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Polysaccharide from black currant (*Ribes nigrum* L.) stimulates dendritic cells through TLR4 signaling

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Black currant (*Ribes nigrum*) has various beneficial properties for human health. In particular, polysaccharide from black currant was found to be an immunostimulating food ingredient and was reported to have antitumor activity in a mouse model. We named it cassis polysaccharide (CAPS). In a previous study, CAPS administration caused tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) production *in vitro* and *in vivo*, but the immunological mechanism of CAPS was not demonstrated. In this study, we revealed the CAPS immunostimulating mechanism *in vitro*. First, we found that CAPS activated dendritic cells (DCs). Second, we investigated whether it depends on Toll-like receptor 4 (TLR4) and myeloid differentiation primary response (Myd). We concluded that CAPS stimulates DCs through Myd88 depending TLR4 signaling and activates Th1-type cytokine release.

Key words: black currant, polysaccharide, immunostimulation, dendritic cell

INTRODUCTION

Black currant is well known as a healthy fruit and is used to make various foods and beverages such as jams and juices. Various reports described beneficial functions of black currant for human health [1], vasodilatation [2], eyestrain [3], and as an antivirus agent [4]. These properties are mainly due to the anthocyanins in black currant. In previous studies, we found immunostimulating effects of a polysaccharide, which we named cassis polysaccharide (CAPS), derived from black currant [5–7] and we identified its antitumor activity and ability to induce tumor necrosis factor-α (TNF-α) production in a mouse study [5, 6]. We also found that CAPS has an effect on macrophage activation in *in vitro* experiments [5, 6]. To understand the efficacy of CAPS in terms of its immunostimulating effects, it was very important to reveal the molecular mechanism of CAPS immunostimulating effects in detail. However, we were not able to determine that CAPS has an effect on DC activation and molecular recognition patterns.

Antigen-presenting cells (APCs) have an important role in immunomodulation [8–11]. In particular, dendritic cells (DCs) which are APCs, have a strong antigen presenting activity [12]. They are located in various organs, such as the stomach, intestine, and skin, and have the capacity to induce a primary immune response in inactive T cells. Inactive T cells differentiate into T helper 1 (Th1)-type T cells as a result of IL-12 stimulation [11]. Th-1-type T cells (natural killer T cells or killer T cells) have important roles in cancer therapy; for example, they remove virus and cancer cells [12]. Therefore, DCs are the most important APCs in the innate immune system.

There are various Toll-like receptors (TLRs) that recognize antigens according to their chemical structure [13]. TLR2 recognizes lipoteichoic acids, lipoproteins, and peptidoglycans of gram-positive bacteria [14]. TLR4 recognizes lipopolysaccharide (LPS) from gram-negative bacteria [14]. TLR9 recognizes bacterial CpG-DNA. These TLRs have important roles in the innate immune system [15], promote several signal transductions, such as transduction of nuclear factor-kappa B (NF-κB), and induce various cytokines. Myeloid differentiation primary response 88 (Myd88) is the key adaptor molecule used by several TLRs related to pathogenic microbial infections [16].

Some food ingredients such as lactic acid bacteria and polysaccharides (lignin-carbohydrates) have immunostimulating effects [17–20]. For example, orally administration of lactic acid bacteria (*Lactobacillus paracasei* KW-3110) improves allergy [21] and atopic dermatitis [22] through a TLR signaling pathway [23]. β-glucan also has an immunostimulating effect caused by TLR signal transduction

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[24].

In this study, we revealed the immunostimulating mechanism of CAPS. First, we found that CAPS activated DCs. Second, we investigated whether it depends on TLR4 and Myd88 signaling. Taken together, our results indicate that CAPS stimulates DCs through TLR4 signaling and activates Th1-type cytokine release.

