

Combination of Poly-Gamma-Glutamate and Cyclophosphamide Enhanced Antitumor Efficacy Against Tumor Growth and Metastasis in a Murine Melanoma Model

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Received: June 28, 2013
Revised: July 17, 2013
Accepted: July 18, 2013

First published online
July 19, 2013

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pISSN 1017-7825, eISSN 1738-8872

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Conventional chemotherapeutic regimens often accompany severe side effects and fail to induce complete regression of chemoresistant or relapsing metastatic cancers. The need for establishing more efficacious anticancer strategies led to the development of a combined modality treatment of chemotherapy in conjunction with immunotherapy or radiotherapy. It has been reported that poly-gamma-glutamate (γ -PGA), a natural polymer composed of glutamic acids, increases antitumor activity by activating antigen-presenting cells and natural killer (NK) cells. Here, we investigated the antitumor effect of γ -PGA in combination with cyclophosphamide in a murine melanoma model. Whereas cyclophosphamide alone directly triggered apoptosis of tumor cells *in vitro*, γ -PGA did not show cytotoxicity in tumor cells. Instead, it activated macrophages, as reflected by the upregulation of surface activation markers and the secretion of proinflammatory factors, such as nitric oxide and tumor necrosis factor α . When the antitumor effects were examined in a mouse model, combined treatment with cyclophosphamide and γ -PGA markedly suppressed tumor growth and metastasis. Notably, γ -PGA treatment dramatically increased the NK cell population in lung tissues, coinciding with decreased metastasis and increased survival. These data collectively suggest that γ -PGA can act as an immunotherapeutic agent that exhibits a synergistic antitumor effect in combination with conventional chemotherapy.

Keywords: Poly-gamma-glutamate, cyclophosphamide, antitumor effect, natural killer cells, metastasis

Introduction

Chemotherapy is used to treat diverse cancers, but chemotherapy alone is insufficient to cure many advanced cancers, owing to side effects and the limited efficacy against chemoresistant or relapsing tumors. The chemotherapeutic agent cyclophosphamide shows strong cytotoxicity against a broad range of cell types, but it has not shown long-term or effective therapeutic responses in patients when given as a monotherapy [12, 29]. Thus, many clinical studies have assessed the co-application of other therapies, including

immunotherapy and radiotherapy, to complement the defects of chemotherapy [21, 22].

Poly-gamma-glutamate (γ -PGA) is a polymer composed of glutamic acids, which is naturally synthesized by a few gram-positive bacteria [30]. γ -PGA is considered to be safe as it is produced by generally regarded as safe (GRAS) microorganisms and has long been taken up as an ingredient of fermented soybean foods [20]. It has been reported that high-molecular-weight γ -PGA is capable of modulating the immune response by activating dendritic cells (DCs) through Toll-like receptor 4 (TLR4), and

consequently enhancing natural killer (NK) cell activity and exhibiting antitumor effects [14, 17]. In addition to its antitumor activity, γ -PGA also shows immunomodulatory effects on diverse immune-related diseases, including asthma and multiple sclerosis, in animal models [15, 16]. Recently, owing to its safe, biodegradable, and immunostimulatory characteristics, γ -PGA has gained increasing interest in the fields of adjuvants, drug delivery systems, and immunotherapy [4, 9, 24, 27].

In this study, we investigated the combined antitumor effect of γ -PGA and cyclophosphamide in a murine melanoma model. Whereas cyclophosphamide exhibited direct cytotoxic effects on tumor cells, γ -PGA did not affect the cell viability or proliferation *in vitro*. Instead, γ -PGA stimulated macrophages to upregulate surface activation markers and proinflammatory factors. *In vivo*, co-treatment with cyclophosphamide plus γ -PGA retarded tumor progression and pulmonary metastasis to a greater degree than that seen with γ -PGA or cyclophosphamide alone. In addition, administration of γ -PGA significantly increased the NK cell population in the lung. In conclusion, our combined chemioimmunotherapy with cyclophosphamide and γ -PGA exhibited synergistic antitumor efficacy *in vivo*, suggesting that γ -PGA could be a potential immunotherapeutic candidate.

