Mechanism of Immunosuppressive Effect of a Folk Medicine Sasa Veitchii by Analyzing the Cytokine Synthesis of Splenocytes in Mice In Vitro

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Summary
Sasa veitchii (Japanese folk medicine Kumazasa), genus Sasa, Poaceae, is a folk medicine, the extract of which exhibits various biological activities that are beneficial for human health, including anti-tumor, anti-allergic, anti-inflammatory, anti-ulcer, antimicrobial, and immune stimulatory activities as well as blood-pressure-lowering effects and the amelioration of hyperglycemia and hyperlipidemia. Our research group has been examining the immunomodulatory effects of Sasa. In order to analyze these effects precisely, we prepared a water-soluble methanol-insoluble fraction of bamboo (BWMP). We previously demonstrated that BWMP suppressed the production of IFN-γ. In the present study, we attempted to elucidate the molecular mechanism underlying the inhibition of IFN-γ. The polysaccharide fraction, BWMP, was prepared by methanol precipitation of the S. veitchii extract (KE). Mouse CD3+ T-cells were isolated using negative selection from mouse splenocytes. Splenocytes or T-cell fractions were stimulated with pathogen-associated molecular patterns (PAMPs), and the inhibition of cytokine syntheses by BWMP was assessed. SCG, a ligand of the C-type lectin receptor dectin-1, was used as a PAMP. BWMP inhibited the production of IFN-γ and IL-4 from CD3+ T-cells stimulated by a mouse CD3ε antibody. BWMP also inhibited the production of IL-2 and cell proliferation. However, it did not inhibit cell proliferation in the presence of rm-IL-2.

Keywords: Sasa veitchii; immunomodulation; T-cell; CD3ε; IL-2

Introduction
Functional foods, also known as folk medicines, are produced from various materials including mushrooms, yeasts, fungi, algae, and plants. The extracts of plants are known to be beneficial for human health, and exhibit anti-tumor, anti-allergic, and anti-inflammatory activities. This field of research has been significantly enlarged by the application of mixtures of plant extracts to the treatment of various chronic diseases that have developed resistance to western medicines. Sasa veitchii (Japanese folk medicine Kumazasa), genus Sasa, Poaceae, is a folk medicine, the extract of which exhibits various biological activities that are beneficial for human health, including anti-tumor, anti-allergic, anti-inflammatory, anti-ulcer, antimicrobial, and immune stimulatory activities as well as blood-pressure-lowering effects and the amelioration of hyperglycemia and hyperlipidemia [1-7]. S. veitchii contains antioxidant substances such as orientin and vitexin [8], antimicrobial substances including phytoncide, vitamin K, and chlorophyll [9], and anti-viral substances such as tricin [10, 11]. The polysaccharides of S. veitchii (named bamfolin), soluble hemicelluloses, and lignin also exhibit anti-tumor activities [12], while arabinoxylan has been shown to display immune stimulatory activity [13].

Recently, our research group extensively analyzing S. veitchii both from chemical and immunological point of views. Tsuibo et al. has reported the chemical and immune chemical characterization of polysaccharides of S. veitchii leaves by analyzing the hot water extract. It was clarified that S. veitchii contained two polysaccharide fractions (NPS and APS) separated by DEAE-Sephadex chromatography. HMF strongly reacted with human immunoglobulin. Sugar analyses indicated both of the fractions contained similar component such as, rhamnose, arabinose, xylose, mannose, glucose, and galactose in the molar ratio of 1.0: 2.3: 1.5: 3.8: 0.6: 5.3 for NPS and 1.0: 3.0: 2.6: 0.8: 6.3: 3.0 for APS [14].

For immunological analysis we have prepared macromolecular fraction, BWMP (bamboo water-soluble methanol precipitation), major component of BWMP is polysaccharides analyzed by Tsuibo et al. as described above. Yoshida previously examined the cooperative effects of SCG (a β-glucan preparation extracted from Sparassis crispa), dectin-1, a ligand of the C-type lectin receptor (CLRs), and BWMP and demonstrated the selective suppression of the production of interferon gamma (IFN-γ) [15].

