Proteoglycan isolated from *Phellinus linteus* inhibits tumor growth through mechanisms leading to an activation of CD11c$^+$CD8$^+$ DC and type I helper T cell-dominant immune state

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Abstract **Dendritic cells (DC) are known to not only induce the activation of T cells, but are also associated with the polarization of T cells. This study investigated whether or not proteoglycan (PG) isolated from *Phellinus linteus* induces the phenotypic and functional maturation of CD11c$^+$ DC in vitro and in vivo. PG was found to induce the phenotypic and functional maturation of bone marrow-derived DC via Toll-like receptors (TLR) 2 and 4 in vitro. Administration of PG in vivo strongly inhibited the MCA-102 tumor growth and increase in vivo. The ratio of CD8$^+$ DC to CD8$^-$ DC increased, and PG enhanced IL-12 and IFN-γ production, and expression of surface molecules including major histocompatibility complexes (MHC) classes I, MHC II, CD80, and CD86 in MCA-102-challenged mice. PG also caused a marked increase in the production of Th (helper T cells)-1 cytokine (IFN-γ) and a decrease in the production of Th-2 cytokine (IL-4) by splenic cells and inguinal lymph node cells in MCA-102 tumor-bearing mice. Furthermore, PG stimulated the proliferation of CD4$^+$ and CD8$^+$ T cells. In addition, a combination of PG and tumor lysate-pulsed DC inhibited completely the growth of MCA-102 cells in tumor-bearing mice. These results indicate that the administration of PG inhibited the tumor growth through a mechanism leading to a Th-1 dominant immune state and the activation of CD11c$^+$CD8$^+$ DC.**

Keywords: Proteoglycan; Dendritic cell; Interleukine-12; T cell; *Phellinus linteus*

1. Introduction

There is much attention currently focused on the use of dendritic cells (DC) in immuno-cell therapy against hematopoietic and solid tumors. DC are professional antigen-presenting cells (APC) with a unique and potent capacity to induce innate and adaptive immune responses [1] as well as an ability to regulate the T cell-mediated immune responses [2,3]. DC can also prime naive T cells to antigens and dictate the development of T cell-mediated immune responses into either the helper T cells (Th)-1 or Th-2 type [4]. Activation of the CD4$^+$ Th-1 cells that produces primarily interleukin-2 (IL-2) and interferon-γ (IFN-γ) is necessary in order to induce cellular immunity in mice. The Th-2 cells that produce IL-4, IL-5, IL-10 and IL-13 control humoral immunity [5,6]. The development of Th-1 or Th-2 types from naive cells to effector cells is regulated by the presence of specific cytokines in the microenvironment at the time of T cell priming. For Th-1 cells, IL-12 is an essential cytokine of differentiation, whereas for Th-2 cells, IL-4 and IL-10 are critical [7,8]. In mice, the CD8$^+$ DC, but not the CD8$^-$ DC subset, has the capacity to produce a large amount of IL-12, which is the most important cytokine in the induction of Th-1 cells [9].

A variety of biological response modifiers that modulate the host biological responses against tumors have been developed as adjuvants for applications in cancer therapy. Such agents have been capable of potentiating the host immune responses without direct cytotoxicity to cancer cells [10]. In a comparative study of anti-tumor activities by polysaccharides from basidiomycetes, a more than 80% growth inhibition of Sarcoma 180 transplanted into immunocompetent ICR mice was observed [11]. Most notably, the hot water-crude extract of *Phellinus linteus* was the most potent, the growth-inhibiting properties of which were significantly apparent in that system [12]. It has been reported that the polysaccharides purified from the mycelial culture of *P. linteus* stimulate both the proliferation of T lymphocytes and the humoral immune function via the polyclonal activation of B cells [13,14]. It has also been shown that the culture filtrate of *Phellinus* sp. contains cyclophellitol. This compound suppresses the metastatic potential of tumor cells by interfering with the synthesis of the cell surface carbohydrates [15,16].

We previously reported that proteoglycan (PG) isolated from *P. linteus* enhanced the proliferation of splenic lymphocytes [17]. PG increased the proliferation of B lymphocytes via

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protein tyrosine kinase and protein kinase C, but did not affect T lymphocytes [18]. PG also induced the phenotypic and functional maturation of bone marrow-derived DC through increased surface molecules, IL-12p70, mixed lymphocyte reactions, and migration into the lymphoid organs [19]. However, the reciprocal relationship between DC and PG in tumor-bearing mice is unknown. Therefore, this study investigated the roles of splenic and draining lymph node DC in MCA-102 tumor-bearing mice administered with PG. The study also investigated whether or not the administration of PG has an effect on tumor rejection through the activation of $CD4^+$ Th cells and cytotoxic $CD8^+$ T cells. Notably, regarding anti-tumor activities, these immunostimulating activities of PG might be related to the maturation of $CD11c^+CD8^+$ DC in lymphoid organs.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice (6–8 weeks old) were obtained from the Korean Institute of Chemistry Technology (Daejeon, Korea). They were maintained under pathogen-free conditions until used. Sterilized food and water were supplied ad libitum, and the mice were housed under a 12-h day/night cycle.

2.2. Preparation of PG

PG was isolated from the mushroom *P. linteus* by using ethanol precipitation methods followed by DEAE-cellulose and gel filtration chromatography [17]. The molecular weight of PG was estimated to be approximately 150 kDa. It contained 72.2% polysaccharide and 22.3% protein. It was determined to be an acidic proteoheteroglycan. The main components of PG are polysaccharides that consist of mannose (41.6%), glucose (23.4%) and galactose (23.8%). Analysis by gas chromatography showed that PG was free of lipids. Endotoxin was assayed under endotoxin-free experimental conditions by using a Limulus Amebocyte Lysate (LAL) Pyrogen Kit (Biowhittaker, Walkersville, MD). The experiments were conducted according to the manufacturer's protocol: 100 μl of standards, PG or controls was mixed with 100 μl of the LAL reagent and incubated for 1 h at 37 °C. Each tube was then examined for gelation. The quantity of endotoxin in PG was $\leqslant 0.01$ ng/mg.

2.3. Administration of PG and tumor growth analysis

MCA-102 tumor cells (1×10^6) were implanted in the right flank of female C57BL/6 mice (6–8 weeks old). Next, PG (100 mg/kg/day) or saline (control) was administered to the tumor-bearing mice intraperitoneally (i.p.) for 19 consecutive days. On day 20, in PG- or PBS-administered mice, the spleens or the inguinal lymph nodes (iLN) were removed and dissociated with collagenase in Ca^{2+}-free media in the presence of EDTA. They were separated into low- and high-density fractions on a Nycodenz gradient.