Materials and Methods

Mice, Reagents, and Cell Lines

Six- to eight-week-old female C57BL/6 mice were purchased from Koatech (Koatech, Korea) and housed under a specific pathogen-free facility in the Korea Research Institute of Bioscience and Biotechnology (KRIBB, Korea). γ -PGA (average molecular mass, 2,000 kDa) was kindly provided by BioLeaders Corporation (Korea). The B16, B16F10, and RAW264.7 cells were purchased from the American Type Culture Collection (ATCC, USA) and maintained in DMEM (Life Technologies, USA) and RPMI 1640 (Life Technologies) supplemented with 10% FBS (Life Technologies) and 100 U/ml penicillin–100 μ g/ml streptomycin (Life Technologies). This study was performed according to the guidelines of the Animal Experiments Ethics Committee at KRIBB.

Proliferation Assays

B16 cells (2×10^3 cells/well) were seeded in 96-well culture plates and treated with cyclophosphamide (0 to 20 mg/ml) or γ -PGA (0 to 2.0 mg/ml) for 72 h. After the treatment, 20 μ l of WST-1 solution (Roche, Switzerland) was added to each well. After incubation for 2 h, the optical density was measured at 420 nm using a microplate reader (Molecular Devices, USA).

Cell Culture and Treatment of RAW264.7 Cells

RAW264.7 (5×10^5 cells/well) cells were seeded on 24-well culture plates, and treated with γ -PGA (1.0 mg/ml) or LPS (100 ng/ml). After 48 h incubation, the cells were stained with F4/80, CD11b, CD40, and CD86 for flow cytometric analysis.

Measurement of Cytokines and NO Production

RAW264.7 cells (8.4×10^4 cells/well) were seeded in 96-well culture plates and treated with PBS, γ -PGA (0.5 and 1.0 mg/ml), or LPS (100 ng/ml). After incubation for 72 h, the culture media were harvested and stored at -80°C until use. The concentrations of cytokines and nitric oxide (NO) were measured using the Bio-Plex Cytokine Assay system (Bio-Rad, USA) and the Griess reagent system (Promega, USA), respectively, according to the manufacturer's instructions.

Tumor Challenge and Treatment

A dose of 1×10^4 B16 cells in 100 μ l of RPMI 1640 was injected subcutaneously into the right hind flank of each mouse. The tumor size was measured two or three times per week; a digital caliper was used to measure the two-dimensional longest axis (L in mm) and shortest axis (W in mm), and the tumor volume was calculated according to the formula: volume in $\text{mm}^3 = (L \times W^2)/2$. For the lung metastasis model, 2×10^5 B16F10 cells were injected into the tail vein. In both cases, 1 week after the tumor challenge, the mice were intraperitoneally injected with a single dose of cyclophosphamide (100 mg/kg; Sigma-Aldrich, USA) and/or orally dosed with γ -PGA (2,000 kDa, 800 μ g/mouse) daily for 3 weeks.

Preparation of Lung Cells

Lymphocytes were isolated from lung tissues by mechanical chopping and enzymatic digestion of the lung tissue, as previously described [2]. Briefly, lung tissues were chopped into small pieces and digested with 150 unit/ml of collagenase type IV (Life Technologies) and 5 μ g/ml of DNase I (Sigma-Aldrich) for 90 min at 37°C . The enzymatic digestion was stopped by adding EDTA solution (final concentration, 1 mM), and the lung fragments were crushed on a nylon mesh with a glass pestle. The red blood cells were removed, and the remaining cells were washed and used for subsequent analysis.

Flow Cytometry

B16 cells were harvested and stained with 7-AAD (eBioscience) and Annexin-V FITC (eBioscience); RAW264.7 cells were stained for F4/80, CD11b, CD40, and CD86; and isolated lung cells were stained for CD3e and NK1.1. After fixation, samples were acquired on a Gallios (Beckman Coulter, USA) and data were analyzed using the FlowJo software (TreeStar, USA).