MATERIALS AND METHODS

Sample preparation

Ribes nigrum was purchased from Central Chemical Co., Ltd. (Tokyo, Japan). Crude black currant juice was obtained, and glucanase enzymes were added as follows: 0.01% Sclase S (Mitsubishi-Chemical Foods Corporation, Tokyo, Japan) and 0.01% Viscozyme (Novozymes Japan Ltd., Chiba, Japan). After incubation at 48°C for 5 hr, inactivation was carried out at 80°C for 10 min. Then, the supernatant was recovered by centrifugation (8,000 rpm for 10 min). Ethanol precipitation was performed with a double volume of black currant juice, and the precipitant was collected by centrifugation (8,000 rpm for 10 min). Ethanol precipitation was repeated three times before lyophilization. Finally, the precipitant was dried with a freeze dryer and dissolved in a moderate volume of phosphate-buffered saline (PBS).

Endotoxin measurement

Endotoxin was analyzed with an Endozyme endotoxin test kit (Hyglos GmbH, Germany), according to the manufacturer's instruction.

Molecular weight distribution

The molecular weight of CAPS was analyzed by gel-filtration chromatography on a high-performance liquid chromatography system (Shimadzu Corporation, Kyoto, Japan) equipped with a Shodex OHpak SB-805 HQ column (Showa Denko, Tokyo, Japan) equilibrated with PBS at a flow rate of 1 ml/min. The detection was performed with an RID-20A refractive index detector (Shimadzu Corporation, Kyoto, Japan). A calibration curve was prepared with a dextran standard solution consisting of T-2000 (MW: 2,000,000), T-500 (MW: 473,000), T-70 (MW: 67,200), T-40 (MW: 43,000), T-10 (MW: 10,000), saccharose (MW: 342), and glucose (MW: 180). These markers were purchased from Pharmacosmos (Holbaek, Denmark).

Dietary fiber quantification

The dietary fiber content of CAPS was measured with the Proskey method by Japan Food Research Laboratories.

Mice

Specific pathogen-free male ICR wild-type, TLR2−/−, TLR4−/−, TLR9−/−, and MyD88−/− mice were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). The mice were housed under a 12:12-hr light/dark cycle with lights on at 600 hr in a thermoregulated room maintained at 24 ± 2.0°C. Humidity was maintained in the range of 55 ± 10%, and AIN-93G food (CLEA Japan, Inc., Tokyo, Japan) and water were provided. Dissection was performed at the age of 8 to 10 weeks. The animals were treated in accordance with Kirin Company's ethical guidelines for animal care, handling, and termination.

Preparation of DCs derived from bone marrow (BM)

FMS-like tyrosine kinase 3 ligand (Flt-3L) induces dendritic cell differentiation. Flt-3L-induced DCs were generated as previously described [25]. In berief, BM cells were extracted from ICR mice, and erythrocytes were removed by brief exposure to 168 mM NH_4Cl. Cells were cultured at a density of 5×10^5 cells/ml for 7 days in RPMI 1640 medium supplemented with 1 mM sodium pyruvate (Thermo Fisher Scientific, Yokohama, Japan), 2.5 mM HEPES (Thermo Fisher Scientific, Yokohama, Japan), 100 unit/ml penicillin (Thermo Fisher Scientific, Yokohama, Japan), 100 µg/ml streptomycin (Thermo Fisher Scientific, Yokohama, Japan), 50 µM 2-mercaptoethanol (Thermo Fisher Scientific, Yokohama, Japan), 10% fetal calf serum, and 100 ng/ml Flt-3L (R&D Systems, Inc., Minneapolis, MN, USA). CAPS was added at concentrations of 10–100 µg/ml, and cultures were maintained for 24 hr. LPS (1 µg/ml, Merck Millipore, Darmstadt, Germany) was used as a positive control.

Cell culture assay

BM-derived Flt-3L-induced DCs were cultured using 96-well microplates at a density of 1×10^5 cells/ml BM-derived Flt-3L-induced DCs were seeded in the same medium as used for cell preparation. LPS (1 µg/ml) or CAPS (10 or 100 µg/ml) was added to the medium, and cells were cultured for 24 hr at 37°C. Culture supernatants were assayed for cytokine concentration.

Cell viability measurement

Cell vitality was analyzed with Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer's instructions.

Cytokine measurement

TNF-α levels in the supernatant were measured with a Ready-SET-Go!® ELISA kit (eBioscience Inc., San Diego, CA, USA), according to the manufacturer's instructions.