2.4. Assessment of cell viability

Cell viability was assessed using methyl thiazolyl tetrazolium (MTT) staining. 100 μl of cell culture containing 1×10^4 cells was plated in the wells of 96-well culture plates. After incubation with PG for 48 h, 100 μl of MTT solution (2.5 mg/ml) was added. After incubation at 37 °C for 4 h, 100 μl of acid-isopropanol (0.04 N HCl in isopropanol) was added and mixed gently with the cell suspension and OD_{560} was determined with ELISA reader.

2.5. Flow cytometric analysis

Whole spleen cells or lymph node cells were first blocked with 10% (v/v) normal goat serum for 15 min at 4 °C and stained with PerCP-conjugated anti-CD8 (clone: 53-6.7), PE-conjugated anti-$H2K^b$ (AF6-88.5), anti-IA^b (AF6-120.1), anti-CD80 (16-10A1), and anti-CD86 (GL1) with fluorescein isothiocyanate (FITC)-conjugated anti-CD11c (HL3) antibody (PharMingen, SanDiego, CA) for 30 min at 4 °C. In parallel experiments, the cells were stained with PerCP-conjugated anti-CD3 (145-2C11), phycoerythrin (PE)-conjugated anti-CD8 (53-6.7), and FITC conjugated anti-CD4 (GK1.5) antibody (PharMingen). The stained cells were analyzed using a FACSCalibur (Becton Dickinson, San Jose, CA) with the CellQuest program.

2.6. Intracellular cytokine staining

Cells were first blocked with 10% v/v normal goat serum for 15 min at 4 °C and then stained with FITC-conjugated anti-CD11c antibody for 30 min at 4 °C. The cells stained with the appropriate isotype-matched Ig were used as the negative controls. The cells were fixed and permeated with the Cytofix/Cytoperm kit (PharMingen), according to the manufacturer's instructions. For intracellular IL-12 p40/p70 and IFN-γ detection in $CD11c^+$ DC, DC were stained with PE-conjugated antibodies (PharMingen) in a permeation buffer. In a parallel experiment, for intracellular IL-4 and IFN-γ detection in $CD3^+$ T cells, they were stained with PE-conjugated anti-IL-4 and FITC-conjugated anti-IFN-γ with PerCP conjugated anti-CD3 antibodies (PharMingen). The cells were analyzed using a FACSCalibur.

2.7. Generation of bone marrow (BM)-derived myeloid DC and tumor growth

Bone marrow cells were collected from the marrow of femurs and tibiae of mice under aseptic conditions. The BM cells were cultured in a culture medium (Opti-MEM, 2 mM L-glutamine, 10% fetal bovine serum, 50 μM mercaptoethanol and 100 U/ml penicillin) supplemented with 20 ng/ml recombinant murine (rm) GM-CSF and 20 ng/ml rmIL-4 at 1×10^6 cells/ml. The DC were harvested from 4-day culture, washed, and resuspended at 5×10^6 cells/ml. The DC populations were enriched by 14.5% metrizamide. Viable MCA-102 tumor cells were suspended at 1×10^7 cells/ml. The tumor cell lysates were generated by five rapid freeze-thaw cycles as described in [20]. The tumor cell lysates were collected and incubated overnight with purified DC at a tumor-cell-to-DC ratio of 3:1. The tumor cell lysates-pulsed DC (TP-DC) or unpulsed DC (Normal-DC) (1×10^6 cells) were introduced subcutaneously into C57BL/6 mice. One week later, 1×10^6 MCA-102 tumor cells were intradermally (i.d.) inoculated into the left flank of the mice. One group of TP-DC mice was treated with PG for 19 consecutive days starting from that day. In order to determine whether PG has a direct effect on the MCA-102 introduced tumors, C57BL/6 mice were i.d. implanted with 1×10^6 MCA-102 tumor cells per mouse on day 0. After 24 h, saline or PG (100 mg/kg/day) was administered to the tumor-bearing mice i.p. for 19 consecutive days. The sizes of the tumors were determined every 7 days using the microcaliper: tumor volume (mm^3) = (major axis) × (minor axis)^2 × 0.5236 [21].

2.8. Statistical analysis

The results were expressed as means ± SD of the indicated number of experiments. The statistical significance was estimated using a Student's t test for unpaired observations. A P value of <0.05 was considered to be significant.

3. Results

3.1. Administration of PG in vivo inhibits growth of MCA-102 tumor cells

The effects of PG on tumor growth in mice implanted with MCA-102 tumor cells are shown in Fig. 1A. The MCA-102 cells were implanted in the mice on day 0. Saline (control) or PG 100 mg/kg/day was administered intraperitoneally for 19 consecutive days. PG significantly delayed tumor growth (Fig. 1A) and suppressed tumor weight (Fig. 1B). Tumor formation was somewhat inhibited in all mice administered with PG. The final tumor weight on day 20 was reduced by about 41% by the PG treatment. These results suggest that the administration of PG may enhance anti-tumor capacity through immune-modulatory activity, because PG is not directly cytotoxic to MCA-102 cells (Fig. 1C). Also, the administration of PG did not show any apparent side effects such as a decrease of body weight (Fig. 1D). These results clearly indicate that PG was effective in preventing the experimental tumor growth of MCA-102 cells.

