of changes in Mip-2 concentration in blood following PLAG treatment using ELISA for each week. Significance compared with negative control $*P<0.033$, $***P<0.001$, with tumor control $\#\#P<0.002$, $\#\#\#P<0.001$ ($n=3$ in FACS analysis, $n=4$ in IHC, $n=6$ in CBC and ELISA). Results are shown as the mean ± SD.

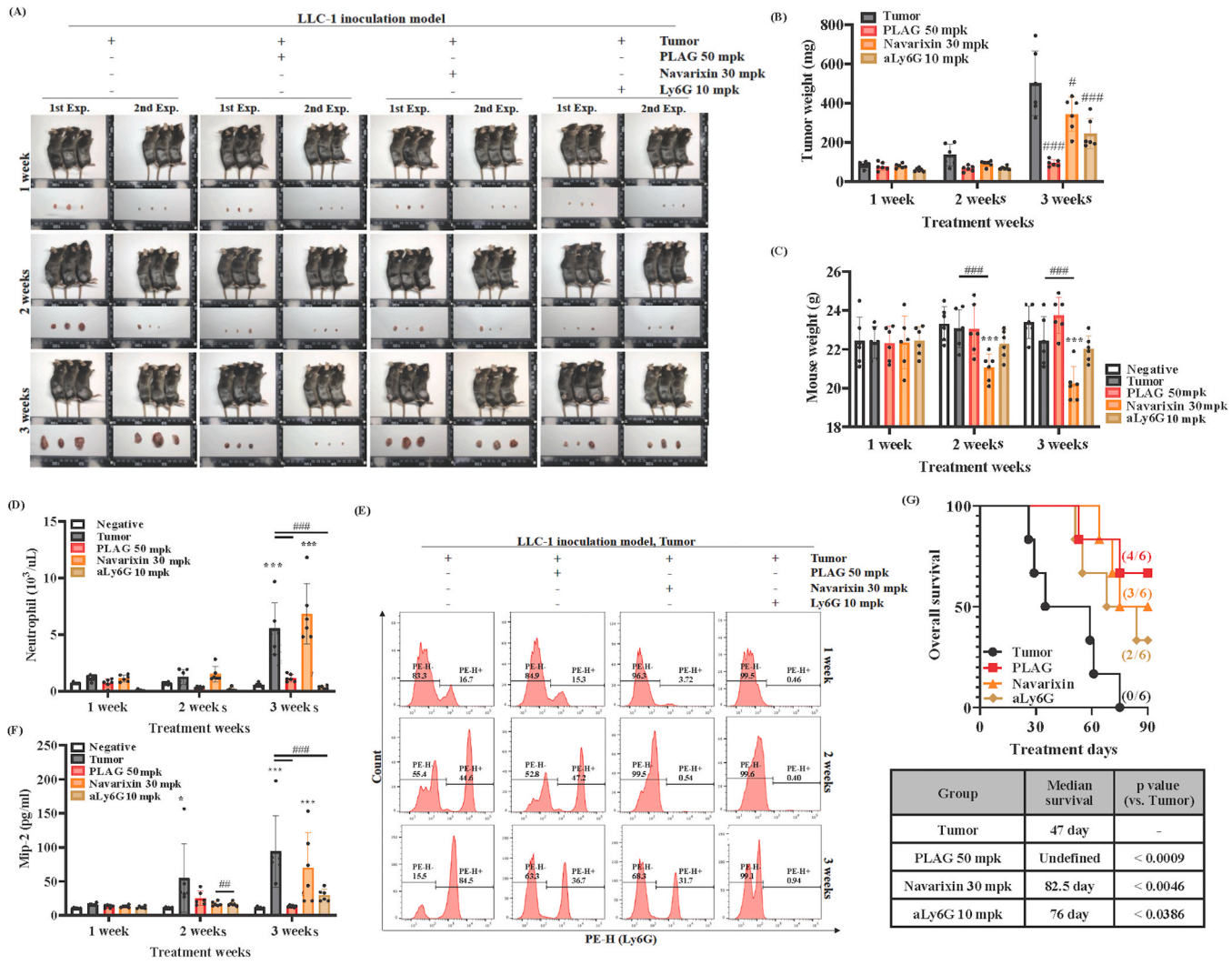
tumor growth was observed to be dependent on TXNIP expression. PLAG suppressed tumor growth by an average of 86.52 % as compared to the TNXIP-WT tumor control, whereas TXNIP-KD cell-implanted mice inhibited tumor growth by an average of 3.24 % compared to the tumor control (Fig. 5**A, B** / **Supplement** Fig. 5), and the effect of PLAG on survival rate was attenuated in the absence of TXNIP expression (Fig. 5**C**).