Quantitative real-time PCR

Total RNA was purified with QIAshredder and RNeasy Mini kit (QIAGEN Japan, Tokyo, Japan) according to the manufacturer's directions. cDNA was synthesized with a PrimeScript II 1st strand cDNA Synthesis Kit (Takara Bio Inc., Kusatsu, Shiga, Japan). The expression levels were monitored by PCR with SYBR Premix Ex Taq II (Takara Bio Inc., Kusatsu, Shiga, Japan), according to the manufacturer's instructions. The cDNA mixture (250 ng) was added to 20 µl reaction mixture. The primers were as follows: GAPDH (tctgctgatgcccccatgttcg and tgggtggcagtgatggcatgga),

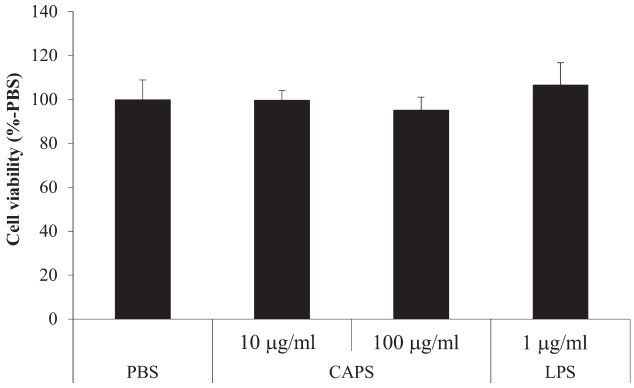

Fig. 1. Cell viability of dendritic cells from bone marrows (1.0×10^5 cells/ml) incubated with CAPS or LPS for 24 h, and cell viability were measured by Cell counting kit-8. The results are expressed as mean ± SD (n=6). There are no significantly difference by Dunnett test ($p<0.05$).

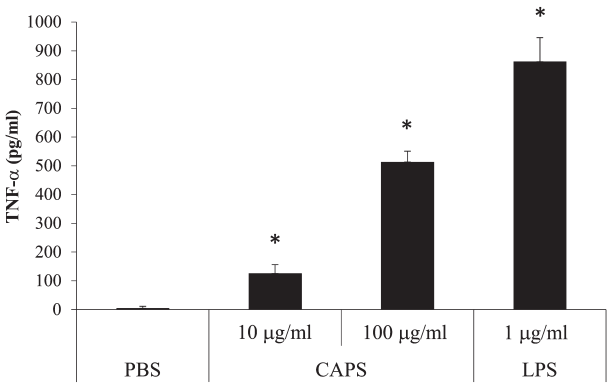

Fig. 2. Dendritic cells from bone marrow activation by CAPS administration. Effects of CAPS on TNF-α release. Dendritic cells from bone marrow s (1.0×10^5 cells/ml) were incubated with CAPS or LPS for 24 h, and cytokine levels were measured by ELISA. The results are expressed as mean ± SD (n=6). The differences between PBS and each sample were statistically significant by Dunnett test ($p<0.05$).

TNF-α (ctgtagcccacgtcgtagc and tgagatccatgccgttg), interleukin 1β (IL-1β; ccgtggaccttccaggatgaggaca and atgggtccgacagcacgaggcttt), and IL-12p40 (tctgctgatgcccccatgttcg and tgggtggcagtgatggcatgga). Reactions were performed using a LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany). Samples were quantified by comparison with a standard curve generated by cDNA templates using the respective primers. The value of the target gene was divided by the value of the GAPDH gene, and relative gene expressions were normalized to the value of the control.

Statistics

The statistical analysis was performed using the Ekuseru-Toukei 2010 statistical software (Social Survey Research Information Co., Ltd., Tokyo, Japan), and the results are presented as the mean ± standard deviation of the mean. Results were analyzed by Dunnett test.

RESULTS

CAPS samples

We obtained 13.5 g of freeze-dried CAPS powder from 3,454 g of black currant juice; its average molecular weight was 27,643. CAPS powder does not contain any endotoxin. The dietary fiber content was 828 mg per 1 g of CAPS powder.