Statistical Analysis

Statistical analyses were performed using two-tailed Student's *t*-tests and log-rank tests, and a *p*-value < 0.05 was considered statistically significant.

Results

Treatment with γ -PGA Does Not Affect Tumor Cell Proliferation or Viability *In Vitro*

Cyclophosphamide is a cytotoxic chemical that irreversibly alkylates DNA molecules in proliferating cells [13]. To compare the influence of cyclophosphamide and γ -PGA on the survival and proliferation of tumor cells, we treated B16 melanoma cells with cyclophosphamide and/or γ -PGA, and assessed the proliferation using a WST-1-based colorimetric analysis. When the cells were incubated with cyclophosphamide (0 to 20 mg/ml) for 72 h, the proliferations of B16 cells were dramatically and dose-dependently reduced (Fig. 1A, left panel). Treatment with γ -PGA alone did not affect the proliferation of B16 cells (Fig. 1B, left panel). When the B16 cells were treated with both cyclophosphamide (2.5 mg/ml) and γ -PGA (1.0 mg/ml), the cytotoxic effects were similar to those seen in cells treated with cyclophosphamide alone (Fig. 1C). To examine apoptotic cell death more precisely, B16 cells treated with cyclophosphamide and/or γ -PGA were harvested and

analyzed by flow cytometry. Consistent with the results from WST-1 colorimetric analysis, γ -PGA alone did not induce apoptosis, whereas cyclophosphamide administered alone or with γ -PGA exhibited direct and dose-dependent cell killing effects (Figs. 1A and 1B, right panels). These results suggest that γ -PGA does not have direct tumoricidal activity *in vitro*.

γ -PGA Induces Macrophage Activation

Since γ -PGA has been reported to activate DCs through TLR4, and macrophages also constitutively express the receptor [17], we examined the effect of γ -PGA on macrophages using a RAW264.7 cell line and primary peritoneal macrophages. When RAW264.7 cells were incubated with γ -PGA (1.0 mg/ml) for 48 h, the activation markers CD40 and CD86 were upregulated compared with the PBS-treated control (Fig. 2A). Furthermore, γ -PGA-treated macrophages produced various proinflammatory cytokines, including IL-1 β , IL-6, IFN- γ , CCL5, and tumor necrosis factor (TNF)- α , as well as the anti-inflammatory cytokine, IL-10 (Fig. 2B). Nitric oxide (NO) was also detected from

