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International Immunopharmacology 1 (2001) 1969–1978

International Immunopharmacology

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Polysaccharide isolated from the radix of *Platycodon grandiflorum* selectively activates B cells and macrophages but not T cells

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Received 20 April 2001; received in revised form 29 May 2001; accepted 17 June 2001

Abstract

Many polysaccharides isolated from plants are considered to be biological response modifiers and have been shown to enhance various immune responses in vivo and in vitro. Here, we demonstrate that polysaccharide isolated from the radix of *Platycodon grandiflorum* (PG) has a unique mode of immunostimulation with regard to its cell-type specificity. PG was found to markedly increase polyclonal IgM antibody production and the proliferation of B cells, and to activate iNOS transcription and NO production in macrophages. Moreover, the intraperitoneal administration of PG in mice resulted in increased IgM antibody production in B cells, which were immunized by using T-dependent antigen sheep red blood cells (sRBCs). However, PG did not affect the proliferation of T cells, the IL-2 expression of Th1 cells, or the IL-4 expression of Th2 cells. Although PG and lipopolysaccharide (LPS) had a similar mode of action in B cells and macrophages, they were differentiated by the fact that PG-induced cellular activation was not inhibited by polymyxin B, a specific inhibitor of LPS. Anti-CD19 or anti-CD79b antibody blocked B cell proliferation and anti-CD14 or anti-CD11b antibody decreased macrophage NO production, indicating the possible cellular binding sites of PG. Our results demonstrate that PG is a specific activator of B cells and macrophages but not of T cells, and suggest that PG is quite distinct from other well-known immunostimulants, such as lentinan and schizophyllan, which mainly act upon macrophages and T cells.

Keywords: *Platycodon grandiflorum*; Polysaccharide; B cells; Macrophages; T cells

Abbreviations: AFC, antibody forming cell; BCR, B cell receptor; cDNA, complementary deoxyribonucleic acid; Con A, concanavalin A; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; mRNA, messenger ribonucleic acid; NO, nitric oxide; PFC assay, plaque forming cell assay; PG, *Platycodon grandiflorum*; PMB, polymyxin B; RNA, ribonucleic acid; RT-PCR, reverse transcription-polymerase chain reaction; SH assay, suspension hemolytic assay; SPF, specific pathogen free; sRBC, sheep red blood cell; Th cells, helper T cell.

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1567-5769/01/$ - see front matter
PII: S1567-5769(01)00124-2

1. Introduction

During the past three decades, many polysaccharides and polysaccharide–protein complexes have been isolated from mushrooms, fungi, yeasts, algae, lichens and plants. The biological activities of these polysaccharides have attracted more attention recently in the biochemical and medical areas because of their immunomodulatory and antitumor effects [1]. The search for novel polysaccharides with antitumor properties stems from the basic shortcomings of existing cancer chemotherapy and radiotherapy. In particular, the great majority of chemical compounds, which have been identified as cytotoxic to cancer cells, are also toxic to normal cells [2,3]. Hence, the discovery and identification of new safe drugs, without severe side effects, has become an important goal of research in the biomedical sciences. The enhancement or potentiation of host defense mechanisms has been recognized as a possible means of inhibiting tumor growth without harming the host, and from this standpoint, extensive study has been undertaken on polysaccharides isolated from natural sources. Recently, lentinan, schizophyllan and krestin have been accepted as immunoceuticals in several oriental countries [3,4]. More recently, our group isolated polysaccharides from *Phellinus linteus* and *Angelica gigas* Nakai, and demonstrated their antitumor and immunomodulatory activities [2,5–7]. Specifically, these polysaccharides were found to increase the survival time of tumor-bearing mice, without showing cytotoxicity to B16F10 melanoma cells [5,8].

Platycodon grandiflorum (Campanulaceae) has been used traditionally as an expectorant and as a remedy for bronchitis, tonsillitis, laryngitis and suppurative dermatitis. Moreover, it has been reported that the radix of *P. grandiflorum* prevents obesity, hypercholesterolemia, hypertension, diabetes, and hyperlipidemia [9–14]. Recently, saponin, one of the major components of *P. grandiflorum*, was shown to inhibit the intestinal absorption of dietary fat by inhibiting pancreatic lipase-induced fat hydrolysis [13]. In the present study, we investigated the effects of a polysaccharide isolated from *P. grandiflorum* on the immune system, especially at the cellular level, and demonstrated that this polysaccharide selectively activated B cells and macrophages but not T cells.