Very recently, we also examined the cooperative effects of various pathogen-associated molecular patterns (PAMPs) and BWMP in order to generalize their effects. Our findings suggested that BWMP inhibited the signaling pathways of PAMPs, but not ligand-receptor interactions, which indicated that the inhibition of IFN-γ by BWMP was mediated through the cell-to-cell interactions of splenic cells during cultivation [16].

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In the present study, we attempted to elucidate the mechanism underlying BWMP-mediated cytokine synthesis.

**Materials and Methods**

**S. Veitchii Extract**

Hoshi Kumazasa extract was supplied by Hoshi Pharmaceutical Co., Ltd. (Japan), and was obtained by the hydrothermal extraction of *S. veitchii* using hot water at 103°C.

**Preparation of BWMP**

The polysaccharide fraction, BWMP, was prepared by methanol precipitation of the *S. veitchii* extract as follows. The *S. veitchii* extract was suspended in methanol, centrifuged at 4,000 rpm for 10 min, and the supernatant was discarded. A small amount of water was added to solubilize the precipitate. Methanol was again added to the resulting solution, which was centrifuged at 4,000 rpm for 10 min, and the supernatant was discarded. The precipitant was dried using ethanol and acetone [15].

**Preparation of SCG**

The fruit bodies of *S. crispa* were cultured by Mina health Co. (Saitama, Japan). SCG was prepared as described previously. In brief, air-dried and powdered *S. crispa* was extracted with ice-cold alkali (10% NaOH/5% urea, 4°C, 2d). The extract, dissolved in 8 M urea, was applied to a diethylaminoethyl (DEAE) Sephadex A-25 (Cl⁻) column equilibrated with 8 M urea, and the pass-through fraction was collected, extensively dialyzed against tap and distilled water, and then lyophilized (elemental analysis C:H:N = 40.06:6.77:0.08). SCG solution was prepared by dissolving the lyophilized powder in 0.5 N NaOH, followed by immediate dialysis against saline for 3 d. After dialysis, the dialyzed fraction was autoclaved and frozen until use [17].

**Animals**

DBA/2 and C57BL/6N male mice between 7 and 9 weeks of age were purchased from Japan SLC (Shizuoka, Japan). The experimental protocol was approved by the Committee for Animal Care and Use (Tokyo University of Pharmacy and Life Science). Mice were maintained under specific pathogen-free (SPF) conditions, at 23 ± 2°C, with constant humidity of 55 ± 5%, under a cycle of 12 h of light and 12 h of dark, and had free access to food and tap water according to the Guidelines for Experimental Animal Care issued by the Prime Minister’s Office of Japan.

**Preparation of splenocytes**

Splenocytes were prepared as previously described. Briefly, the spleen was teased apart in RPMI 1640 medium, and after centrifugation, the single cell suspension was treated with Ammonium-Chloride-Potassium (ACK)-lysing buffer (8.29 g/L NH₄Cl, 1 g/L KHCO₃, and 37.2 mg/L EDTA/2Na) to lyse red blood cells. After centrifugation, the splenocytes were maintained in RPMI 1640 medium supplemented with 50 μg/mL gentamicin sulfate and 10% fetal bovine serum (FBS). Cells were cultured at 5 ×10⁶ cells per 1 mL of culture medium. Splenocytes were cultured at 37°C in a humidified 5% CO₂, 95% air atmosphere.

**Preparation of Cluster of Differentiation 3, CD3, + T-Cells**

CD3+ T-cell fractions were isolated from the splenocytes of DBA/2 or C57BL/6N mice by negative selection using a Mouse CD3+ T-cell enrichment column (R&D systems, Minneapolis, MN). T-cells were maintained in RPMI 1640 medium supplemented with 50 μg/mL gentamicin sulfate and 10% FBS. Cells were cultured at 1 ×10⁶ cells per 1 mL of culture medium. T-cells were cultured at 37°C in a humidified 5% CO₂, 95% air atmosphere.