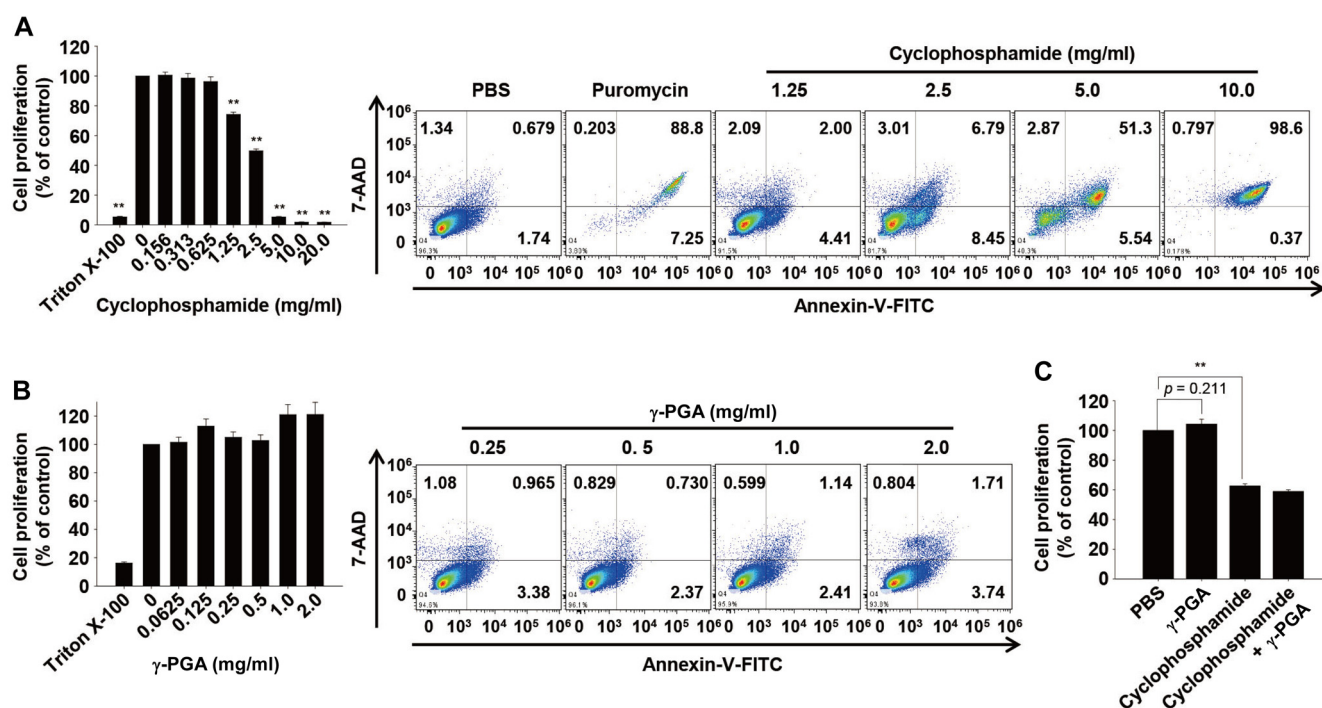


Fig. 1. Effects of cyclophosphamide and γ -PGA on tumor cell proliferation and viability *in vitro*

(A) B16 cells were treated with 0 to 20 mg/ml of cyclophosphamide, (B) and 0 to 2.0 mg/ml of γ -PGA for 72 h. After incubation, the supernatants and cells were harvested and subjected to WST-1 assays and 7-AAD/annexin V staining, respectively. (C) B16 cells were cultured in the presence of γ -PGA (1.0 mg/ml), cyclophosphamide (2.5 mg/ml), or both for 72 h. Three independent experiments were performed in triplicate and the data show the average cell proliferation \pm SEM; ***p* < 0.01 and **p* < 0.05 versus the untreated control.