The number of active infiltrating neutrophils was significantly reduced in proportion to tumor size (14.1 % reduction) in WT tumor controls after PLAG treatment, whereas the effect of PLAG treatment was attenuated in tumors lacking TXNIP expression (Fig. 5**D-H**). In addition, the inhibitory effect of PLAG on MIP-2 expression, a factor associated with increased neutrophil activity and migration in positive controls, was attenuated in tumors lacking TXNIP expression (Fig. 5**I-K**).

3.6. The effect of PLAG on neutrophil infiltration increases the antitumor efficacy of aPD-1

Excessive intra-tumoral neutrophil infiltration inhibits the invasiveness and activity of cytotoxic T cells. Therefore, the proper regulation of neutrophil infiltration is crucial for enhancing the activity of ICIs and allowing cytotoxic T cells to restrict tumor growth. The inhibitory effects of PLAG on neutrophil infiltration significantly increased the antitumor efficacy of ICI against NSCLC cells (Fig. 6**A**). Treatment with aPD-1 alone reduced tumor growth by 58.6 % as compared to the positive controls, whereas treatment with PLAG reduced tumor growth by 87.1 % on average (25 mg/kg: 76.2 %, 50 mg/kg: 88.6 %, 100 mg/kg: 96.6 %). When aPD-1 and PLAG (50 mg/kg or 100 mg/kg) were administered as a combination therapy, one tumor was eliminated within each group (Fig. 6**B-D**). In addition, two individuals survived in the aPD-1 single treatment group, whereas all individuals survived for 90 days in the PLAG 50 mg/kg concurrent treatment group (Fig. 6**E**).

The increase in neutrophils in the blood and NLR levels following



Group	Median survival	p value (vs. Tumor)
Tumor	47 day	-
PLAG 50 mpk	Undefined	< 0.0009
Navarixin 30 mpk	82.5 day	< 0.0046
aLy6G 10 mpk	76 day	< 0.0386

Fig. 4. PLAG has a superior tumor progression inhibitory effect compared to conventional neutrophil infiltration inhibitors. (A) Comparison of tumor burden and size changes following weekly PLAG, Navarixin, and anti-Ly6G antibody treatment (B, C) Tumor and mouse weight of control group and treatment groups for each week. (D) Analysis of changes in blood neutrophil count according to each weekly substance treatment. (E) Quantitative analysis of tumor-infiltrating neutrophil changes following each treatment using FACS for each week. (F) Quantitative analysis of blood concentration of Mip-2 in each treatment group. (G) Comparison of the 90-day survival rate improvement effect according to each substance treatment. Comparison of 90-day survival rates between control and treatment groups. Significance compared with negative control *P<0.033, **P<0.002, ***P<0.001, with tumor control #P<0.033, ##P<0.002, ###P<0.001 (n=3 in FACS analysis, n=6 in other experiment). Results are shown as the mean ± SD.

tumor growth was reversed in a dose-dependent manner in the PLAG-treated groups (Fig. 6**F-H**). In particular, modulation of infiltrating neutrophils significantly increased cytotoxic T-cell infiltration within the tumor (Fig. 6**J-L**).

4. Discussion

Neutrophils function as a double-edged sword, making it difficult to develop strategies to treat disease. Although neutrophil infiltration is necessary for the treatment of certain conditions, the role of excessive neutrophil infiltration in various diseases shows that proper regulation of neutrophil infiltration is a significant factor in the maintenance of normal immune responses [34–36]. Excessive neutrophil infiltration in tumors promotes tumor progression by establishing a tumor-friendly immune environment and activating various signaling pathways that create optimal conditions for tumor growth [37–41]. Therefore, controlling neutrophil infiltration is an important strategy for antitumor therapy.