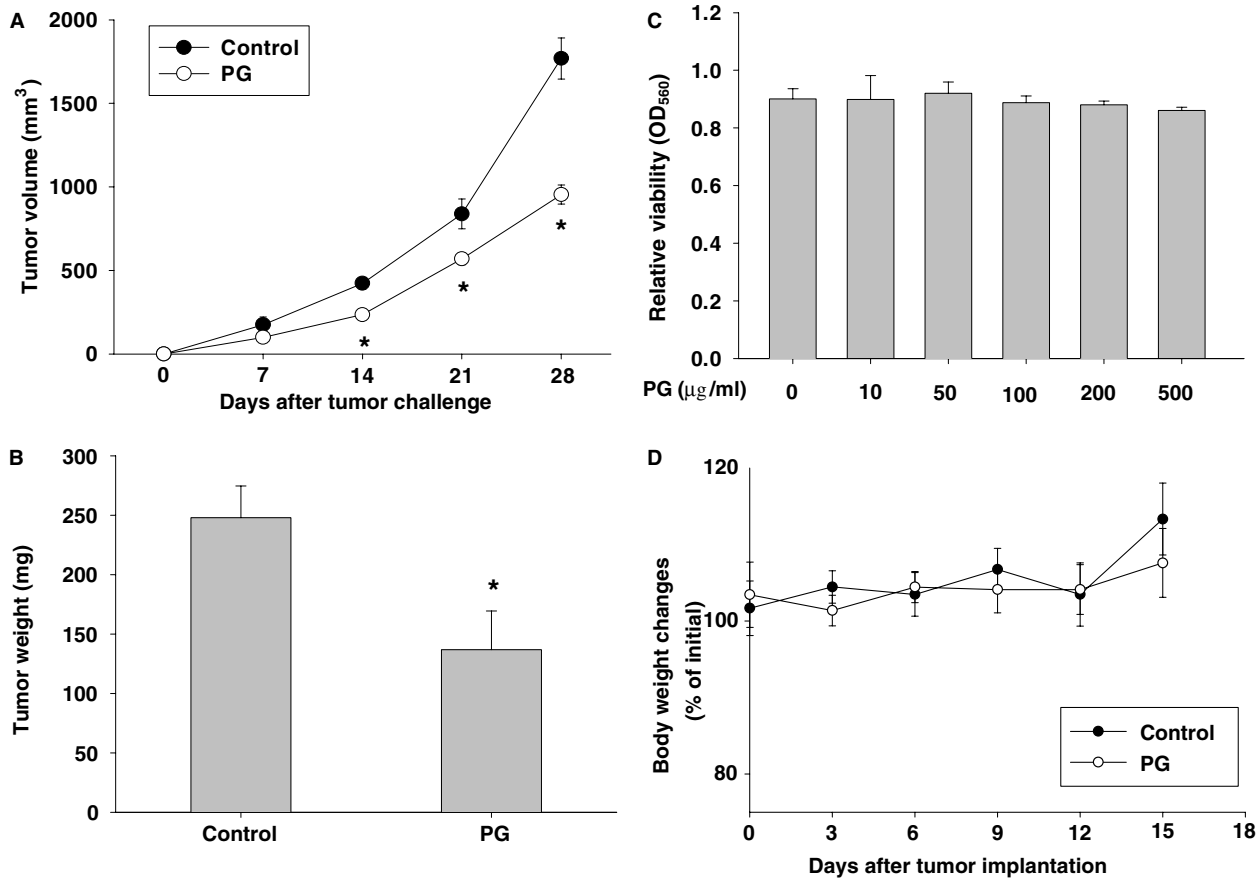

Fig. 1. Administration of in vivo PG inhibits the growth and increase of MCA-102 tumor cells. Mice were subcutaneously implanted with MCA-102 cells on day 0. PG was intraperitoneally administered at 100 mg/kg/day for 19 consecutive days (open circle). The mice of the control group were given saline (closed circle). Tumor size was measured from day 7 (A). The tumors were excised and weighed on day 20 (B). Assessment of cytotoxicity in PG-treated MCA-102 cells was incubated with various concentrations of PG (C). The body weight of mice was measured (D). The number of mice per group was five ($n = 5$). The significance was determined by Student's t test (*, $P < 0.05$).

3.2. PG induces the phenotypic and functional maturation of BM-DC via Toll-like receptors (TLR) 2 and 4

To evaluate whether PG influences the phenotypic maturation of DC, BM-derived immature DC from day-6 murine bone marrow cultures were cultured with PG or LPS (positive control) for 24 h and then analyzed for any surface expression of MHC class II or costimulatory molecule, CD86. LPS was included in these experiments as a positive control for DC maturation. An analysis of the cell surface markers demonstrated that most cells expressed CD11c (~84%), but not CD3 or CD19 (data not shown). Fig. 2A (thick lines) shows that PG, similarly to LPS, induced a dramatic upregulation of MHC II and costimulatory molecule, CD86, on the surface of $CD11c^+$ DC. The upregulation of these molecules was already observed after 6 h of stimulation and was not due to LPS contaminating the PG preparation, since PG did not show any effect in the presence of polymyxin B (PB), an LPS inhibitor (thin line). PB effectively inhibited the membrane expression of MHC II and costimulatory molecules, CD86, increased by LPS, but had no effect on the untreated- and PG-treated DC. Also, we analyzed the intracellular IL-12 p40/p70 production by PG-treated DC. As shown in Fig. 2B, intracellular staining of FITC-conjugated $CD11c^+$ DC with PE-conjugated IL-12 p40/p70 mAbs revealed that PG- or LPS-treated DC significantly enhanced IL-12 p40/p70 production compared to the control DC. Also, the increasing effect of PG treated DC on IL-12 p40/p70 expression was independent of LPS contamination because PB was strongly blocking IL-12 p40/p70 production in LPS-stimulated DC, but not in PG-treated DC. Additionally, to assess whether PG-induced maturation of DC is initiated through TLRs 2 and 4, we examined the effects of anti-TLR2 and anti-TLR4 Ab treatment on the PG-induced phenotypic maturation of DC to further characterize the membrane receptors of PG. As shown in Fig. 3, anti-TLR2 and anti-TLR4 significantly decrease the PG-induced CD86 and MHC class II expression, and IL12 p40/p70 production. Thus, these observations indicate that PG increases the costimulatory molecule CD86 and the MHC class II molecule, and that IL-12 production in DC in vitro and PG-induced phenotypic maturation of DC are possibly mediated by TLR 2 and 4.

3.3. PG administration induces phenotypic maturation of $CD11c^+CD8^+$ DC in lymphoid organs

To determine whether or not the administration of PG induces the phenotypic maturation of the $CD11c^+$ DC in tumor-bearing mice, the study investigated the costimulatory and MHC molecules of the $CD11c^+$ cells of the spleen and lymph nodes. As shown in Fig. 4, the PG administration was sufficient to increase the expression of CD80, CD86, MHC I, and MHC II in $CD11c^+$ cells in tumor-bearing mice. The mean