2. Methods and methods

2.1. Materials

Specific pathogen free (SPF) BDF1 mice (female, 5–7 weeks old) were obtained from DaeHan Biolink (Chungbuk, Korea), and maintained under SPF conditions until used. RPMI 1640 medium was purchased from GIBCO (Grand Island, NY, USA). Antibodies against CD11b, CD14, CD19, CD38, and CD79b were purchased from PharMingen (San Diego, CA). The roots of *P. grandiflorum*, which had been cultivated for 20 years, were kindly provided by Sungho Jang Saeng Doragi, Korea. The dried root of *P. grandiflorum* (250 g) was extracted with hot water for 4 h, and the extract filtered, and subjected to Diaion HP-20 chromatography (Mitsubishi Chemical, Japan). The eluant so obtained, was lyophilized, treated with a solvent mixture of H_2O/acetone (1:5), and centrifuged to obtain the H_2O/acetone-insoluble fraction (64 g). This light yellow powder was identified as an inulin-type polysaccharide, which was named PG. It has previously been reported that the polysaccharide isolated from the aqueous extracts of the radix of *P. grandiflorum* is an inulin-type polyfructose with a $\beta(2 \rightarrow 1)$ linked D-fructose [13,15]. PG will be made available to qualified investigators who may be interested in utilizing this material.

2.2. Proliferation assay

Cellular proliferation was determined, as described previously [5]. B and T cells were fractionated from whole spleen cell suspensions, which were freed of red blood cells by treating them with lysis buffer (0.15 M NH_4Cl, 0.01 M $KHCO_3$, and 0.1 mM Na_2EDTA, pH 7.4). To remove adherent cells, such as macrophages, the spleen cells were incubated for 1 h in petri dishes at 5×10^6 cells/ml. B and T cells were prepared using the nylon–wool methods according to the manufacturer's instructions (Polysciences, Warrington, PA). Cells were cultured at

$1–5 \times 10^6$ cells/ml in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), glutamine and 2-mercaptoethanol (Sigma, St. Louis, MO). PG was added at concentrations of 1–100 μg/ml on day 0. Specific lymphocyte mitogens, such as concanavalin A (Con A, T cell mitogen) and lipopolysaccharide (LPS, B cell mitogen) were used for reference purposes at a final concentration of 5 μg/ml. Cells were pulsed with 1 μCi/well of ^{3}H-thymidine (113 Ci/nmol, NEN, Boston, MA) for the last 18 h of the 3-day incubation and harvested with an automated cell harvester (Inotech, Dottikon, Switzerland). The amount of ^{3}H-thymidine incorporated into the cells was measured using a Wallac Microbeta scintillation counter (Wallac, Turku, Finland) [16].

2.3. Antibody production of B cells

Elevated IgM levels were used to determine polyclonal B cell activation. Spleen cells were cultured with PG (1–100 μg/ml) for 3 days. The IgM level was determined by suspension hemolytic (SH) assay and antibody forming cells (AFCs) were counted using the plaque forming cell (PFC) assay, as described previously [5,17]. The SH assay was performed by mixing immunized cells (100 μl) with complement (8 μl) and trinitrophenyl-conjugated sheep red blood cells (sRBC, 25 μl) and incubating at 37 °C in a water bath for 1 h. The amount of hemoglobin released from the sRBCs was measured at 540 nm. The PFC assay involved mixing immunized cells with complement, trinitrophenyl-conjugated sRBC, and agarose (350 μl), plating the mixture on a petri dish and incubating for 2 h in a CO_2 incubator. The number of plaques was counted and expressed as AFCs/10^6 cells. The immunomodulatory effect of PG in vivo was investigated using a model of T-dependent immunization and specific IgM antibody production [2]. Briefly, BDF1 mice were immunized with sRBCs on day 0 and PG was injected intraperitoneally at doses ranging from 10 to 100 mg/kg/day from day 0 to day 3. On day 4, PFC assay was performed on spleen cells.

