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Immunomodulatory Effects of *Aureobasidium pullulans* SM-2001 Exopolymers on Cyclophosphamide-Treated Mice

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The immunomodulatory effects of *Aureobasidium pullulans* SM-2001 exopolymers containing β-1,3/1,6-glucan were evaluated in cyclophosphamide (CPA)-treated mice. To induce immunosuppression, 150 and 110 mg/kg of CPA were intraperitoneally injected 3 days and 1 day, respectively, before beginning administration of the test material. Exopolymers were delivered subcutaneously or orally, four times, in a volume of 10 ml/kg at 12-h intervals beginning 24 h after the second CPA treatment. Changes in thymus and spleen weights, splenic amounts of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-10, and numbers of CD3+, CD4+, CD8+, and TNF-α+ thymus and spleen cells were monitored in CPA-treated mice. As a result of CPA treatment, dramatic decreases in the number of CD3+, CD4+, CD8+, and TNF-α+ cells were detected in the thymus and spleen, along with decreases in thymus and spleen weights. In addition, splenic TNF-α, IL-1β, and IL-10 contents were also decreased on observation with flow cytometry. However, oral and subcutaneous treatments with exopolymers effectively reduced the immunosuppressive changes induced by CPA. Therefore, it is concluded that exopolymers of *A. pullulans* SM-2001 can effectively prevent immunosuppression through, at least partially, the recruitment of T cells and TNF-α+ cells or enhancement of their activity, and can provide an effective component of prevention or treatment regimens for immunosuppression related to cancer, sepsis, and high-dose chemotherapy or radiotherapy.

Keywords: Immunomodulatory effects, exopolymers, β-glucan, *Aureobasidium pullulans* SM-2001, cyclophosphamide, mouse

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Sepsis is a major cause of mortality in intensive care units, accounting for more than 200,000 deaths per year in the United States alone [17]. Increasing evidence suggests that sepsis impairs immune function by inducing defects in innate immunity and excessive lymphocyte apoptosis. The resulting immunosuppression has been suggested to be a major contributing factor in sepsis-induced mortality. Accordingly, activation of macrophages with interferon (IFN)-γ in septic patients, blockade of complement-induced neutrophil dysfunction, and inhibition of lymphocyte apoptosis in animals with experimentally induced sepsis have all been reported to have beneficial effects [39]. Recovery from immunodeficiency caused by high-dose chemotherapy or radiotherapy remains a major clinical problem. Typically, hematological reconstitution is medically considered to have occurred when neutrophil and platelet counts have been restored to a defined normal level; however, cellular interactions in lymphoid organs are required for recovery of immune system function and should also be considered [1]. This issue may have important implications in the design of therapeutic approaches to improving antiinfective, antisepsis, and/or anticancer treatments [12, 28].

Many polysaccharides are known to activate cellular constituents of host immune responses [36]. β-Glucan is a component of fungal cell walls that modulates many processes *in vivo* and *in vitro* [10]. It possesses several major immunopharmacological activities, including antitumor effects [7, 24], radioprotective action [13], and increased host resistance to viral, bacterial, and parasitic infections [3, 7, 8, 23] as well as ancillary effects [2, 5, 14]. The exact mechanism of β-glucan action remains unclear. Some experiments have shown that it does not directly activate leukocyte functions such as the oxidative burst [25] or

secretion of cytokines [30]. Although a few investigators have reported β-glucan-mediated down-modulation [26, 35], most of the research to date describes a positive modulatory role. Similarly, exopolymers derived from *Aureobasidium pullulans* SM-2001, an ultraviolet (UV)-induced mutant of the wild-type strain, also contain -1,3/1,6-glucans showing various pharmacological activities [32]; we have reported their anti-osteoporotic [34], antiinflammatory [20, 21], and fracture-healing [22] effects.