In the present study, we present evidence supporting the efficacy of PLAG in suppressing tumor growth through the effective inhibition of intra-tumoral neutrophil infiltration. In addition, complete tumor remission was achieved through combination therapy with PLAG and aPD-1. PLAG effectively regulated neutrophil infiltration and reduced tumor growth by more than 80 % compared with the positive controls, as shown in Figs. 1 and 2. In addition, four mice survived for 90 days in the PLAG 50 mg/kg treatment group, whereas all mice died in the positive control group (Fig. 1**E**). This effect was due to PLAG-mediated inhibition of the expression and release of MIP-2 that induces neutrophil infiltration (Fig. 2**F, G**). According to previous studies, the expression of proteins that induce neutrophil migration, such as MIP-2, increases with mitogen-activated protein kinase (MAPK) signaling activity. The activation of the mitogen-activated protein kinase (MAPK) signaling pathway by the adenosine receptor family increases the release of factors that induce intra-tumoral neutrophil infiltration [42–47]. In recent papers, we suggested that PLAG induces the expression of TXNIP, a member of the α-arrestin family, leading to the degradation of receptors such as adenosine receptor 2B (A2BR), protease-activated receptor 2 (PAR2), and epidermal growth factor receptor (EGFR), thereby

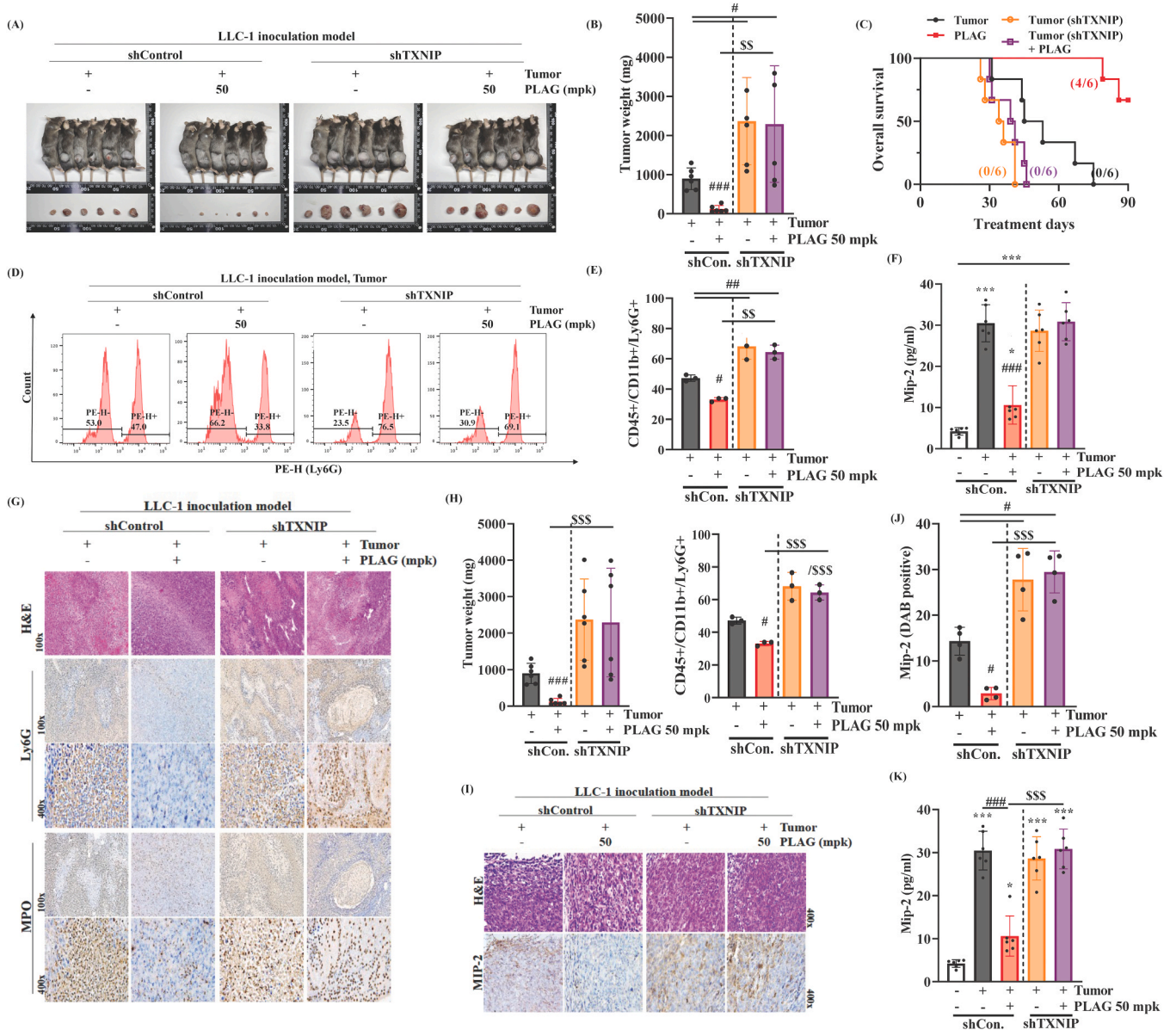


Fig. 5. The tumor growth inhibitory effect of PLAG through regulating neutrophil infiltration is dependent on TXNIP expression. (A) Measurement of tumor burden and size in PLAG-treated group and tumor control group according to TXNIP expression conditions. (B) Quantitative analysis of tumor weight change in the PLAG treatment group and tumor control group according to the TXNIP expression conditions. (C) Comparative analysis of the effect of improving the 90-day survival rate of the PLAG-treated group and the tumor control group according to TXNIP expression conditions. The PLAG administration group was significant at $p<0.0015$ compared to the Tumor group. (D, E) Quantitative