2.4. Cytokine gene expression

Spleen cells were cultured in the presence or absence of PG for 24 h and total RNA was extracted using an Ultraspec II RNA isolation Kit (Biotech Lab., Houston, TX). RT-PCR was then performed to determine changes in cytokine gene expression, as described previously [5]. Briefly, cells were lysed by vigorous pipetting in Ultraspec II RNA (1×10^7 cells/ml). Reverse transcription (RT) of the RNA was performed using a GeneAmp RNA PCR kit with 100 ng of total cellular RNA (Perkin Elmer, Branchburg, NJ, USA). The mixture was incubated for 1 h at 37 °C, and for 5 min at 99 °C. PCR was then carried out with 2.5 units of AmpliTaq DNA polymerase and 10 pmol of cytokine primers for IL-2 and IL-4, in the same tube. The sequences of the primers used were as follows: for IL-2, sense 5′-CTT GCC CAA GCA GGC CAC AG-3′, antisense 5′-GAG CCT TAT GTG TTG TAA GC-3′; for IL-4, sense 5′-GAA TGT ACC AGG AGC CAT ATC-3′, antisense 5′-CTC AGT ACT ACG AGT AAT CCA-3′. PCR was performed in a Bio-Rad Cycler (Bio-Rad Lab., Richmond, CA, USA) starting with a 105-s incubation step at 95 °C, then 30 cycles of: − 30 s at 94 °C, 1 min at 55 °C, and 2 min at 72 °C, followed by a 7-min incubation step at 72 °C to complete the polymerization. PCR products were electrophoresed on a 3% Nusieve 3:1 agarose gel and photographed after staining with ethidium bromide.

2.5. Nitrite quantification and iNOS gene expression

Peritoneal macrophages were isolated from the abdominal cavity and grown in RPMI 1640 supplemented with, 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were plated and incubated in the presence of 5% CO_2 at 37 °C. NO_2^- accumulation was used as an indicator of NO production in the medium, as previously described [6,7]. Cells were plated at 5×10^5 cells/ml in 24-well culture plates and stimulated with PG for 24 h. The isolated supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid) and incubated at room temperature for 10 min. Nitrite production was determined by measuring absorbance at 540 nm versus an $NaNO_2$-based standard curve. iNOS gene expression level was determined by RT-PCR using the primers: sense 5′-CTG CAG CAC TTG GAT CAG GAA CCT G-3′ and antisense 5′GGG AGT AGC CTG TGT GCA CCT GGA A-3′.

The relative ratios of iNOS to β-actin mRNA were calculated from the band areas obtained using an image analysis system (Multi-Analyst, Bio-Ras, CA).

3. Results

3.1. PG increased the functions of B cells, but not T cells

As a first step towards understanding the immunomodulatory activity of PG, we investigated the effect of PG on lymphocyte proliferation. As shown in Fig. 1, PG was found to increase the proliferation of unfractionated spleen cells in a dose dependent manner. PG at 100 μg/ml increased cellular proliferation 44.5-fold. We then investigated the cell-type specificity of PG using fractionated T and B cells, and found that PG selectively increased the proliferation of B cells, but not the proliferation of T cells (Fig. 2A). After PG stimulation at 100 μg/ml, B cell proliferation was found to be five times that of the T cells. The mode of action of PG was then compared with other lymphocyte mitogens, such as LPS and Con A. Fig. 2B shows the characteristic action profiles of LPS and Con A, which are well known B and T cell mitogens, respectively. In particular, PG like LPS was found to have a mitogenic effect on B cells, but not on T cells. Although, at 100 μg/ml, PG increased the proliferation of fractionated T cells, this might have resulted from the effect of PG on the small proportion of contaminating B cells present.

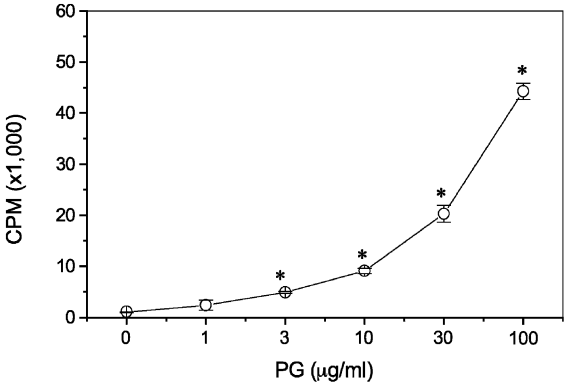

Fig. 1. The effect of PG on the proliferation of whole spleen cells. PG was added at concentrations from 1 to 100 μg/ml to whole spleen cells for 72 h. After incubation, the degree of lymphocyte proliferation was measured by incorporating ^{3}H-thymidine into the cellular DNA. Significance was determined using the Student's *t*-test versus the control group ($^{*}p < 0.01$).