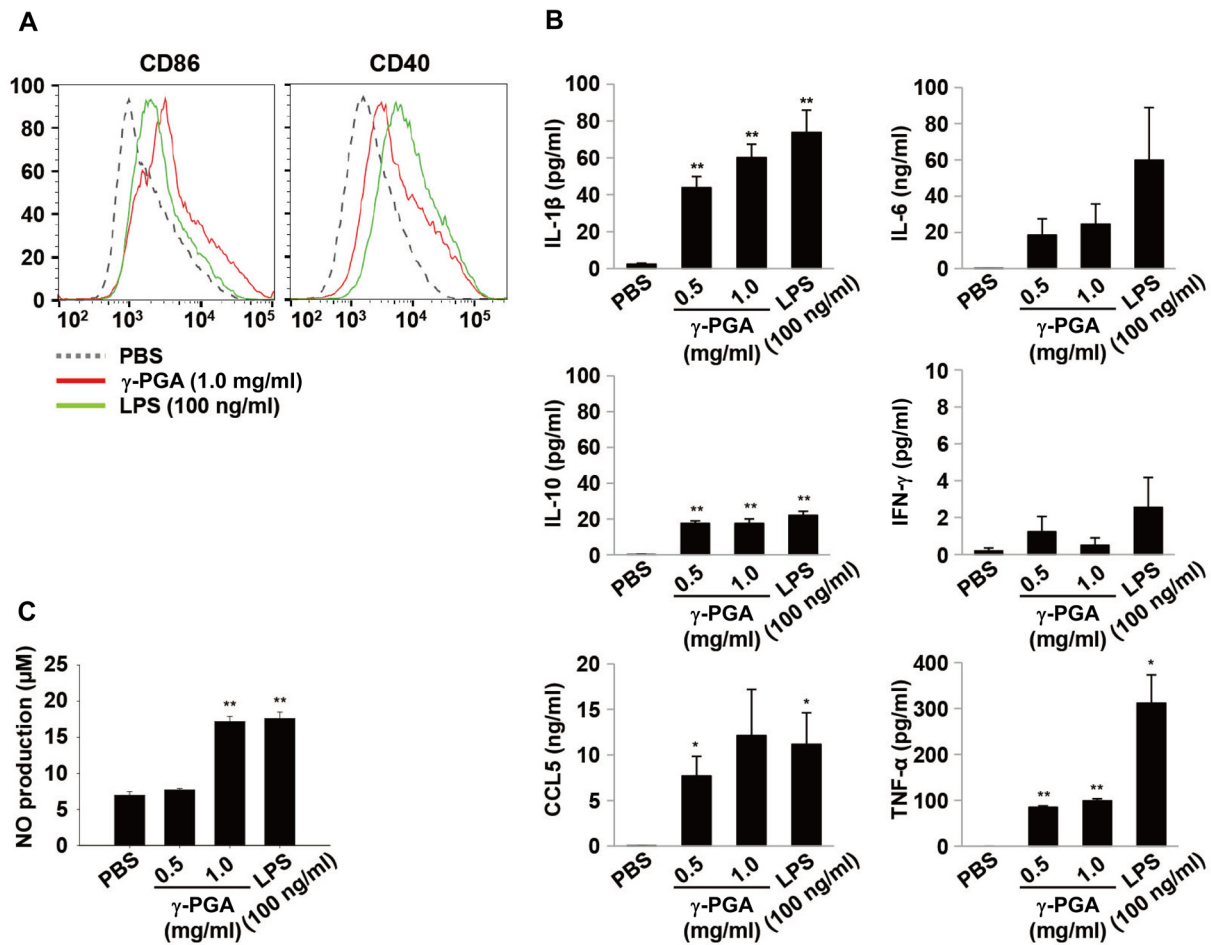


Fig. 2. Macrophage activation and cytokine productions induced by γ -PGA

(A) RAW264.7 cells were incubated with γ -PGA or LPS for 48 h, and the cells were harvested and stained for F4/80, CD11b, CD86, and CD40. (B and C) After incubation for 72 h, the cytokine levels in the culture media were determined using a multiplex assay, and NO production was measured by the Greiss reaction. Experiments were performed in triplicate and the data are presented as the mean \pm SEM; ** p < 0.01 and * p < 0.05 versus the untreated control.

the macrophages incubated with γ -PGA (Fig. 2C). Primary peritoneal macrophages exhibited similar patterns of cytokine, chemokine, and NO production (data not shown). These results indicate that γ -PGA directly activates macrophages, leading to the expression of diverse proinflammatory mediators.

Co-Treatment with Cyclophosphamide and γ -PGA Synergistically Inhibits the Growth of Solid Tumors *In Vivo*

Previously, Kim *et al.* [14] showed that γ -PGA exerts an antitumor effect in mouse melanoma models. To test whether oral administration of γ -PGA could synergistically improve the antitumor efficacy of cyclophosphamide, we

performed *in vivo* combination therapy. Mice were subcutaneously inoculated with B16 melanoma cells (1×10^4 cells/mouse) on the right flank. One week later, they were given a single intraperitoneal injection with 100 mg/kg of cyclophosphamide and/or orally dosed with γ -PGA (2,000 kDa, 800 μ g/mouse) daily for 3 weeks. As shown in Fig. 3A, tumor growth was dramatically retarded in the cyclophosphamide-injected mice, whereas moderate tumor suppression was observed in mice treated with γ -PGA alone. Notably, co-treatment with cyclophosphamide plus γ -PGA further inhibited tumor growth (Fig. 3A) and dramatically enhanced the survival rate (100% survival by day 35) and percentage of tumor-free mice compared with those receiving cyclophosphamide or γ -PGA alone (Figs. 3B