In the present study, the immunomodulatory effects of *A. pullulans* SM-2001 exopolymers were evaluated in cyclophosphamide (CPA)-treated mice, based on changes in thymus and spleen weights, white blood cell (WBC) numbers, splenic amounts of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-10, and numbers of CD3+, CD4+, CD8+, and TNF-α+ thymus and spleen cells.

MATERIALS AND METHODS

Experimental Animals

Thirty-six male ICR mice (6 weaks old upon receipt; SLC, Japan) were used after acclimatization for 7 days. Four or five animals were allocated per polycarbonate cage in a temperature (20–25°C) and humidity (40–45%) controlled room. The light–dark cycle was 12:12 h and feed (Samyang, Korea) and water were supplied with free access. The animals were distributed into four groups with nine mice per group.

Test Materials, Grouping, and Dosing

Exopolymers from *A. pullulans* SM-2001 were supplied by Glucan Corp. (Korea) and were stored in a refrigerator at 4°C to protect from light and degradation. Based on a previously reported analysis [32], the exopolymers are known to consist of water (86%), β-1,3/1,6-glucan (2%), glucose (5%), starch (1%), cellulose (3%), and ash (3%). The exopolymers were subcutaneously or orally administered four times in a volume of 10 ml/kg at 12-h intervals, beginning 24 h after the second administration of CPA. For intact and CPA-treated control mice, 10 ml/kg of saline was subcutaneously injected at the same intervals.

Induction of Immunosuppression

To induce immunosuppression, 150 and 110 mg/kg of CPA (Sigma, MO, U.S.A.) were dissolved in saline and intraperitoneally injected 3 days and 1 day, respectively, before beginning administration of the test material. In intact control mice, an equal volume of saline was administered instead of CPA, using the same methods.

Organ Weight Measurement

At sacrifice, the wet-weights of spleen and thymus were measured and regarded as absolute weights, and then relative organ weights (% of body weight) were calculated to reduce individual differences in body weight.

Histopathology

After measuring organ weights, the thymus and spleen were sampled, fixed in 10% neutral buffered formalin, and embedded in paraffin. Three- to four-micron sections were prepared, and representative sections were stained with hematoxylin and eosin (H&E) for examination by light microscopy. The histological profiles of individual organs were then observed. The number of white pulps in spleen were calculated as the number/histological section using an automated image analyzer (DMI-300; DMI, Korea). In addition, the frequency of thymic cortex atrophy (number showing atrophic changes in thymic cortex/total number of observed thymus) was also determined.

Immunohistochemistry (IHC)

Sections were deparraffinized, and then CD3 epitope retrieval was conducted in 10 mM Tris/1 mM EDTA buffer (pH 9.0), CD4 and CD8 in 1 mM EDTA buffer (pH 8.0), and TNF-α in 10 mM citrate buffer (pH 6.0), according to previously described methods [29, 33]. Briefly, a staining dish containing buffer was preheated in a water bath until the temperature reached 95–100°C. Slides were immersed in the staining dish, upon which a lid was then loosely placed, and then incubated for 20 min. The staining dish was then returned to room temperature and the slides were left to cool for 20 min. After epitope retrieval, sections were immunostained using ABC kits (Vector Labs Inc., CA, U.S.A.) and the primary antisera listed in Table 1. Among 1,000 spleen or thymus cells, the number of each type of immunoreactive cell was determined as the number/1,000 splenocytes or thymocytes, using the automated image analyzer. In thymus, the counts were separately conducted in both the cortex and medulla.

Flow Cytometry

Splenocyte preparation. Pieces of sampled spleen (10–20 mg) were washed twice in RPMI-1640 medium (Gibco BRL, NY, U.S.A.) at 4°C, homogenized in the same medium, and then filtered using a cell dissociation sieve (mesh No. 1000; Sigma, MO, U.S.A.). Following dissociation, splenocytes were incubated in hypotonic buffer, washed twice in Hanks balanced salt solution (HBSS; Gibco BRL, NY, U.S.A.), and then resuspended in RPMI-1640 medium containing 10% fetal bovine serum (FBS; Gibco BRL, NY, U.S.A.).