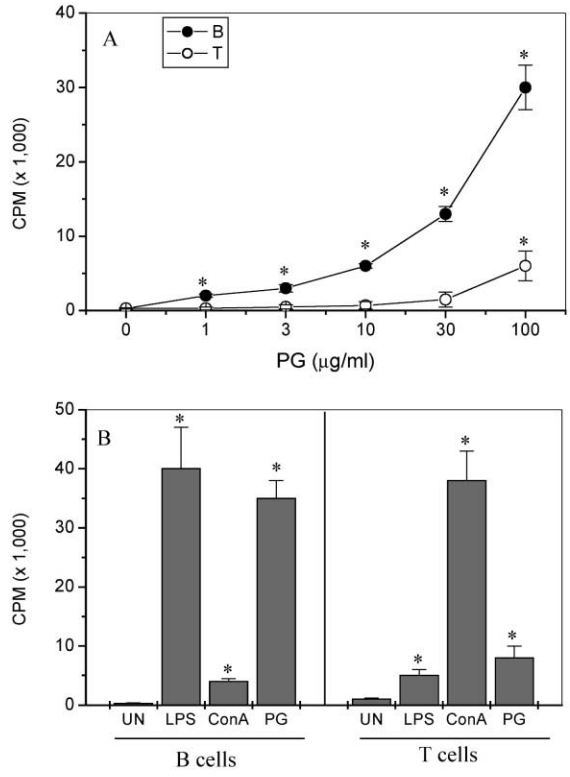

Fig. 2. The effect of PG on the proliferation of fractionated B and T cells. PG at concentrations from 1 to 100 μg/ml was added to fractionated B or T cells (A). The mitogenic pattern of PG (100 μg/ml) was compared to those of the reference mitogens, LPS (B cell mitogen, 5 μg/ml) and Con A (T cell mitogen, 5 μg/ml) (B). After incubating for 72 h, the degree of lymphocyte proliferation was measured by incorporating ^{3}H-thymidine into the cellular DNA. Significance was determined using the Student's *t*-test versus the control group ($^{*}p < 0.01$). UN, chemically untreated control group.

The effects of PG on B cell functions were further examined by determining the increased level of polyclonal IgM antibody production in B cells, and PG was found to activate B cells into antibody producing plasma cells (Fig. 3A and B). The effects of PG on Th cells were then investigated to confirm our previous results, i.e., that PG does not activate T cells. T cells are characterized as either CD8+ or CD4+ T cells on the basis of their functional capabilities and cytokine profiles. Among these,

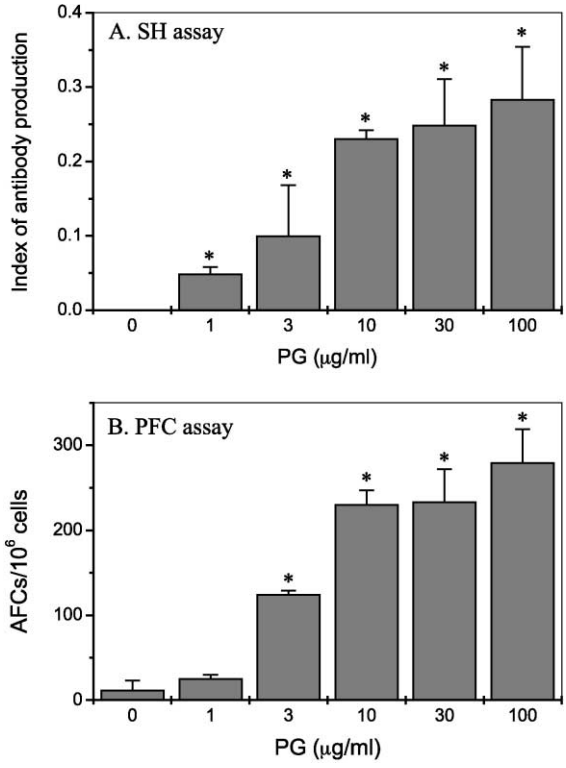

Fig. 3. The effect of PG on the in vitro polyclonal IgM antibody production of B cells. Spleen cells were activated with PG at concentrations of 1–100 μg/ml for 3 days, and polyclonal IgM antibody production by B cells was determined by SH (A) and PFC assay (B). Significance was determined using Student's *t*-test versus the control group ($^{*}p < 0.01$).