analysis of changes in the regulatory effect of PLAG on tumor neutrophil infiltration according to differences in expression of TXNIP using FACS. (F) Analysis of the effect of PLAG on the modulation of changes in neutrophil count in blood according to differences in TXNIP expression. (G, H) Analysis of tumor-infiltrating neutrophil changes and activity following PLAG treatment using anti-Ly6G/anti-MPO antibodies. (I, J) IHC Analysis of tumor-infiltrating neutrophil changes and activity following PLAG treatment with anti-Mip-2. (K) Quantitative analysis of changes in Mip-2 concentration in blood following PLAG treatment using ELISA. Significance compared with negative control *$P<0.033$, **$P<0.002$, ***$P<0.001$, with tumor control #$P<0.033$, ##$P<0.002$, ###$P<0.001$ (n=3 in FACS analysis, n=6 in other experiment). Results are shown as the mean ± SD.

attenuating the over-activation of tumor signaling pathways [30,33,48]. Based on these data, we propose that PLAG suppresses the expression of factors that promote neutrophil infiltration and controls neutrophil migration through the degradation of pro-tumor receptors. As shown in Supplement Figs. 1 and 2, PLAG treatment not only increased TXNIP expression in tumors, but also suppressed the expression of A2BR and Erk phosphorylation. In particular, PLAG induces the formation of a TXNIP–A2BR protein complex, which leads to A2BR degradation through ubiquitination. A2BR degradation attenuated ERK phosphorylation and resulted in ERK cytosolic translocation, potentially inhibiting the translation of factors that promote neutrophil infiltration [30,33]. PLAG effectively regulated the expression of factors that induced neutrophil migration, which played an important role in preventing excessive intra-tumoral neutrophil infiltration (Fig. 3, Supplement Fig. 3).

The unique mechanism of action of PLAG not only enables more effective tumor control than conventional neutrophil infiltration inhibitors but also inhibits tumor growth much better than Navarixin (CXCR2 antagonist) and Ly6G (neutrophil inhibitor) antibodies (Fig. 4).