Fluorescent antibody staining. For immunofluorescence staining of splenocytes, 1×10^6 cells suspended in 10 μl of minimal essential medium (MEM) containing 1% bovine serum albumin (BSA) and 0.02% azide (MEM/BSA/azide) were incubated at 5°C for 1 h with the fluorescein isothiocyanate (FITC)-conjugated primary antisera listed in Table 1. Subsequently, the cells were washed twice in MEM/BSA/azide.

Detection. After immunolabeling, cells were analyzed on a FACStar flow cytometer (Becton Dickinson, CA, U.S.A.) equipped with an argon laser (488 nm excitation) and appropriate filter settings for observation of FITC fluorescence. Among 1×10^6 spleen cells, the percentage of each type of fluorescent cell was calculated.

Tissue Cytokine Detection

Splenic concentrations of TNF-α, IL-1β, and IL-10 were measured by ELISA using the commercially available kits listed in Table 1, as previously described [16]. Approximately 10–15 mg of tissue was homogenized in a tissue grinder containing 1 ml of lysis buffer [PBS containing 2 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mg/ml of aprotinin, leupeptin, and pepstatin A] as described by Clark *et al.* [6]. One hundred ml of standard (diluted in lysis buffer) or 10, 50, or 100 ml of tissue homogenate was analyzed in duplicate, and a portion of the sample was assayed for protein content. Data were expressed as pg/mg of protein. A standard curve was generated

Table 1. Primary antisera used in this study.

Immunohistochemistry			Dilution
Antiserum[a]	Code	Source	
Anti-mouse CD3 (17A2) monoclonal antibody	CBL-1317	Chemicon Inc., CA, U.S.A.	1:100
Anti-mouse CD4 (GK1.5) monoclonal antibody	CBL-13583	Chemicon Inc., CA, U.S.A.	1:100
Anti-mouse CD8 (Ly-2) monoclonal antibody	CBL-1318	Chemicon Inc., CA, U.S.A.	1:100
Anti-TNF-α polyclonal antibody	HP8001	Hycult Biotechnology b.v., The Netherlands	1:100

[a]All antisera were raised in rat.

Flow cytometry		
Antiserum[a]	Code	Source
FITC-conjugated anti-mouse CD3 (17A2) monoclonal antibody	555274	BD Biosciences/Pharmingen, CA, U.S.A.
FITC-conjugated anti-mouse CD4 (L3T4) monoclonal antibody	553651	BD Biosciences/Pharmingen, CA, U.S.A.
FITC-conjugated anti-mouse CD8 (Ly-2) monoclonal antibody	553031	BD Biosciences/Pharmingen, CA, U.S.A.
FITC-conjugated anti-mouse TNF-α monoclonal antibody	554418	BD Biosciences/Pharmingen, CA, U.S.A.

[a]All antisera were raised in rat.

ELISA kits
TNF-α: Mouse TNF-α ELISA kit; BD Biosciences/Pharmingen, CA, U.S.A.
IL-1β: Mouse IL-1β ELISA kit; Genzyme, MA, U.S.A.
IL-10: Mouse IL-10 ELISA kit; Genzyme, MA, U.S.A.

for each assay based on replicate absorbance measurements, and the average coefficient of variance was <10%.

Statistical Analyses

All data were calculated as the mean ± SD, and statistical analyses were conducted using the Mann–Whitney U-Wilcoxon rank sum W test (MW test) with SPSS for Windows (Release 6.1.3; SPSS Inc., IL, U.S.A.).

Results

Changes in Organ Weights

Significant decreases ($p<0.01$) in relative and absolute spleen and thymus weights were detected in CPA-treated control mice compared with the intact control group. However, dramatic increases in spleen and thymus weights were detected in both exopolymer-treated groups compared with the CPA-treated control group (Table 2).