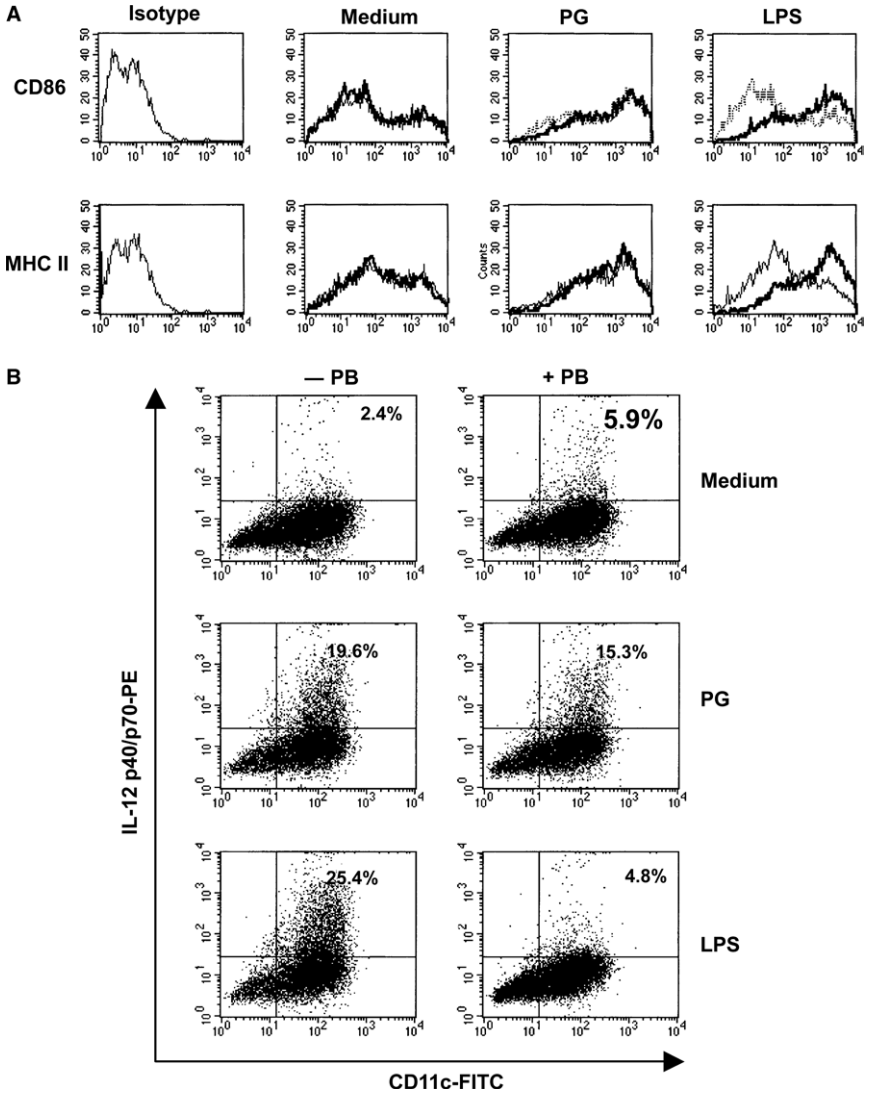

Fig. 2. PG enhances the expression of costimulatory and MHC class II molecules, and the intracellular IL-12 production of BM-DC. DC were generated as described in Section 2. On day 7, the cells were harvested and analyzed by two-color flow cytometry. The cells were gated on $CD11c^+$. The DC were stimulated for 24 h with 100 μg/ml PG or 200 ng/ml LPS on day 6 in the absence (thick line) or presence (thin line) of the LPS inhibitor, polymyxin B (PB) (A). Analysis of IL-12 p40/p70 expression in $CD11c^+$ DC by intracellular cytokine staining after PG or LPS stimulation (24 h) in the absence or presence of PB (B). The numbers indicate the percentages of $CD11c^+$ cells expressing IL-12 p40/p70. The histograms show fluorescence values on gated large cells. The data are from one representative experiment out of 3 performed.

fluorescence intensity (MFI) of the surface molecules of the $CD11c^+$ cells was stronger in the lymph node cells than in the splenic cells (Fig. 4A). The population of surface molecule-positive cells was unchanged in the splenic cells, compared with that in the control mice (Fig. 4B), although a strong MFI was shown. However, the PG-treated mice significantly increased the frequency (%) of $CD80^+$ and $CD86^+$ in the $CD11c^+$ cells in the lymph nodes, compared with the control mice (Fig. 4C). In addition, the effects of PG on $CD11c^+$ DC subclasses were investigated by a flow cytometric analysis. $CD8^+$ DC were reported to involve the activation and differentiation of Th cells by providing costimulatory signals, by which $CD8^+$ DC have been the development of Th-1 cells [21]. Accordingly, we investigated whether PG induces maturation of $CD8^+$ DC. As shown in Fig. 5, the frequency of $CD11c^+CD80^+CD8^+$ and $CD11c^+CD86^+CD8^+$ cells in the splenic and lymph node DC dramatically increased upon the administration of PG. These results suggest that activated $CD8^+$ DC are involved in priming the Th-1 subset to respond to PG for the inhibition of tumor growth.

3.4. PG administration increases the level of intracellular IL-12 and IFN-γ in $CD11c^+$ DC

Next, we investigated whether or not the administration of PG can induce the production of cytokines such as IL-12 and IFN-γ in $CD11c^+$ DC in tumor-bearing mice. As shown in Fig. 6, $CD11c^+$ DC from PG-treated mice dramatically enhanced the intracellular levels of IL-12 and IFN-γ in the spleen and lymph nodes. These results suggest that PG might enhance the ability of DC to produce some cytokines and develop the differentiation to the Th-1 cells of the $CD4^+$ T cells in the lymphoid tissues of tumor-bearing mice.