CD4 + Th1 cells secrete cytokines, such as IL-2, and are associated with cell-mediated immune responses, and CD4 + Th2 cells produce IL-4, which

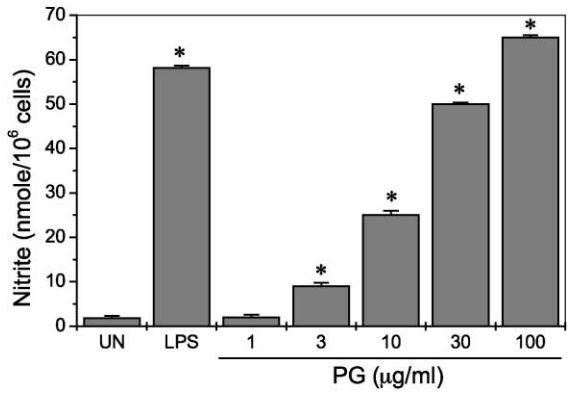

Fig. 5. The effect of PG on macrophage nitrite production. PG (1–100 μg/ml) and LPS (0.2 μg/ml) were added to peritoneal macrophages (5×10^5 cells/ml) for 24 h, respectively, and the supernatant nitrite levels determined. Significance was determined using the Student's *t*-test by comparison with the chemically untreated control ($^{*}p < 0.01$). UN, chemically untreated control group.

helps B cells proliferate and differentiate. In the present study, splenic lymphocytes were incubated with PG for 24 h and the expressions of IL-2 and IL-4 mRNAs were determined. As shown in Fig. 4, the mRNA expressions of IL-2 and IL-4 were not altered by the PG treatment, whereas Con A, used as a reference T cell activator, increased the gene expressions of IL-2 and IL-4. In summary, these results suggest that PG selectively activates B cells, but does not affect Th cells.

3.2. PG increased macrophage functions

We also examined the effect of PG on macrophages since macrophages are known to be

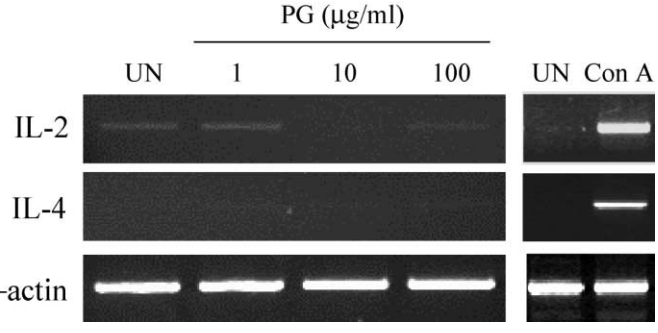

Fig. 4. The effect of PG on IL-2 cells and IL-4 expression in splenocytes. Spleen cells were incubated with PG (1–100 μg/ml) or Con A (5 μg/ml) for 24 h. After incubation, total RNA was isolated and the mRNA expression levels of IL-2 and IL-4 were determined by RT-PCR. UN, chemically untreated control group.

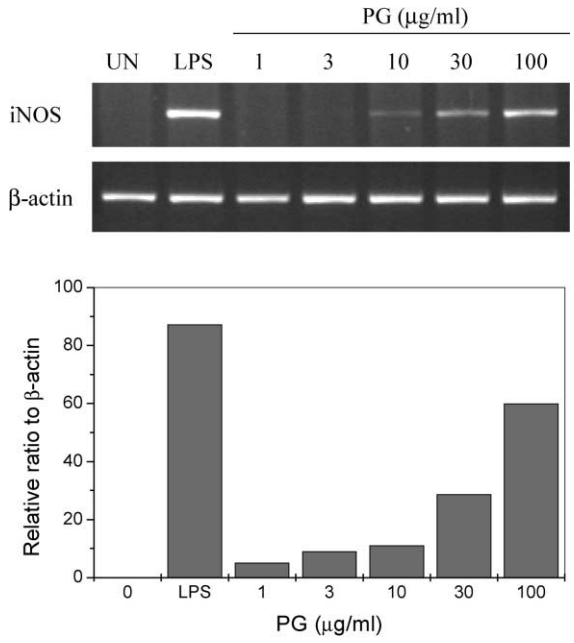

Fig. 6. The effect of PG on macrophage iNOS gene expression. PG (1–100 μg/ml) and LPS (0.2 μg/ml) were added to macrophages (5×10^5 cells/ml) for 24 h, respectively. Total RNAs were isolated and the iNOS mRNA expressions determined by RT-PCR. After analyzing the band areas using an image analysis system (Multi-Analyst), the relative ratios of iNOS to β-actin mRNA were calculated. The result shown is representative of three separate experiments. UN, chemically untreated control group.