Cytotoxicity of CAPS against bone marrow-derived dendritic cells (BMDCs)

In order to examine whether CAPS had cytotoxity in BMDCs, BMDCs were cultured for 24 hr with 10 or 100 μg/ml, CAPS and cell viability was assayed with a cell counting kit. As shown in Fig. 1, CAPS did not show cytotoxity in BMDCs at 10 or 100 μg/ml.

Production of TNF-α by Flt-3L–induced DCs in response to CAPS

In order to dissect the effects of CAPS on DC activation, we evaluated the production of TNF-α by BM-derived Flt-3L-induced DCs. LPS was used as positive control. As a result of supernatant analysis, we found that CAPS stimulated TNF-α production in a concentration-dependent manner (Fig. 2). In the same experiment, we collected the cells and extracted their mRNA. The results of RT-PCR showed that CAPS stimulated TNF-α, IL-1β, and IL-12p40 gene expression (Fig. 3).

Cytokine production by BMDCs derived from knockout mice

In order to determine the responsible recognition system leading to TNF-α induction by CAPS, Flt-3L-induced DC cultures were evaluated in selected TLR or MyD88 knockout mouse strains. As shown in Fig. 4, TNF-α production induced by CAPS was completely abrogated both in TLR4−/− and MyD88−/− mice.

DISCUSSION

DCs are widely distributed in peripheral tissues such as the skin, nasal cavity, and intestine [8–12]. They are antigen-presenting cells that play an important immunological role in mammalians [12] and transmit antigen signals to other immunological cells through lymphocytes [8–12]. In BMDC activation experiments, CAPS did not affect cell viability (Fig. 1), but it stimulated BMDCs and induced Th1-type cytokine release (Fig. 2). The results of endotoxin measurement confirmed that CAPS powder did not contain any endotoxins. The results of dietary fiber analysis revealed that CAPS contains 82.3% (w/w) dietary fiber. We strongly believe that the dietary fiber of black currant has an effect

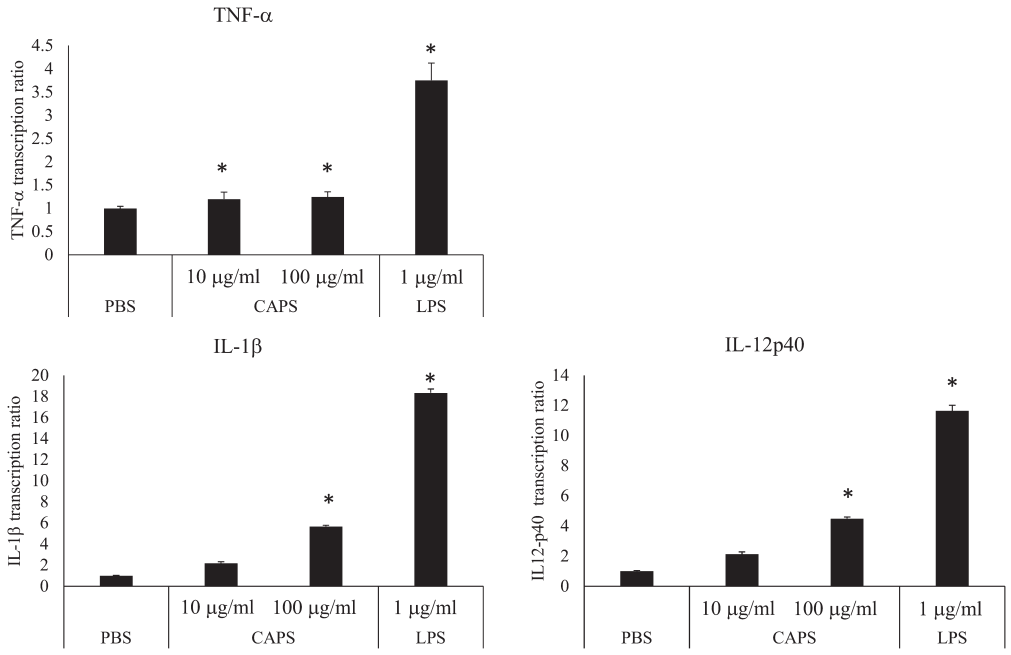

Fig. 3. BMDC transcriptional changes of TNF-α, IL-1β, and IL-12p40 by RT-PCR. BMDCs (1.0×10^5 cells/ml) were incubated with CAPS or LPS. The results are expressed as mean ± SD (n=6). The differences between PBS and each sample were statistically significant by Dunnett test ($p<0.05$).