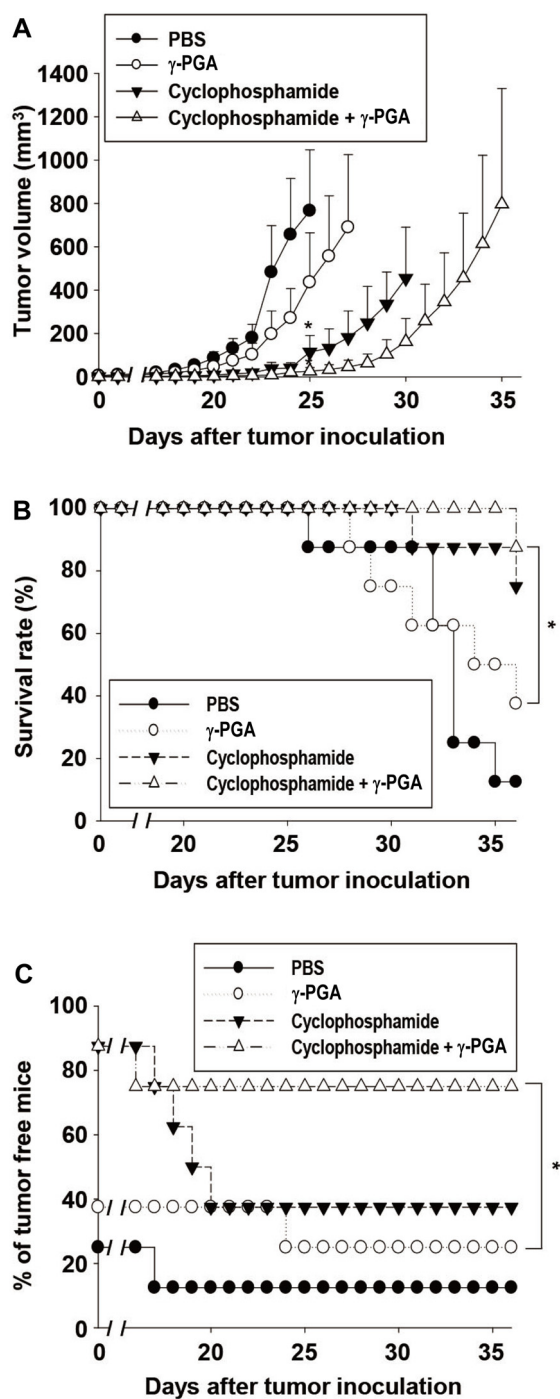


Fig. 3. Inhibition of tumor growth by co-treatment with cyclophosphamide and γ -PGA

B16 cells (1×10^4 cells/mouse) were injected subcutaneously. After a week, mice were injected with a single dose of cyclophosphamide (100 mg/kg) and/or daily doses of γ -PGA (2,000 kDa, 800 μ g/mouse). Tumor volume (A), percentage of tumor-free mice (B), and mouse survival (C) were monitored 2-3 times/week for 3 weeks ($n = 8$ /group). Results are given as the mean tumor volume (mm^3) \pm SEM; ** $p < 0.01$ and * $p < 0.05$.

and 3C). These results demonstrate that orally administered γ -PGA potentiates the antitumor effects of cyclophosphamide against B16 melanoma cells in the mouse model.

Co-Treatment with Cyclophosphamide and γ -PGA Completely Suppresses Pulmonary Metastasis of B16F10 Tumor Cells

The synergistic antitumor effect of the cyclophosphamide- γ -PGA combined therapy was also assessed using a pulmonary metastasis model. Consistent with the results obtained in the solid tumor setting, mice treated with cyclophosphamide or γ -PGA alone showed significantly fewer tumor nodules in the lung compared with the PBS control (9.25 ± 2.67 and 15.0 ± 5.28 , respectively, *vs.* 42.8 ± 1.76 ; Fig. 4A). More importantly, co-treatment with cyclophosphamide plus γ -PGA almost completely inhibited tumor metastasis to the lungs (0.500 ± 0.267 , Fig. 4A). Examination of the immune cell populations in the lungs revealed more NK cells in the cyclophosphamide- γ -PGA combination group than in the cyclophosphamide-treated group (Fig. 4B). These results suggest that γ -PGA significantly enhances the therapeutic effects of cyclophosphamide in both solid and pulmonary melanoma models.