Histopathology of Thymus and Spleen

Dramatic splenic atrophy and a decrease in the number of lymphoid cells in the thymic cortex were detected in CPA-treated control mice. However, the atrophic changes were effectively inhibited by oral and subcutaneous treatments with exopolymers (Fig. 1 and 2). On histomorphometry, the number of white pulps in the spleen was significantly increased ($p<0.01$) in CPA-treated control mice compared with intact control mice. However, the number of white

Table 2. Changes in organ weights detected in CPA-treated mice.

Organ weight	Thymus		Spleen	
	Absolute (g)	Relative (%)	Absolute (g)	Relative (%)
Control				
Intact	0.074±0.010	0.178±0.023	0.133±0.026	0.322±0.061
CPA-treated	0.033±0.008*	0.084±0.019*	0.049±0.009*	0.127±0.022*
Exopolymer treatment				
Oral	0.040±0.004*,##	0.105±0.009*,##	0.061±0.010*,##	0.158±0.033*,##
Subcutaneous	0.044±0.008*,##	0.112±0.026*,##	0.085±0.020*,#	0.216±0.048*,#

Values are expressed as mean±SD of nine mice; *$p<0.01$ compared with intact control group; #$p<0.01$ and ##$p<0.05$ compared with CPA-treated control.

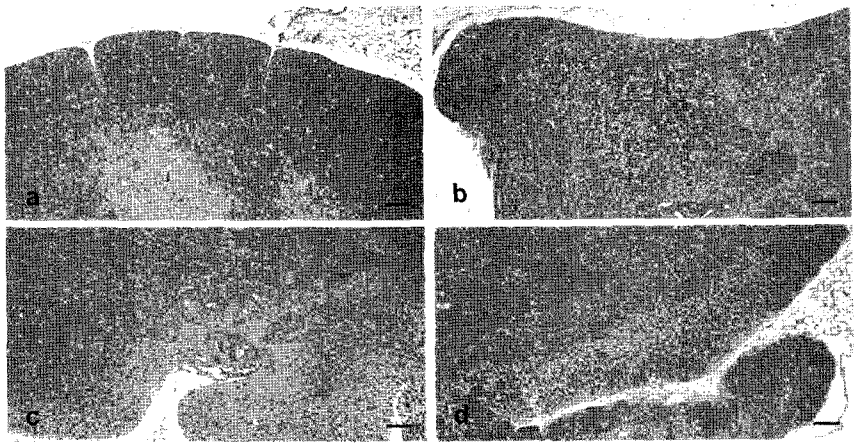

Fig 1. Changes in histopathological profiles of thymus detected in intact (**a**) and CPA-treated (**b**) control groups, and in oral (**c**) and subcutaneous (**d**) exopolymer-treated groups of CPA-treated mice.
Note that decreases in lymphoid cells in the thymic cortex were detected in the CPA-treated control group; however, these atrophic changes were effectively inhibited in both exopolymer-treated groups. All samples were stained with H&E; scale bars=100 μm.

pulps was significantly increased ($p<0.01$) in both exopolymer-treated groups compared with the CPA-treated control group. In addition, the incidence of lymphoid cell depletion in the thymic cortex was dramatically inhibited in the exopolymer-treated groups (Table 3).

IHC of Thymus and Spleen

Significant decreases ($p<0.01$) in the number of CD3+, CD4+, CD8+, and TNF-α+ spleen cells were detected in CPA-treated control mice compared with the intact control group, and significant decreases ($p<0.01$) in CD3+ and TNF-α+ cells were also detected in the cortex and medulla of the thymus. However, the number of these cells in both the thymus and spleen were significantly increased ($p<0.01$ or $p<0.05$) in the exopolymer-treated groups compared with the CPA-treated control group (Table 4).