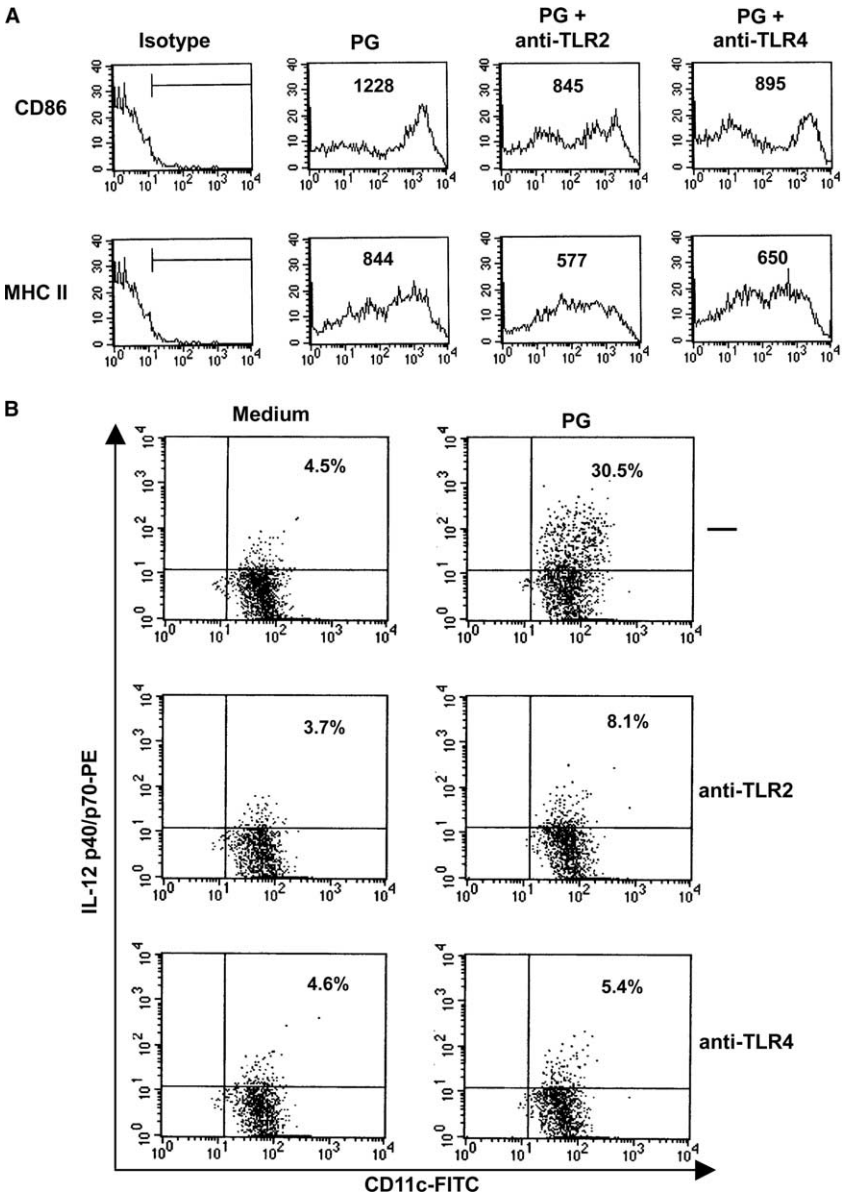

Fig. 3. PG induces the phenotypic and functional maturation of BM-DC via Toll-like receptors (TLR) 2 and 4 in vitro. DC were generated as described in Section 2. On day 7, the cells were harvested and analyzed by two-color flow cytometry. The cells were gated on $CD11c^+$. The DC were stimulated for 24 h with 100 μg/ml PG on day 6 in the absence or presence of anti-TLR 2 or anti-TLR 4 Abs (A). Analysis of IL-12 p40/p70 expression in $CD11c^+$ DC by intracellular cytokine staining after PG stimulation (24 h) in the absence or presence of anti-TLR 2 or anti-TLR 4 Abs (B). The numbers indicate the percentages of $CD11c^+$ cells expressing IL-12 p40/p70. The histograms show fluorescence values on gated large cells. The data are from one representative experiment out of 3 performed.

3.5. PG administration activates $CD4^+$ and $CD8^+$ T cells in vivo

Fig. 7 shows the population of $CD4^+$ T cells in the splenic cells and lymph node cells of C57BL/6 treated with PG. The populations of $CD3^+CD4^+$ in the control and PG treated mice were 12.7% and 34.2% in the spleen cells, and 24.1% and 45.8% in the lymph node cells, respectively (Fig. 7A). The $CD4^+$ cells proliferated in response to PG in a concentration-dependent manner. The $CD3^+CD4^+$ cell population for a half-dosage of PG (50 mg/kg) increased, respectively, by 23.4% and 35.7% in the splenic and lymph node cells (data not shown). The $CD3^+CD8^+$ cells of the spleen and lymph nodes treated with PG increased by 13.2% and 14.4%, respectively (Fig. 7B). These results suggest that PG increases antigen-presentation activities in $CD11c^+CD8^+$ DC.

We also investigated whether or not PG is linked to the CD28/B7 pathways related to T-cell activation via a non-specific signal resulting from the binding of the B7 ligand on the APC with its receptor, CD28, on the T cells. As shown in Fig. 4, PG increased the expression of the CD28 ligands, B7.1 and B7.2, on $CD11c^+$ DC in the spleen and lymph nodes. To investigate whether PG enhances the activation of DC in priming antigen-specific Th cells and CTL in vivo, the expression of CD28 on T cells, which bind B7 costimulatory molecules to DC, was examined by flow cytometryic analysis. PG dramatically enhanced $CD4^+$ and $CD8^+$ T cell subpopu-