one of the target cells of polysaccharides. The stimulation of murine macrophages by LPS resulted in the expression of an inducible NO synthase (iNOS), which catalyzed the production of large amounts of NO from L-arginine and molecular oxygen [18]. In the present study, the basal level of nitrite in unstimulated peritoneal macrophages was 2 nmol/10^6 cells (Fig. 5). After PG stimulation at 3, 10, 30, and 100 μg/ml, nitrite synthesis increased in peritoneal macrophages to 9, 25, 50, and 65 nmol/10^6 cells, respectively. RT-PCR confirmed that the expression level of the iNOS gene was increased dose-dependently (Fig. 6).

3.3. PG and LPS had different properties

Since PG and LPS were found to activate B cells and macrophages in a similar manner, the modes of action of PG and LPS were compared using polymyxin B (PMB), a selective inhibitor of LPS action. PMB is known to inhibit the LPS-induced activation of B cells and macrophages by binding the lipid A moiety of LPS [19]. As shown in Fig. 7A and B, PMB abolished the LPS-induced B cell proliferation and macrophage NO production, whereas it did not inhibit the action of PG on B cells and macrophages.

3.4. PG might bind with the membrane receptors expressed on B cells or macrophages

To investigate membrane receptors of PG, B cells were preincubated in the presence of anti-CD19,

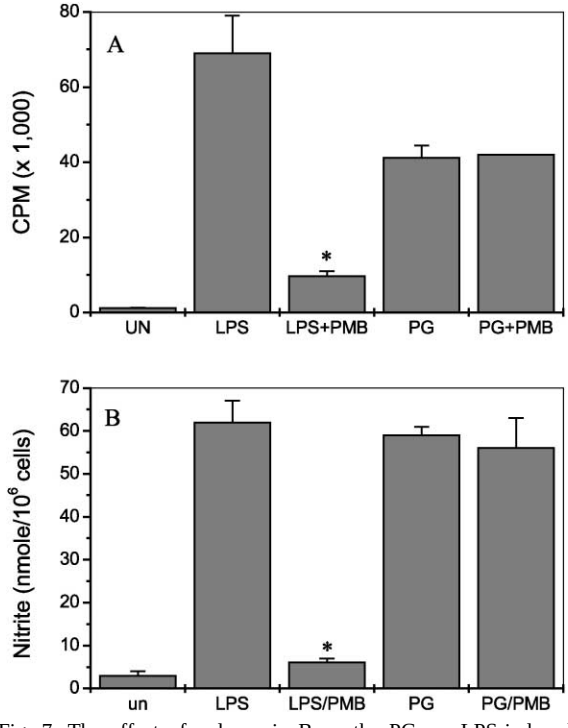

Fig. 7. The effect of polymyxin B on the PG- or LPS-induced activation of B cells (A) and macrophages (B). PG (100 μg/ml) or LPS (5 μg/ml) were preincubated with polymyxin B (PMB, 1000 unit/ml). After incubating for 2 h, the samples were added to B cells and macrophages. The proliferation of B cells (A) and the NO production of macrophages (B) were then measured. Significance was determined using the Student's *t*-test versus the PG- or LPS-treated group ($^*p < 0.01$). UN, chemically untreated control group.