Flow Cytometric Changes in Spleen

Significant decreases ($p<0.01$) in the number of CD3+, CD4+, CD8+, and TNF-α+ spleen cells were detected in the CPA-treated control group compared with the intact control group. However, the number of these cells was significantly increased ($p<0.01$ or $p<0.05$) in the exopolymer-treated groups compared with the CPA-treated control group (Table 5).

Splenic Cytokine Content

Significant decreases ($p<0.01$) in splenic TNF-α, IL-1β, and IL-10 contents were detected in CPA-treated control

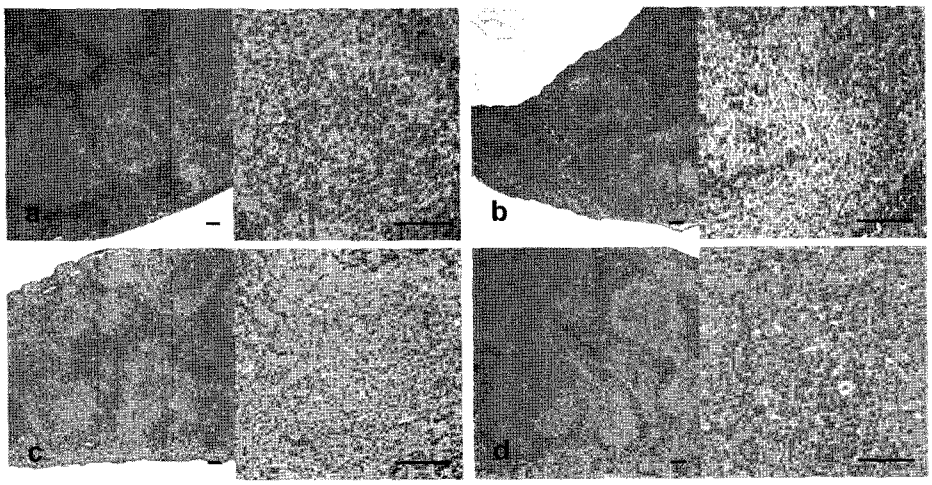

Fig 2. Changes in histopathological profiles of spleen detected in intact (**a**) and CPA-treated (**b**) control mice, and in oral (**c**) and subcutaneous (**d**) exopolymer-treated groups of CPA-treated mice.
Note that dramatic splenic atrophy and a decrease in lymphoid cells were detected in the CPA-treated control group; however, these atrophic changes were effectively inhibited in exopolymer-treated groups. All samples were stained with H&E; scale bars=100 μm.

Table 3. Histomorphometric changes in spleen and thymus detected on histopathology in CPA-treated mice.

Histopathology	Spleen: Number of white pulps[a]	Thymus: Incidence of thymic cortex atrophy[b]
Control		
Intact	18.67±2.12	0/9 (0%)
CPA-treated	6.22±1.20*	9/9 (100%)
Exopolymer treatment		
Oral	12.22±1.99*,#	8/9 (88.89%)
Subcutaneous	21.33±2.35**,#	6/9 (66.67%)

Values are expressed as mean±SD of nine mice; [a]number/histological section; [b]number of atrophic changes in thymic cortex/total number of observed thymus (% of observation); *$p<0.01$ and **$p<0.05$ compared with intact control mice; #$p<0.01$ compared with CPA-treated control mice.

mice compared with intact control mice. However, the levels of these cytokines were significantly increased ($p<0.01$ or $p<0.05$) in both exopolymer-treated groups compared with the CPA-treated control group (Table 6).

Discussion

We found that subcutaneous administration of exopolymers had relatively significant immunomodulatory effects in CPA-treated mice based on changes in thymus and spleen weights, splenic TNF-α, IL-1β, and IL-10 contents, and number of CD3+, CD4+, CD8+, and TNF-α+ thymus and spleens cells.