Discussion

Chemotherapy is widely used against diverse cancers, including breast cancers, lymphomas, and melanomas. Despite the efficacy, conventional chemotherapies are often limited by side effects and fail to induce complete eradication of tumors. To overcome these obstacles, chemotherapies are often combined with immunotherapies. In this study, we investigated the effect of γ -PGA in combination with cyclophosphamide, and assessed the immunological mechanism of action of γ -PGA.

We found that γ -PGA significantly increased the antitumor efficacy of cyclophosphamide *in vivo* (Fig. 3). Since TLR signaling plays a critical role in the induction of innate and adaptive immune responses, diverse TLR agonists have been studied as antitumor immunotherapeutic agents and adjuvants for cancer vaccines. TLR4 agonists, such as LPS and synthetic lipid A derivatives, have been shown to enhance antitumor efficacy in bladder and breast cancer models [6, 18, 26]. In addition, activation of TLR3, -7, and -9 signaling has been reported to suppress tumor progression [7, 19, 25], suggesting that stimulation of TLRs could confer beneficial antitumor effects. In particular, co-treatment with cyclophosphamide and LPS was shown to exhibit a synergistic effect against lung cancer [11]. These

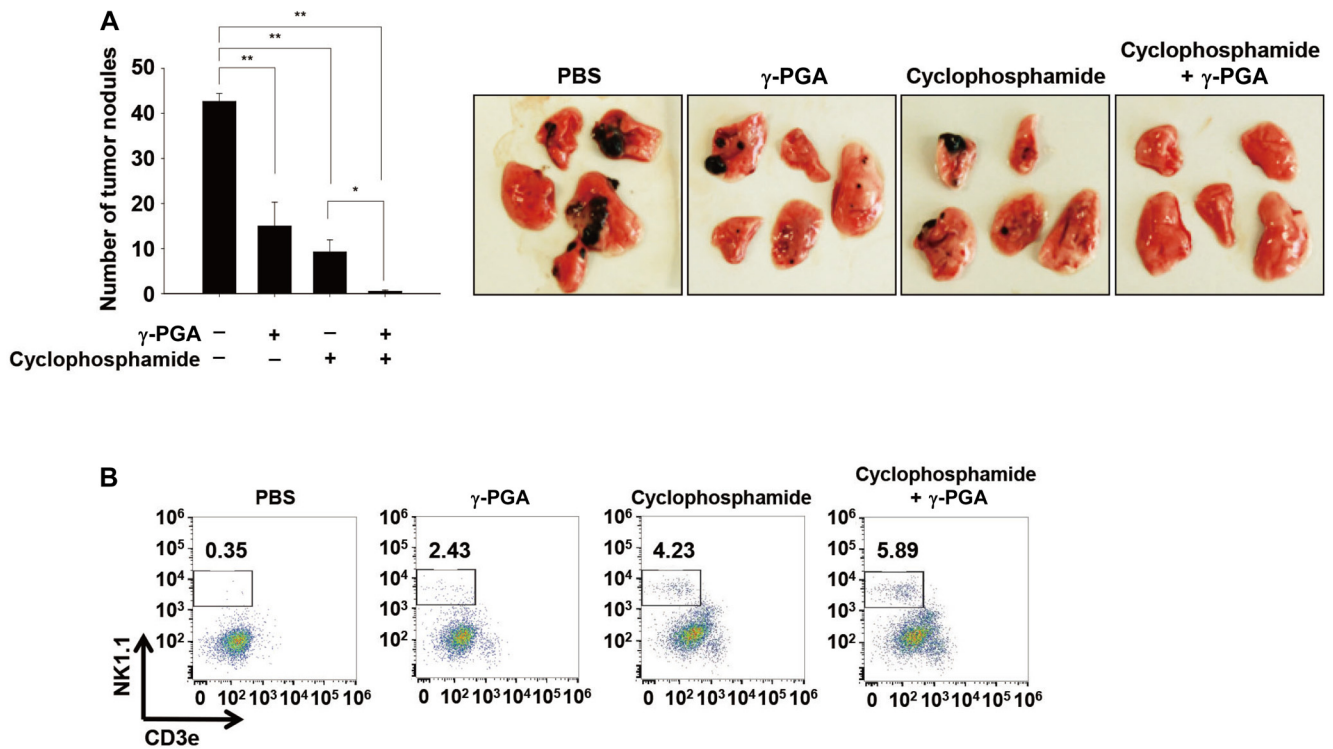


Fig. 4. Pulmonary metastasis was completely blocked by co-treatment with cyclophosphamide and γ -PGA.

B16F10 cells (2×10^5 cells/mouse) were injected intravenously and mice were treated with cyclophosphamide and/or γ -PGA. (A) Twenty-eight days after the tumor injection, tumor nodules were counted in each lobe of the lungs; $**p < 0.01$ and $*p < 0.05$. (B) The NK cell populations in the lungs were analyzed by flow cytometry.

reports are in good agreement with our observation that cyclophosphamide– γ -PGA co-treatment has a synergistic effect against tumors.

Although the *in vitro* cytokine profiles induced by γ -PGA and LPS treatment appeared similar in terms of the sort, the levels of IL-1 β , IL-6, and TNF- α were lower in γ -PGA-stimulated macrophages than in the LPS-treated group (Fig. 2B). As these cytokines are critical inflammatory mediators in LPS-induced septic shock [1], these results suggest that γ -PGA may be safer than LPS, even though they target the same receptor. Interestingly, the levels of