**Measurement of Cytokines**

Cytokine levels in cell culture supernatants were determined using commercial enzyme-linked immune sorbent assay (ELISA) kits: Mouse Tumor Necrosis Factor-alph (TNF-α), IFN-γ, and Granulocyte Macrophage Colony-stimulating Factor (GM-CSF) (Bio Legend, San Diego, CA), as well as interleukin (IL)-2 and IL-4 (BD Biosciences, San Jose, CA).

**Cell Proliferation Assay**

Cell proliferation was determined using BrdU (chemiluminescent) (Roche Diagnostics, Mannheim, Germany).

**Statistical Analysis**

Results are expressed as means and standard deviation (SD). The significance of differences between means was measured by the Student’s t-test.

**Results and Discussion**

Cytokine and Chemokine Production Induced by SCG in Splenocytes Treated with BWMP.

In the previous study, we have examined the cooperative effects of PAMPs and BWMP in vitro using the spleen or bone marrow cells of mice. The splenocytes of male DBA/2 and C57BL/6 mice were cultured with BWMP in the presence or absence of PAMPs, and responses were assessed by measuring cytokines, TNF-α, IFN-γ, GM-CSF, and IL-6. Among these cytokines, BWMP most strongly inhibited the production of IFN-γ by not only toll like receptors (TLRs), but also the C-type lectin receptors (CLRs) dendit-1 and deictin-2. In contrast, TNF-α, and IL-6 were scarcely inhibited. BWMP also inhibited the autologous production of IFN-γ and IL-6 in the splenocyte culture [15, 16]. From the data shown in the previous study, we decided to use SCG, dectin-1 ligand, in the present study.

In order to precisely examine the profile of cytokine synthesis by SCG and the inhibition profile of BWMP, cytokine concentrations in the culture supernatant were examined using the multiplex cytokine assay kit (Mouse Cytokine / Chemokine Panel : MCYTMA: 70K- PX32; Merck Millipore, MA, USA). Splenocytes from DBA/2 mice were cultured for 48 h with SCG (100 μg/mL) in the presence or absence of BWMP (100 μg/mL). The resulting culture supernatant
was collected and analyzed by the multiplex assay kit (Figure 1). As shown in Figure 1, BWMP inhibited the production of IFN-γ, GM-CSF, monokine induced by gamma interferon (MIG (CXCL9)), interferon gamma-induced protein 10 (IP-10 (CXCL10)), IL-3, and leukemia inhibitory factor (LIF) by SCG. The production of IFN-γ, IL-3, and MIG (CXCL9) was markedly decreased by BWMP. These cytokines were most likely produced by activated T-cells. From this, we thought that BWMP effected on the T-cell.

Effects of BWMP on Cytokine Production in CD3+ T-Cells

In the next experiment, T-cells were purified by the T-cell enrichment column and the effects of BWMP on cytokine production were examined. CD3+ T-cells isolated from DBA/2 male mouse splenocytes were cultured for 48 h with BWMP (100 μg/mL) in the presence of Phorbol 12-Myristate 13-acetate (PMA; Sigma-Aldrich Co., St. Louis, MO) (500 ng/mL) + Ionomycin (20 nM), the mouse CD3ε antibody (250 ng/mL), and mouse CD28 antibody (Miltenyi Biotec, CA, USA) (100 ng/mL) (Figure 2). The production of TNF-α, IFN-γ, GM-CSF, and IL-4 induced by these stimuli was assessed.

As shown in Figure 2, the production of TNF-α, IFN-γ, GM-CSF, and IL-4 was induced by PMA + Ionomycin and the mouse CD3ε antibody, and markedly induced by the mouse CD3ε antibody. Furthermore, BWMP inhibited the production of TNF-α, GM-CSF, and IL-4, and markedly inhibited that of IFN-γ.