CPA is a widely used antineoplastic drug, employed alone or in combination with other products [11]. When used as an anticancer drug or in bone marrow transplantation conditioning regimens, it severely injures hematopoietic and lymphoid tissues, thereby leading to profound leukopenia [1]. CPA is known to be biologically inactive until it is biotransformed by microsomal enzymes, leading to the production of a number of active metabolites capable of alkylating nucleic acids [27] and damaging chromosomes through the generation of free radicals, thereby inducing mutation [9]. CPA-induced immunosuppression and/or mutagenicity in mice has been used as a valuable animal model for detecting antimutagenic or favorable immunomodulatory effects [15, 18].

The decrease in thymus and spleen weights observed in CPA-treated mice is considered to be the result of the atrophic changes detected on histopathological examination. We found that exopolymer treatment administered by both routes effectively inhibited this decrease.

Histopathologically, thymus and spleen showed atrophic changes related to depletion of lymphoid cells after CPA treatment [27]. T cell antigen receptors are always membrane-bound and noncovalently associated with a set of four invariant glycoproteins collectively called CD3. Thus,

Table 4. Histomorphometric changes in CD3+, CD4+, CD8+, and TNF-α+ cells in spleen and thymus detected on immunohistochemistry in CPA-treated mice.

IHC-Histomorphometry	Control		Exopolymer treatment	
	Intact	CPA-treated	Oral	Subcutaneous
Spleen				
CD3+ cells	177.22±31.99	65.00±13.18*	108.44±17.19*,#	181.89±17.46#
CD4+ cells	274.33±19.43	46.22±12.89*	117.11±11.68*,#	142.67±16.06*,#
CD8+ cells	191.56±14.88	51.22±13.55*	133.78±10.83*,#	149.44±19.60*,#
TNF-α+ cells	96.44±18.82	46.56±12.29*	65.00±11.63*,#	79.22±12.53#
Thymus: Cortex				
CD3+ cells	740.22±92.10	182.00±57.65*	693.67±116.61#	719.22±64.65#
CD4+ cells	1.56±0.73	2.56±1.59	9.22±1.39*,#	12.44±1.42*,#
CD8+ cells	2.33±2.24	3.00±1.73	2.67±1.22	3.44±1.67
TNF-α+ cells	5.22±1.56	2.65±1.13*	5.44±1.33#	5.67±1.32#
Thymus: Medulla				
CD3+ cells	3.33±1.00	0.33±0.50*	16.78±3.31*,#	38.89±13.08*,#
CD4+ cells	2.67±0.71	2.44±0.88	4.67±0.87*,#	4.78±1.09*,#
CD8+ cells	1.56±0.73	2.00±0.71	3.22±0.67*,#	15.56±2.51*,#
TNF-α+ cells	1.56±0.73	0.00±0.00*	1.67±0.50#	6.67±0.87*,#

Values are expressed as mean±SD of nine mice, number/1,000 splenocytes or thymocytes; *$p<0.01$ compared with intact control mice; #$p<0.01$ compared with CPA-treated control mice.

Table 5. Changes in CD3+, CD4+, CD8+, and TNF-α+ spleen cells detected on flow cytometry in CPA-treated mice.

Flow cytometry	Control		Exopolymer treatment	
	Intact	CPA-treated	Oral	Subcutaneous
Percentage (%)				
CD3+ cells	44.00±6.00	24.17±4.78*	34.33±4.50*,#	43.48±4.20#
CD4+ cells	22.38±4.28	9.51±1.36*	16.06±2.44*,#	16.45±2.73*,#
CD8+ cells	17.33±1.68	8.82±1.38*	14.10±2.24*,#	15.06±1.76**,#
TNF-α+ cells	13.76±1.67	5.78±1.87*	9.97±1.19*,#	10.73±1.03*,#

Values are expressed as mean±SD of nine mice, % of splenocytes; *$p<0.01$ and **$p<0.05$ compared with intact control mice; #$p<0.01$ compared with CPA-treated control mice.