We recently found that PLAG inhibited tumor growth and metastasis in a TXNIP-dependent manner [30,33,48]. Upon binding to its receptor, TXNIP induces the formation of an E3 ligase complex, which leads to its

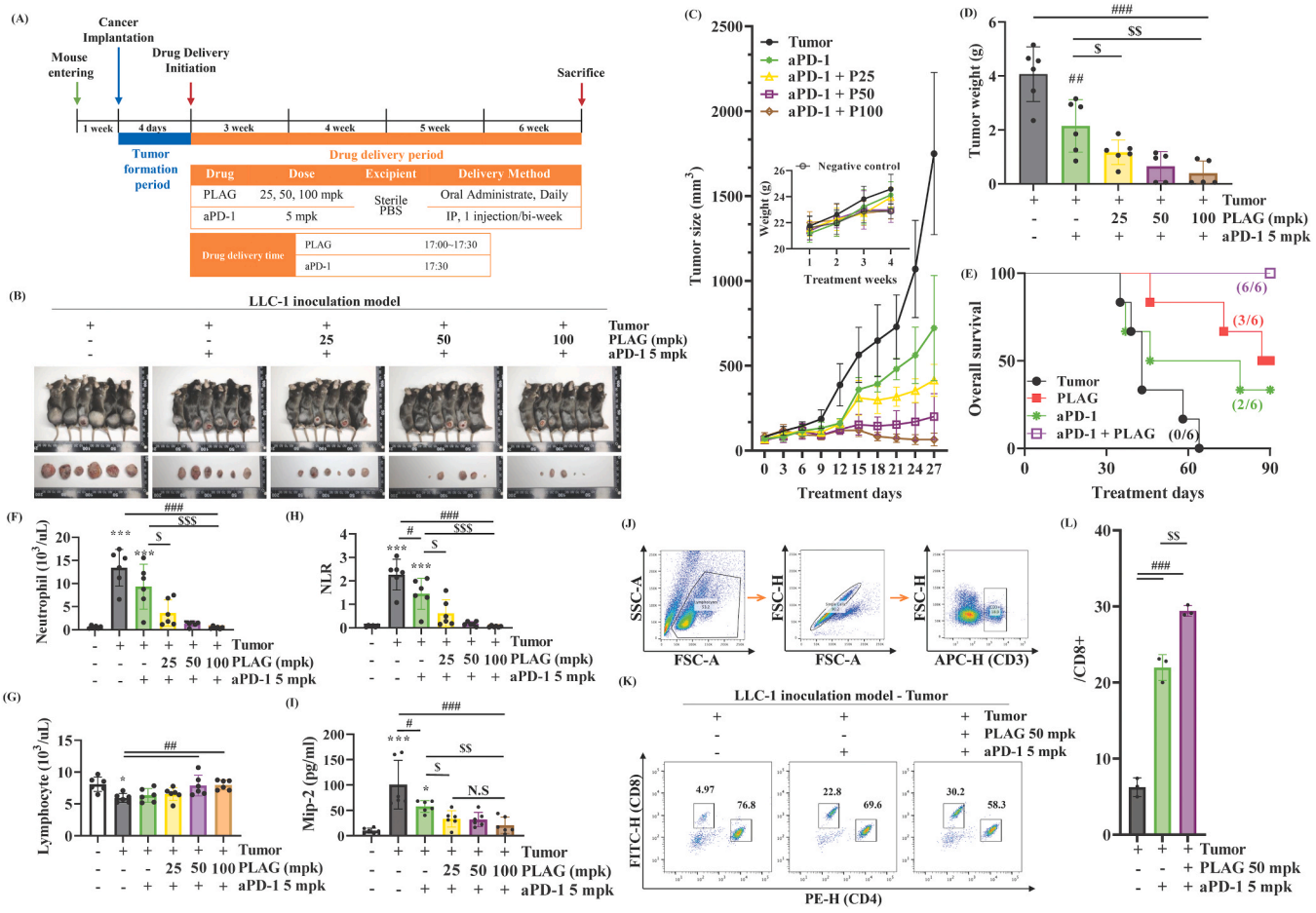


Fig. 6. Co-treatment of PLAG and aPD-1 inhibits NSCLC growth in an LLC-1 inoculation syngeneic model. (A) Schematic diagram of experimental design to evaluate the tumor progression inhibitory effect of aPD-L1 and PLAG co-treatment in LLC-1 implanted mice. (B) Tumor burden and tumor size were measured on the day of sacrifice in control mice and treated with PLAG and aPD-1 groups. (C) Changes in tumor size of mice from each treatment group were measured at 3-day intervals (D) Tumor weight was measured in mice from each treatment group. (E) Comparative analysis of the effect of improving the 90-day survival rate of the aPD-1 alone and PLAG co-treatment groups. The PLAG administration group was significant at $p<0.0108$ compared to the Tumor group. The group administered aPD-1 alone had $p<0.0302$ compared to the tumor alone group, and the group administered aPD-1 and PLAG together had $p<0.0001$. (F, G) Analysis of changes in the effect of regulating the number of blood neutrophils and lymphocytes in aPD-1 treatment with or without PLAG. (H) Quantitative comparative analysis of changes in blood NLR in aPD-1 alone and PLAG co-treatment groups. (I) Quantitative analysis of changes in Mip-2 concentration in blood following PLAG treatment using ELISA. (J) FACS gating strategy for tumor-infiltrating cytotoxic T lymphocyte analysis. (K, L) Quantitative analysis of tumor-infiltrating T-lymphocyte changes in aPD-1 alone and PLAG-treated groups using FACS. Significance compared with negative control *$P<0.033$, **$P<0.002$, ***$P<0.001$, with tumor control #$P<0.033$, ##$P<0.002$, ###$P<0.001$, with aPD-1 only group \$$P<0.033$, \$\$$P<0.002$ (n=3 in FACS analysis, n=6 in other experiment). Results are shown as the mean ± standard deviation (SD).

degradation via ubiquitination. PLAG increases TXNIP expression and inhibits tumor-promoting signaling pathways by inducing receptor degradation. In the present study, we demonstrated that PLAG effectively inhibits tumor growth by controlling neutrophil infiltration in a TXNIP-dependent manner in a TXNIP-knockdown cell line (Fig. 5, Supplement Fig. 5).