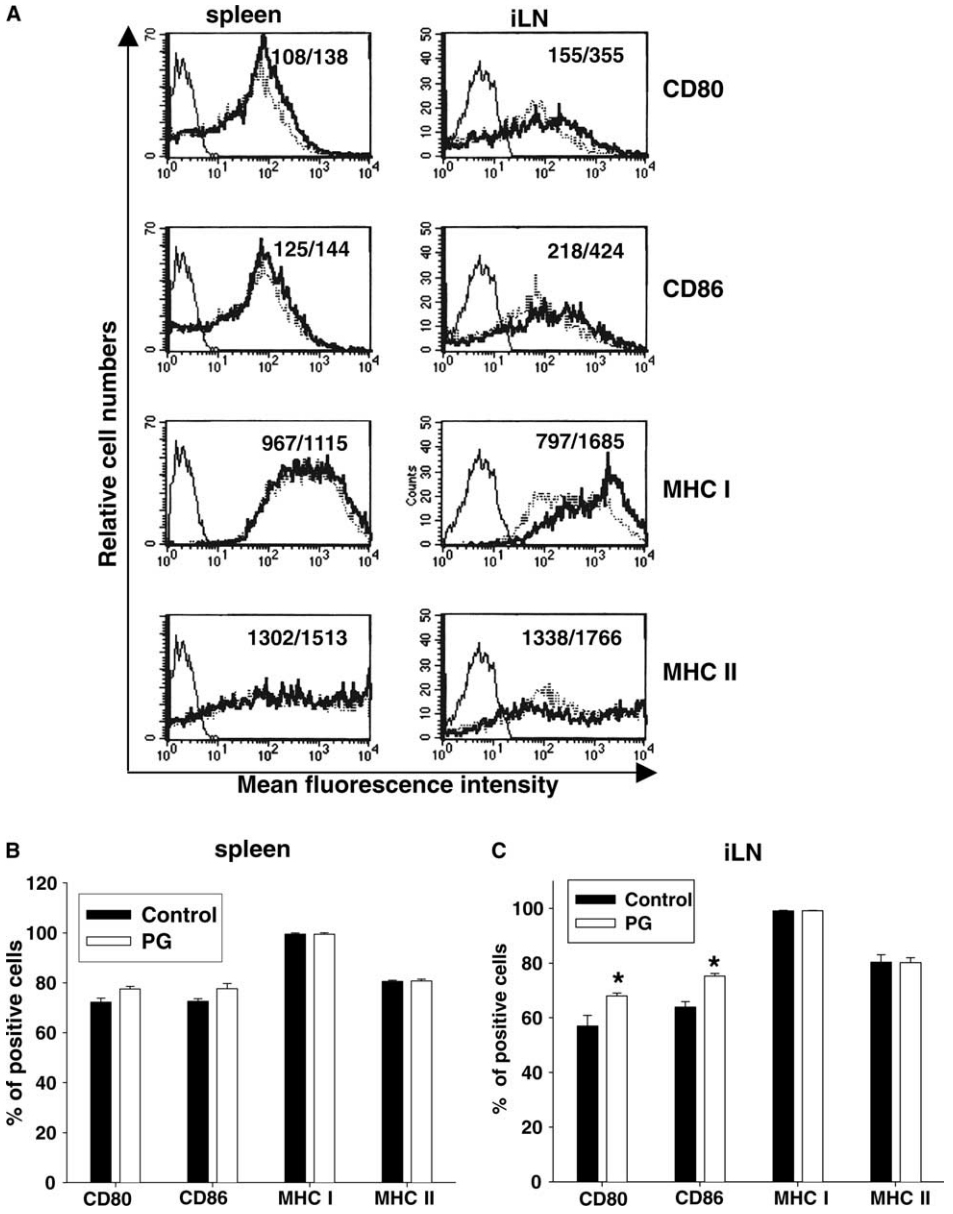

Fig. 4. Administration of in vivo PG increases the expression of costimulatory (CD80 and CD86) and MHC (I and II) molecules on the CD11c$^+$ DC of the spleen and lymph nodes in tumor-bearing mice. On day 20, the cells were harvested and analyzed by two-color flow cytometry. The cells were gated on CD11c$^+$. The mean fluorescence intensity (MFI) of the CD11c$^+$ cells expressing Ag is indicated within each histogram (control MFI/PG-treated MFI: (A) Unstimulated CD11c$^+$ DC (dashed lines) were compared with PG-stimulated CD11c$^+$ DC (thick lines). Thin lines represent isotype controls. The populations of CD11c$^+$ expressing the Ag are depicted in the spleen (B) and lymph node cells (C). The histogram is from one representative experiment out of three performed. The significance was determined by Student's t test (*, $P < 0.05$). iLN, inguinal lymph nodes.

lations, which bind B7 molecules to DC (Table 1). Compared to the level in the control mice, PG induced CD28 expression on CD4$^+$ T cells up to 2-fold and on CD8$^+$ T cells up to 3-fold. These results indicate that PG enhanced CD4$^+$ T cell and CD8$^+$ T cell-generation of tumor-rejection immunity in vivo.

3.6. PG administration modulates Th-1 polarization

IL-12 has multiple immunoregulatory functions, inducing the activation of the Th-1 subset, which plays a pivotal role in the induction of in vivo anti-tumor immunity [20]. PG administration increased IL-12 production on CD11c$^+$ DC in the spleen and lymph nodes (Fig. 6). Therefore, we attempted to examine the production of Th-1- and Th-2-type cytokines in the spleen and lymph node cells of tumor-bearing mice treated with PG. The T cells of the spleen and lymph nodes of the PG-treated mice showed a significant enhancement of intracellular IFN-γ and a decrease in intracellular IL-4 production as compared with those in the control mice (Fig. 8). Thus, the findings indicate that PG administration induces the production of IL-12 and IFN-γ in CD11c$^+$ DC in tumor-bearing mice.

3.7. Combination therapy of PG and tumor lysate-pulsed (TP) DC completely inhibits the growth MCA-102 cells

As shown in Fig. 9, C57BL/6 mice were once administered s.c. through 1×10^6 tumor lysate-pulsed DC (TP-DC) or unpulsed

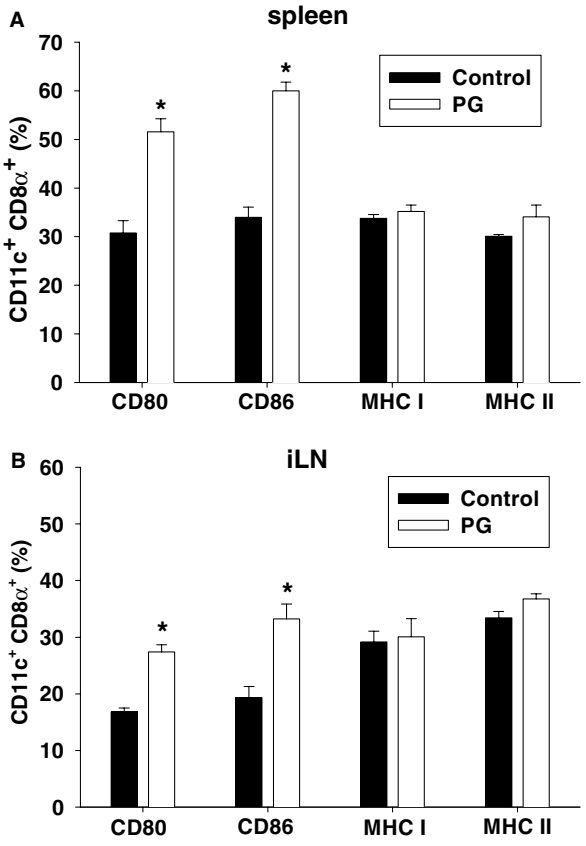

Fig. 5. Administration of PG in vivo increases the population of $CD8^+$ on $CD11c^+$ surface molecules$^+$ DC in tumor-bearing mice. On day 20, the cells were harvested and analyzed by three-color flow cytometry. The cells were gated in $CD11c^+$ $CD80^+$/$CD86^+$/MHC I^+/MHC II^+, and $CD8^+$ cells were measured on the double positive cells in the spleen (A) and lymph nodes (B). The significance was determined by Student's t test (*, $P < 0.05$). iLN: inguinal lymph nodes.

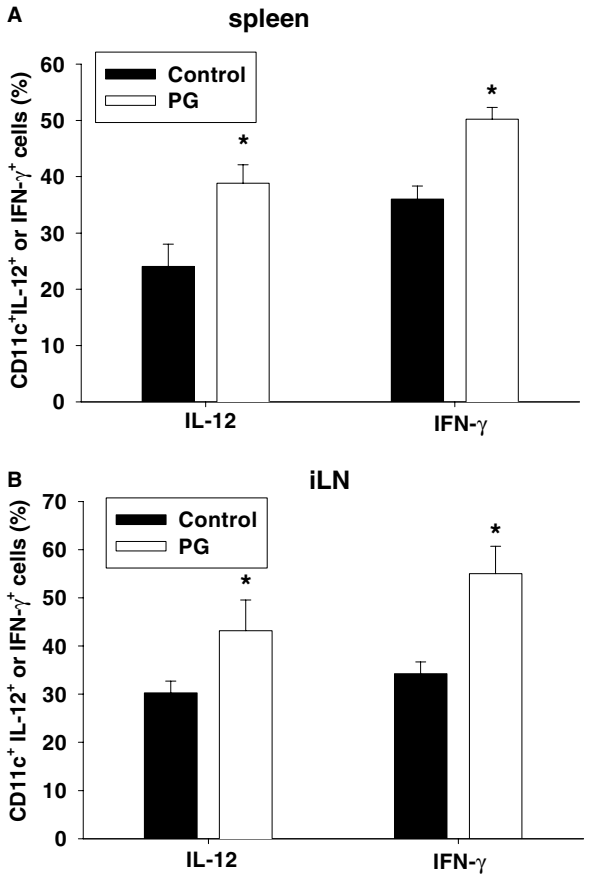

Fig. 6. Administration of PG in vivo induces a large population of IL-12^+ and IFN-γ^+ cells on $CD11c^+$ DC in tumor-bearing mice. On day 20, the cells were harvested and analyzed by two-color staining for flow cytometry. Analysis of IL-12 and IFN-γ expressions in $CD11c^+$ DC by intracellular cytokine staining in the spleen (A) and lymph node cells (B) after PMA and ionomycin (3 h). The results are representative of three separate experiments. The significance was determined by Student's t test (*, $P < 0.05$). iLN: inguinal lymph nodes.