CCL5 were similar in γ -PGA- and LPS-treated macrophages (Fig. 2B). CCL5 does not have direct tumoricidal activity *per se*, but it has shown therapeutic activity against hepatoma *in vivo* [10]. Notably, unlike other proinflammatory cytokines, CCL5 has been reported to be inversely correlated with the severity score of septic shock [5]. Future studies are warranted to investigate the differential cytokine production triggered by γ -PGA and LPS.

Despite the previous report that γ -PGA enhances NK cell activity *in vivo*, the underlying mechanism is not yet

fully understood. Here, we showed that oral administration of γ -PGA increased the NK cell population in the lung (Fig. 4B). This is in good agreement with a previous study showing that NK cell activity was significantly enhanced when CCL5 was co-delivered as an adjuvant [3, 8, 23]. As γ -PGA preferentially induces CCL5 from macrophages (Fig. 2B), we speculate that CCL5 could be a mediator that links antigen-presenting cells and NK cells.

It is also interesting that co-treatment with cyclophosphamide– γ -PGA almost completely inhibited tumor metastasis to the lung (Fig. 4A). The effect of conventional TLR4/9 agonists on tumor metastasis was previously found to be largely dependent on the time point of administration, with only prophylactic delivery of those agonists proving effective in preventing B16F10 metastasis [28]. In the present study, however, we found that the metastasis of B16F10 cells was dramatically attenuated by delivery of γ -PGA or γ -PGA–cyclophosphamide 7 days after tumor injection. These results suggest that γ -PGA can be a potent immunotherapeutic agent that could be particularly applicable to the therapy of a metastatic tumor or relapsing tumor after surgery.

In summary, we herein report that γ -PGA has a synergistic antitumor effect in combination with cyclophosphamide, inducing proinflammatory molecules that can augment antitumor effects *in vivo*. These findings suggest that γ -PGA could be utilized as an antitumor drug and as part of chemoimmunotherapeutic strategies against cancer.

Acknowledgments

This work was supported by the grant of National R&D Program for Cancer Control (0720510), Ministry of Health & Welfare, Republic of Korea and a funding from KRIBB Initiative program to H. Poo.

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