Excessive infiltration of neutrophils is a major barrier to the tumor-suppressive efficacy of ICIs such as aPD-1. Neutrophils inhibit the activation and infiltration of cytotoxic T cells through tumor PD-L1 surface expression and reduce the function of ICIs in tumor control [49–51]. Concurrent treatment with aPD-1 and PLAG reduced tumor size by approximately 30 % as compared to aPD-1 treatment alone (Fig. 6**C, D**) and, in some subjects, facilitated tumor removal. (Fig. 6**B,** Supplement Fig. 6). According to recent studies, the progression of severe lung cancer is accompanied by rapid weight loss in patients, which is another cause of tumor-related death [52,53]. Our results revealed that PLAG effectively restored weight loss in individuals with lung cancer after tumor treatment. In particular, the combination with aPD-1 immunotherapy resulted in complete tumor regression and a return to normal body weight. (Supplement Fig. 6C). Most importantly, all mice in the group treated with both PLAG 50 mg/kg and aPD-1 survived for up to 90 days (Fig. 6**E**).

Targeting neutrophil-mediated tumor overgrowth has been challenging in the development of antitumor therapies. PLAG effectively controlled neutrophil infiltration through the degradation of pro-tumor receptors, increasing the potential to achieve complete tumor remission with ICI treatment. These findings support the potential of PLAG as a novel compound for effective antitumor immunotherapy.

Ethics approval

a. All animal care and experimental protocols were performed in accordance with the Korea Research Institute of Bioscience and Biotechnology (KRIBB) regulations. Animal care and experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC), Korea Research Institute of Bioscience and Biotechnology (approval number: KRIBB-AEC-22018).

b. The authors confirmed that all methods were performed according

to the relevant guidelines and regulations.

c. The study was carried out in compliance with the ARRIVE guidelines.

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CRediT authorship contribution statement

Guentae Kim: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Eun Young Kim:** Methodology, Investigation, Formal analysis. **Hyowon Lee:** Investigation. **Su-Hyun Shin:** Investigation. **Se Hee Lee:** Investigation. **Ki-Young Sohn:** Writing – review & editing. **Jae Wha Kim:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Jae Sam Lee:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.117269.

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