CD3 has been regarded as a marker for T cells. CD4 is a 55-kDa single-chain glycoprotein, and CD8 is a disulfide-linked heterodimer of a 34-kDa subunit. Either CD4 or CD8 is found on mature T cells, although immature T cells may express both glycoproteins. Their function is to determine the class of major histocompatibility (MHC) molecules that is recognized by a T cell. Generally, CD4+ cells are called helper T cells, and CD8+ cells are known as cytotoxic T cells [4]. The cytokine TNF-α is produced by a variety of cells including splenocytes and has been found to be associated with critical events leading to T-lineage commitment and differentiation [31]. TNF-α can enhance the *in vivo* immune response at doses much lower than those that cause weight loss or tissue toxicity. It enhances proliferation of B and T cells and promotes the generation of cytotoxic T cells. In addition, TNF-α enhances IL-2-induced immunoglobulin production and augments IL-2-stimulated natural killer cell activity and proliferation of monocytes [19]. Thymic and splenic T cell subsets, especially CD4 and CD8, were severely depleted following induction of leukopenia and immunosuppression by CPA [27], as were the number of various cytokine-producing cells including TNF-α+ cells [38]. IL-1 is another cytokine released by various cell types including macrophages, dendritic cells, lymphocytes, endothelial cells, fibroblasts, and keratocytes. It exists in two forms, IL-1α and IL-1β, both of which are 17-kDa glycoproteins, the latter being secreted by cells and the former in membrane-bound form. IL-1 is necessary for the successful initiation of some forms of immune response [37]. IL-10 is an immunosuppressive glycoprotein 19–21 kDa in size that is secreted by Th2 cells, some B cells, and activated macrophages. It is now clear that IL-10 primarily acts on activated macrophages to suppress their secretion of IL-1, IL-12, TNF-α, and reactive oxygen radicals [19]. We found that treatment with exopolymers effectively inhibited atrophic changes in the spleen and thymus on histopathological observation and effectively reduced decreases in T cell subsets and TNF-α+ cells on observation with IHC and flow cytometry. Therefore, we suggest that exopolymers of *A. pullulans* SM-2001 can promote the proliferation of immune cells in lymphoid organs or directly inhibit CPA-induced immunosuppressive activities. The observed increases in TNF-α+ cells following exopolymer treatment are considered to be responses secondary to the increases in T cells. However, we do not exclude the possibility of directly promoted TNF-α+ cell proliferation. The increase in splenic TNF-α, IL-1β, and IL-10 contents detected in the present study is considered to be evidence that exopolymers can facilitate the activity of cells producing these cytokines. However, further studies are needed to elucidate the exact mechanism of the immunomodulatory effects of exopolymers observed in this work.

In conclusion, CPA-induced immunosuppression was effectively reduced by subcutaneous treatment with exopolymers of *A. pullulans* SM-2001. This effect was mediated, at least partially, through the recruitment of T cells and TNF-α+ cells or enhancement of their activity. Therefore, it is suggested that these exopolymers may be an effective component in prevention or treatment regimens for

Table 6. Changes in splenic cytokine content detected in CPA-treated mice.

Flow cytometry	Control		Exopolymer treatment	
	Intact	CPA-treated	Oral	Subcutaneous
Splenic content				
TNF-α	163.39±20.17	35.88±8.00*	67.72±13.21*,#	141.23±20.78**,#
IL-1β	46.67±7.62	14.92±4.93*	21.76±1.63*,#	39.37±8.87#
IL-10	207.58±20.05	74.91±16.68*	117.17±19.64*,#	165.03±20.73*,#

Values are expressed as mean±SD of nine mice, pg/mg protein; *$p<0.01$ and **$p<0.05$ compared with intact control mice; #$p<0.01$ and ##$p<0.05$ compared with CPA-treated control mice.

immunosuppression related to cancer, sepsis, and high-dose chemotherapy or radiotherapy.

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