The DBA/2 mouse is known to exhibit strong reactivity to stimuli; therefore, the effects of BWMP on the standard strain, C57BL/6N, were also analyzed. CD3+ T-cells isolated from C57BL/6N male mouse splenocytes were cultured for 48 h with BWMP (100 μg/mL) in the presence of saline, PMA (500 ng/mL) + Ionomycin (20 nM), the mouse CD3ε antibody (250 ng/mL), and mouse CD28 antibody (100 ng/mL) (Figure 3). The effects of BWMP on the production of TNF-α, IFN-γ, GM-CSF, and IL-4 by these stimuli were assessed. As shown in Figure 3, the production of TNF-α, IFN-γ, GM-CSF, and IL-4 was induced by PMA + Ionomycin and the mouse CD3ε antibody. However, a comparison of the concentrations of cytokines induced by these stimuli revealed that they were significantly lower in C57BL/6N mice than in DBA/2 mice. Thus, we used DBA/2 mice in subsequent experiments. Furthermore, cytokines such as IFN-γ and IL-4 are the representative cytokines produced by CD4 positive T-cell, it is strongly suggested that BWMP affected on the CD4 positive T-cell not on the CD8 positive T-cell.

Effects of BWMP on Cell Proliferation and IFN-γ and IL-2 Production in CD3+ T-Cells

In order to analyze T-cell growth, the effects of BWMP on cell proliferation and the production of IL-2 by T-cells were examined. CD3+ T-cells were isolated from DBA/2 male splenocytes, and the
resulting T-cell fraction was cultured for 48 h on a black plate with saline, and mouse CD3ε antibody (250 ng/mL), and mouse CD3ε antibody + BWMP (100 μg/mL) (Figure 4). BWMP inhibited the mouse CD3ε antibody-induced proliferation of T-cells and production of IFN-γ.

The time-dependent effects of BWMP on the production of IL-2 and IFN-γ by T-cells were analyzed. CD3+ T-cells isolated from DBA/2 male mouse splenocytes with the Mouse CD3+ T-cell enrichment column (R&D) were cultured for 1, 2, 4, 8, 20, and 48 h with saline and mouse CD3ε antibody (250 ng/mL), mouse CD3ε antibody + BWMP (100 μg/mL), and mouse CD3ε antibody + tacrolimus (FK506; Sigma-Aldrich Co., St. Louis, MO) (10 ng/mL) (Figure 5). The effects of BWMP on the production of IFN-γ and IL-2 were assessed. Under these experimental conditions, BWMP inhibited the production of IL-2 at earlier time points, such as 4 h, and 20 h. However, at 48 h IL-2 concentration was not inhibited by BWMP, also shown in figure 4. It is probably due to the consumption of IL-2 by lymphocytes in the culture. In contrast, IFN-γ production was inhibited by BWMP during all cultivation stages in this experimental condition. In the previous publications two processes are known to be involved in the activation of T-cells. The first process involves the production of IL-2 through the stimulation of, for example, T-cell receptors (TCR), while the second process consists of the consumption of IL-2 by T-cells through autocrine and paracrine mechanisms in order to induce cell proliferation [18, 19]. It is also previously shown that IFN-γ is produced by activated T-cells [20]. These findings strongly support the results shown in this section that BWMP inhibited the production of IL-2 and IFN-γ in CD3+ T-cell stimulated by mouse CD3ε antibody.