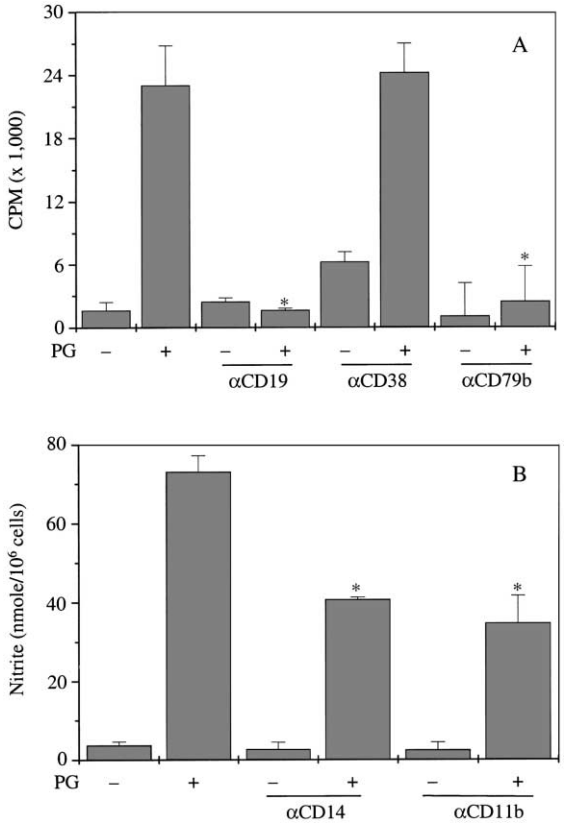

Fig. 8. The possible cellular binding sites of PG in B cells and macrophages. Splenic B cells were preincubated with anti-CD19 (αCD19), anti-CD38 (αCD38), or anti-79b (αCD79b) antibody at 2.5 μg/ml for 1 hr. The degree of B cell proliferation was measured by incorporating ^{3}H-thymidine into the cellular DNA (A). Peritoneal macrophages were preincubated with anti-CD14 (αCD14) or anti-CD11b (αCD11b) antibody at 5 μg/ml for 1 h. The supernatant nitrite levels determined (B). PG was added at a final concentration of 30 μg/ml. Significance was determined using the Student's *t*-test by comparison with the PG-treated group ($^*p < 0.01$).

anti-CD38 or anti-CD79b antibody for 1 h. After 3 days of incubation with 30 μg/ml of PG, B cell proliferation was examined. Anti-CD19 or anti-CD79b antibody significantly inhibited PG-induced B cell proliferation, but anti-CD38 antibody did not (Fig. 8A). In addition, when peritoneal macrophages were pretreated with anti-CD14 or anti-CD11b antibody for 1 h, PG-induced macrophage NO production was significantly decreased by these antibodies (Fig. 8B).

3.5. PG increased in vivo immune functions

The immunomodulatory effect of PG in vivo was investigated using a model of T-dependent immunization and specific IgM antibody production, which require the participation of antibody secreting B cells and accessory cells such as macrophages and Th cells. As shown in Fig. 9A, the number of AFCs in non-immunized mice was undetectable, but this increased to 241 AFCs/10^6 cells 4 days after immunization with sRBC antigen. Moreover, the administration of PG at 10, 30, and 100 mg/kg increased the number of AFCs to 351, 416, and 380, respec-

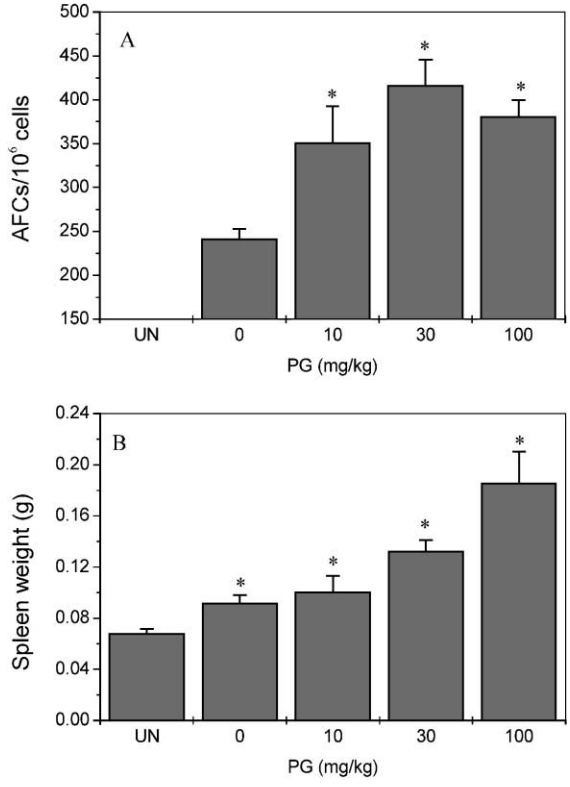

Fig. 9. The effect of PG on in vivo immunization and specific IgM antibody production of B cells. Mice were immunized with sRBCs and PG was injected intraperitoneally at doses ranging from 10 to 100 mg/kg ($n = 5$). After 4 days, spleen cells were isolated and the number of AFCs was determined by PFC assay (A). Spleen weights were also measured (B). Significance was determined using the Student's *t*-test versus a PG-untreated, sRBC-immunized group ($^*p < 0.01$). UN, chemically untreated control group.

tively (Fig. 9A). This result demonstrates that PG has biological activity in vivo, and this was further confirmed by an increase in spleen weights (Fig. 9B).