DC (Normal-DC). One week later, 1×10^6 MCA-102 tumor cells were i.d. inoculated into the left flank. The inoculation with normal-DC failed to decrease the tumor growth. After inoculation with TP-DC in the absence or presence of PG treatment for 19 consecutive days, tumor growth was delayed in all TP-DC immunized groups. No tumor formation was observed in the TP-DC + PG on day 28 of the post-tumor challenge. Moreover, three out of five mice in the TP-DC + PG group achieved complete rejection by day 60 after the MCA-102 tumor challenge (data not shown). These results suggest that the vaccine efficiency of combination therapy completely inhibited tumor growth through the activation of $CD4^+$ Th-1 cells and $CD8^+$ cytotoxic T cells. Antigen-specific DC expressing MHC and costimulatory molecules in PG administration dramatically induced the activation of $CD4^+$ and $CD8^+$ T cells.

4. Discussion

Our study demonstrated that the treatment of PG significantly inhibited the growth of MCA-102 tumor cells in mice without causing any adverse effects such as weight loss, indicating that PG might prevent the growth of MCA-102 tumor cells (Fig. 1). PG administration resulted in a marked augmentation of costimulatory and MHC molecules, as well as the production of pro-inflammatory cytokines, by $CD11c^+CD8^+$ DC in tumor-bearing mice. Notably, enhanced IL-12 and IFN-γ production in $CD11c^+$ DC resulted in an increased ability to induce IFN-γ and a decreased ability to induce IL-4 in $CD4^+$ T cells. These results also suggest that PL administration strongly activates DC loaded with tumor antigen, inducing the activation of anti-tumor protective immunity in $CD4^+$ Th-1 cells and cytotoxic $CD8^+$ T cells.

In a previous study, PG strongly stimulated both cell-mediated and humoral immunity in vivo and in vitro [13]. PG activated most immune cell types such as natural killer cells, macrophages, T cells, and B cells. From these results, it is clear that PG increases the non-specific host defenses that play an important role in eliminating tumor cells and the action profiles of PG might be similar to those of IL-2, one of the well known immunotherapeutics [12,13]. PG can be recommended as an improved candidate over immunochemotherapy with IL-2 [12]. However, the anti-tumoral mechanisms of PG, particularly those in the maturation of DC, have not yet been found. Our study demonstrated that PG induced the phenotypic and functional maturation of BM-derived DC in vitro. Thus, we also investigated whether or not the administration of PG activated $CD11c^+$ DC in tumor-bearing mice.

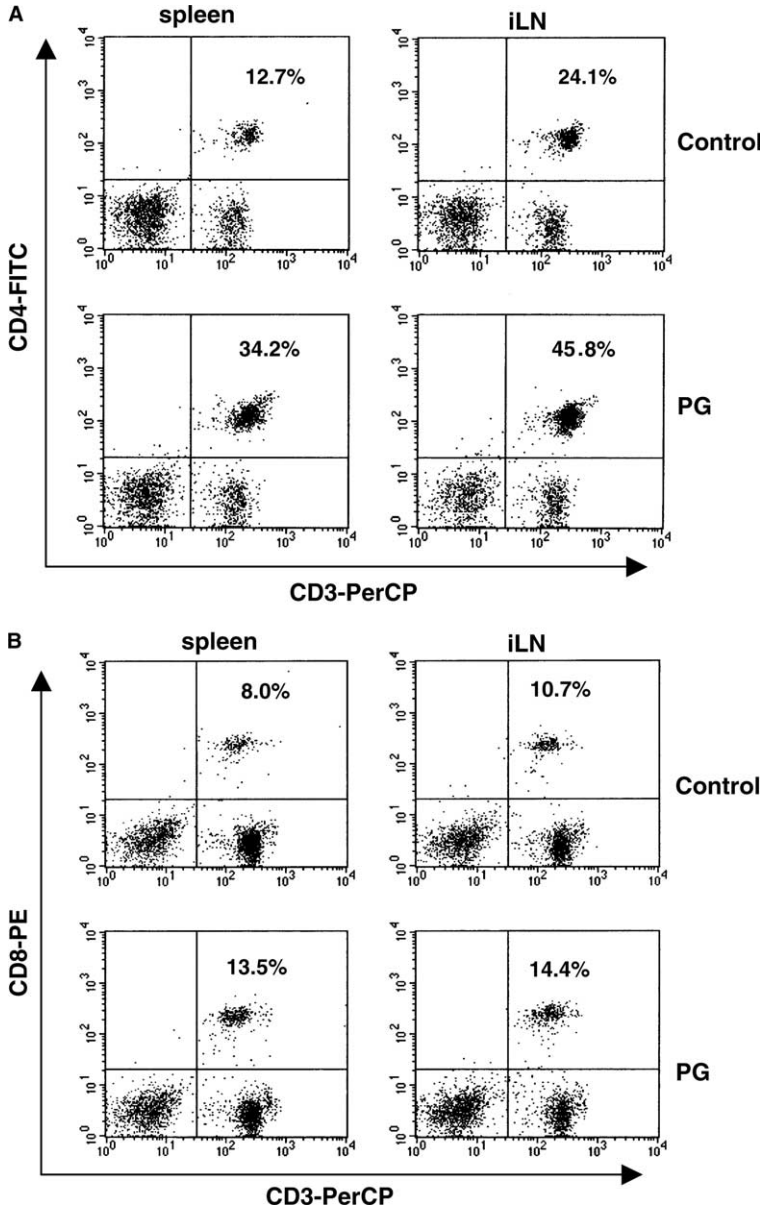

Fig. 7. Administration of PG in vivo induces the activation of $CD4^+$ and $CD8^+$ T cells in the spleen and lymph nodes of tumor-bearing mice. On day 20, the cells were harvested and analyzed by three-color flow cytometry. The incidence of $CD4^+$ (A) and $CD8^+$ (B) T cells within the $CD3^+$ cells in tumor-bearing mice was analyzed. The results are representatives of three separate experiments. iLN: inguinal lymph nodes.