**Effects of Timing of the BWMP Addition on Cell Proliferation and IFN-γ and IL-2 Production by CD3+ T-Cells**

We next demonstrated the kinetics of BWMP action on activated T-cells by cultivating for 24 h in the presence of BWMP and mouse CD3ε antibody. CD3+ T-cells isolated from DBA/2 male mouse splenocytes were cultured for 48 h with saline, and mouse CD3ε antibody (250 ng/mL), and mouse CD3ε antibody + BWMP (100 μg/mL; 0 h or 24 h) (Figure 6). The effects of BWMP on cell
Figure 3: Effects of BWMP on cytokine production by splenic CD3+ T-cells from C57BL/6N mice. T-cells were incubated for 48 h with BWMP (100 μg/mL) in the presence of various stimuli, i.e., PMA (500 ng/mL), Ionomycin (20 nM), a mouse CD3ε antibody (250 ng/mL), and mouse CD28 antibody (100 ng/mL). The concentrations of IFN-γ, TNF-α, GM-CSF, and IL-4 were determined by ELISA. Significant differences, ***p < 0.001.

Figure 4: Effects of BWMP on cell proliferation, IFN-γ production, and IL-2 production by splenic CD3+ T-cells from DBA/2 mice. T-cells were stimulated for 48 h with a mouse CD3ε antibody (250 ng/mL) and mouse CD3ε antibody in the presence or absence of BWMP (100 μg/mL). (A) Cell proliferation was determined by BrdU. The concentration of (B) IFN-γ and (C) IL-2 were determined by ELISA. Significant differences, ***p < 0.001.
proliferation and the production of IFN-γ induced by these stimuli were assessed. Under these experimental conditions, the effects of BWMP were the strongest when mouse CD3ε antibody was added at the beginning of the culture, and inhibited cell proliferation and IFN-γ production. In contrast, the effects of BWMP on T-cells were negligible when it was added after cultivating cells for 24 h.

These results suggested that BWMP was effective against T-cells at the relatively early period of T-cell activation and proliferation. It is also known that not endogenous, but exogenous IL-2 also enhanced proliferation of T-cells. In the case of BWMP, T-cells well responded to IL-2 to proliferate and to produce IFN-γ when a sufficient concentration of exogenous IL-2 was present during BWMP treatment. These findings suggested that the function of T-cells is not inhibited by BWMP, i.e., function of IL-2 receptor, the activation of Aurora B and Survivin mediated by the pathway NF-κB, and function of mTOR during G1/S checkpoint [21].

Effects of BWMP on Cell Proliferation And IFN-γ Production by CD3+ T-Cells in the Presence or Absence of IL-2.

T-cell proliferation is known to be related to TCR-mediated activation of NF-κB [21]. Since BWMP inhibited the production of IL-2, we demonstrated that IL-2 affected the production of IFN-γ in the presence of BWMP. CD3+ T-cells isolated from DBA/2 male mouse splenocytes were cultured for 48 h on a black plate with saline, the mouse CD3ε antibody (250 ng/mL), and mouse CD3ε antibody (250 ng/mL).
CD3ε antibody + BWMP (100 μg/mL) + recombinant (rm)-IL-2 (0, 1, 10, and 100 ng/mL) (Figure 7). The effects of BWMP on cell proliferation and IFN-γ production were assessed, but were not inhibited by IL-2. This may be due to the partial desensitization of IL-2 receptor resulting from the modulation of the immunological signaling between antigen presenting cells and T-cells. The process of IL-2 production was promoted by TCR, such as CD3, CD28 and CD4. The resulting signals were transmitted to NF-κB, NFAT and AP-1, a transcription factor of IL-2 [19, 21].

Conclusion

In the present study, we have demonstrated that BWMP inhibited IFN-γ and IL-4 production from CD3+ T-cell stimulated with CD3ε antibody. BWMP also inhibited IL-2 production and cell proliferation. The inhibitory effect of BWMP disappeared in the presence of excess amount of exogenous IL-2. From these findings, the molecular mechanism of BWMP-mediated T-cell suppression may be due to the inhibition of IL-2 signaling.

BWMP may be useful for the treatment of diseases such as inflammatory bowel disease [22-24] or chronic obstructive pulmonary disease [25], which are autoimmune disease and a certain population of T-cells might be activated during these diseases.

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