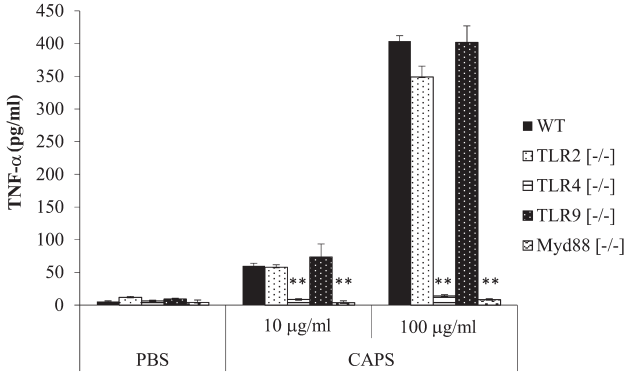

Fig. 4. Toll-like receptor recognition with CAPS. Dendritic cells from bone marrow (1.0×10^5 cells/ml) were incubated with CAPS. TNF-α was measured by ELISA. The results are expressed as mean ± SD (n=6). The differences between WT and each sample were statistically significant by Dunnett test ($p<0.05$).

on immunostimulation. Moreover, DCs promote the innate immune system through release of Th1-type cytokines, such as TNF-α (Figs. 2 and 3). In our previous studies, we detected an antitumor effect of CAPS in mice [5, 6]. Therefore, we assumed that CAPS could activate BMDCs and induce Th1-type lymphocytes due to the immunostimulating effect of CAPS.

TLRs have important roles [13], as they recognize molecular pattern of pathogens, regulate immunological balances, and control cell proliferation. We investigated the BMDC-TLR recognition pattern of CAPS with knockout mouse studies. As shown in Fig. 4, CAPS could not activate BMDCs derived from TLR4 or Myd88 knockout mice; therefore, we assumed that CAPS activated TLR4 on BMDCs. TLR4 recognizes polysaccharide antigens like the exopolysaccharide derived from lactic acid bacteria [26, 27], and CAPS is a polysaccharide purified from black currant juice [5, 6]; therefore, these studies support our conclusion concerning CAPS-TLR4 recognition. TLR4 is located on DC surfaces.

There are two types of TLR4 signaling, one dependent on Myd88 and one not dependent on Myd88. The Myd88-dependent pathway activates NF-κB through TIRAP and stimulates Th1-type cytokine release [15]. The Myd88-independent pathway activates IRF3 through TRIF and stimulates type 1 interferon [16]. In our experiments, CAPS could not activate type 1 interferon (data not shown), but CAPS does stimulate TNF-α release. We assumed that CAPS could stimulate DCs thorough a Myd88-dependent pathway, thus releasing mediators for host defense systems.

Th-1 type cytokine differentiation is very important for inducing oral tolerance [28]. Oral tolerance is also important for food immunology such as for suppressing excess allergic reaction [28]. CAPS stimulated Th-1 type cytokine release in a mouse study [5]. It is possible that intake of CAPS is related to suppression of excessive allergic reaction and induction of oral tolerance.

There are many TLR4 ligands, such as lignin-carbohydrate

[29], LPS [30]. TLR4 was found to be a polysaccharide receptor. In bacterial infections, TLR4 immediately senses the bacterial cell wall, which has an immunostimulating effect through a Myd88-dependent TLR4 pathway. Since the effect of CAPS on the immune system is similar to that of lignin-carbohydrate, the structure of CAPS might be similar to that of lignin-carbohydrate. This provides support for CAPS as a TLR4 ligand.

In conclusion, the immunostimulating mechanism of CAPS involves stimulation of DCs through a Myd88-dependent TLR4 pathway.

Conflict of interest

HA, YK, GW, YK, RY, RT, MM, and YT are employees of Kirin Co. Ltd., the study sponsor. KS is an employee of Mercian Corporation.

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