4. Discussion

The main objective of this study was to characterize the immunomodulatory activity of PG, a polysaccharide isolated from the roots of *P. grandiflorum*. Our results demonstrate that PG has typical immunostimulatory activity, in that it selectively activates B cells and macrophages, but not T cells. In addition, the study shows that PG and LPS have different properties, as indicated by the inability of PMB, an LPS inhibitor, to attenuate the activity of PG, although PG and LPS show similar modes of action on B cells and macrophages.

The polysaccharides obtained from many natural sources represent a structurally diverse class of macromolecules, and this structural variability can profoundly affect the biological activity of the molecules in B cells, T cells, and macrophages. Lentinan, $\beta(1 \rightarrow 3)$-glucan isolated from *Lentinus edodes*, is a stimulator of T cells and macrophages, but does not accelerate B cell antibody formation [3]. The antitumor effect of lentinan is abrogated in neonatal thymectomized mice and also inhibited by pretreatment with anti-macrophage agents, which suggest that the effects of lentinan are due to the potentiation of the responses of T cells and macrophages [20–23]. Another $\beta(1 \rightarrow 3)$-glucan, schizophyllan, isolated from *Schizophyllium commune* appears to be similar to lentinan in terms of its biological activity. Schizophyllan restores and enhances cellular immunity in the tumor-bearing host by functioning as a T cell adjuvant and a macrophage activator [24]. Other types of glucans isolated from *Saccharomyces* activate macrophages [25]. Polysaccharide from *P. linteus* (PL), which is composed of glucose, galactose, mannose, arabinose, xylose, and uronic acid, shows broad action profiles on B cells, T cells, and macrophages [2,26]. In the present study, we demonstrate that PG is an immunostimulant that selectively activates B cells and macrophages, and prove that it has characteristics that are different from those of lentinan, schizophyllan, and PL.

The cell-type selectivity of PG appears to be similar to that of angelan, a pectic polysaccharide isolated from the *A. gigas* Nakai [5–7]. Angelan was reported to directly activate macrophages and B cells, but not T cells. Although PG and angelan are similar in their modes of action, their chemical compositions and structures are different since it has been found that aqueous extracts of the radix of *P. grandiflorum* contains inulin-type polysaccharides [13,15]. Inulin is a glucofructan with a $\beta(2 \rightarrow 1)$-linked D-fructose, and is mainly found in the roots of some members of the Compositae and Campanulaceae. Excepting starch, this is the most abundant carbohydrate reserve in the plant kingdom. Inulin and its degradation products have recently received considerable attention for their potential health benefits, such as their ability to reduce the risk of colonic diseases, diabetes, obesity, osteoporosis, and cancer [27–29]. Although inulin has been proposed to be an immune stimulant, systematic studies have not been performed as yet to assess its lymphocyte activity or investigate its other immune functions [30].

In this study, we demonstrate that PG selectively activates B cells and macrophages, but not T cells. Since PG cannot penetrate cells due to its large molecular mass, this selectivity may be caused by the surface binding of this molecule to receptors specifically expressed on B cells and macrophages, but not on T cells. The B cell receptor (BCR) consists of surface immunoglubulin and CD79a–CD79b. Upon BCR ligation, the BCR-associated kinase Lyn phosphorylates CD79a–CD79b. In addition, co-receptors such as CD19 and CD38 positively regulate BCR signaling [31]. Our results indicated that PG might bind transmembrane glycoproteins including CD19 and CD72b, but not CD38, in B cells. In addition, CD14 and CD11b were assumed to act as PG receptors in macrophages. CD14 is a 55-kDa glycosylphosphatidylinositol-anchored protein, which is expressed on the surfaces of macrophages and binds LPS with high affinity [32]. Complement receptor CD11b (CR3 or Mac-1) is expressed on the surface of macrophages and NK cells, and has been identified as the receptor of β-glucans [33]. Although PG and β-glucans show different specificities on B and T cells, they may use the same receptor on macrophages. A further complete investigation of the membrane receptors of PG

should shed light on its selectivity for B cells and macrophages.

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