Table 1
Effect of PG on T cell activation in tumor-bearing mice

	Control-mice		PG-mice	
	spleen (%)[a]	iLN (%)	spleen (%)	iLN (%)
$CD4^+CD28^+$	5.3 ± 0.8	4.3 ± 1.2	9.3 ± 2.3[b]	9.8 ± 1.1[b]
$CD8^+CD28^+$	0.9 ± 0.2	1.3 ± 0.4	2.5 ± 0.6[b]	4.4 ± 0.8[b]

Data represent means ± SD of three different experiments.
[a] % of positive cells was determined by flow cytometric analysis.
[b] $P < 0.05$, compared with control-mice (Student's t test).

DC are potent antigen-presenting cells capable of capturing antigens and processing and presenting antigenic peptide fragments. Mature DC cells can migrate to lymphoid organs and enhance to prime T cells [2,22]. Due to these properties, DC are considered to be promising in cancer immunotherapy. Also, based on their ability to induce Th-1 versus Th-2 polarization, mature DC have been called DC1 or DC2, respectively [23]. Myeloid DC give rise to DC1 or DC2, depending on the nature of the maturation stimuli influencing IL-12 production [24–27]. Also, IL-12, produced by DC, macrophages, and other antigen-presenting cells, is known to have potent anti-tumor activities [28,29]. IFN-γ appears to be the most potent DC-1-promoting factor and is a cytokine that plays an important role in inducing and modulating a cascade of immune responses [25,28,30]. Recent studies have indicated that the ratio of Th-1 to Th-2 subsets is closely correlated with the outcome of many diseases [31,32], and controlling this Th-1 to Th-2 ratio has been demonstrated as a therapeutic strategy [33]. Notably, the induction of Th-1 immune

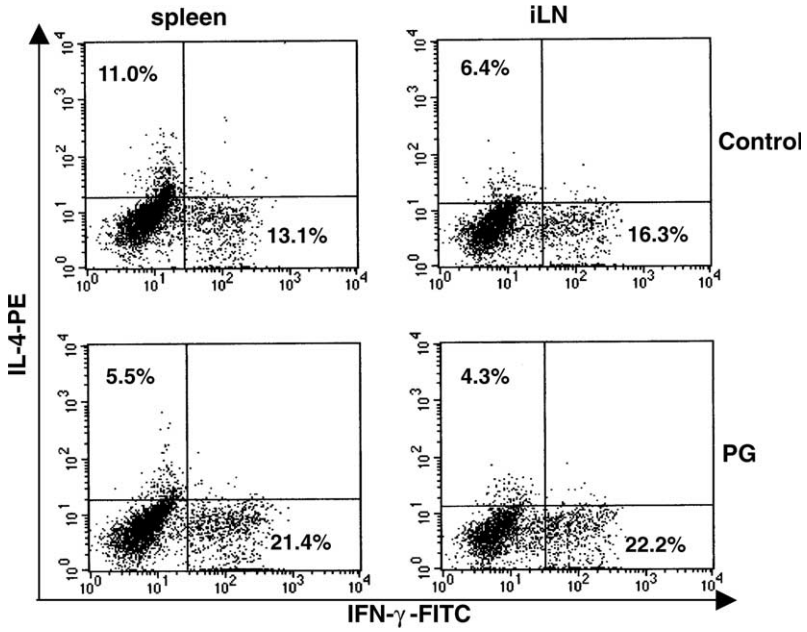

Fig. 8. PG administration in vivo modulates Th-1 type polarization. On day 20, the cells were harvested and analyzed by three-color flow cytometry. The cells were gated on $CD3^+$ T cells. Analysis of intracellular IL-4 and IFN-γ productions in $CD3^+$ T cells by intracellular cytokine staining in spleen and lymph nodes after treatment of PMA and ionomycin (3 h). The results are representatives of three separate experiments. Significance was determined by Student's t test (*, $P < 0.05$). iLN: inguinal lymph nodes.

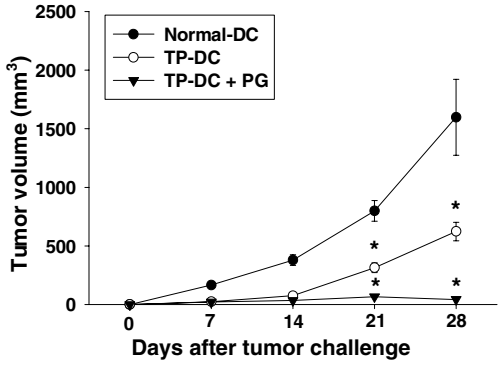

Fig. 9. Combination of PG-based chemotherapy and tumor lysate-pulsed DC (TP-DC) completely inhibits the growth of MCA-102 cells. C57BL/6 mice were immunized once subcutaneously with 1×10^6 DC of TP-DC or unpulsed DC (Normal-DC). One week later, 1×10^6 MCA-102 tumor cells were intradermally inoculated into the left flank. The size of the growing MCA-102 tumor cells was measured every 7 days using microcalipers. Each group consisted of five mice ($n = 5$). The significance was determined by Student's t test (*, $P < 0.05$).

responses plays a critical role in protecting against various types of tumors [34]. The administration in vivo of recombinant IL-12 induced a profound but transient commitment to Th-1-associated patterns of cytokine and antibody production [35]. The repeated injection of recombinant IL-12 at relatively high doses showed severe toxicities, including an increase in transaminase concentration, pulmonary toxicity, and leukopenia [36]. Therefore, it might be better to use activated and mature DC in clinical applications. Based on the results of this study, which found that the administration of PG in vivo stimulates the production of IL-12 and the expression of surface molecules on DC, PG can augment the maturation of DC. This suggests that PG could be used as a novel stimulatory adjuvant of DC in vivo, instead of, or in addition to, TNF-α, CD40 ligand, or LPS.

In summary, this study found that the administration of PG induced anti-tumor and immunomodulating activities via maturation of $CD11c^+CD8^+$ DC in tumor-bearing mice. The inhibitory effect of PG on the growth of MCA-102 tumor cells is associated with its immunoregulatory properties, including the induction of IL-12 and IFN-γ production leading to a Th-1 dominant state. These effects were made more pronounced by the substantial expression of surface molecules. Therefore, PG would be useful in preventing tumor growth, and it also has the advantage of having no side effects. Attempts to clarify the mechanism responsible for inhibitory effects of PG on tumor growth are now under study.

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