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Type II arabinogalactan from *Anoectochilus formosanus* induced dendritic cell maturation through TLR2 and TLR4

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ABSTRACT

Background: Polysaccharides, considered as immunomodulators with the capacity to activate immunity against microbial pathogens and tumors, have been employed for their dietary and medical benefits.
Purpose: This study investigated the immunomodulatory effect of polysaccharide such as type II arabinogalactan from *Anoectochilus formosanus* (AGAF) on dendritic cell (DC) maturation and the underlying molecular mechanisms.
Methods and results: Exposing DCs to AGAF induces cell maturation, which is characterized by the upregulation of CD86, CD83, CD80, CD40, and MHC class I and class II expression through flow cytometry analysis and morphological change without cytotoxicity. In addition, AGAF-triggered DC2.4 cells were involved in priming T-cell activation *in vitro* and *in vivo*. Transfection of toll-like receptor (TLR) 2 proteins and TLR4 siRNA suppressed DC maturation, suggesting that AGAF induced DC maturation through TLR2 and TLR4.
Conclusion: These findings indicate that AGAF may be a potentially effective immunomodulator in stimulating DC maturation.

Introduction

Various plants that contain major active compounds such as polysaccharides are used in traditional medicine to prevent several diseases. Polysaccharides offer numerous benefits including antioxidant (Liu et al. 2013; Wang et al. 2014b), anti-inflammatory (Chen et al. 2007; Liu and Lin 2012; Popov et al. 2014), antitumor (Yi et al. 2013), and antidiabetic effects (Xu et al. 2014). *Anoectochilus formosanus* (*Orchidaceae*) has been available as a commercial health-preserving tea or been processing to functional foods for several years in Taiwan, and the indigestible polysaccharides isolated from the aqueous extract of the plant have been reported to exert immunomodulatory effects (Yang et al. 2012). Chemical analyses demonstrate that the polysaccharides are composed mainly of type II arabinogalactan (average molecular weight, 29 kDa). We have shown that type II arabinogalactan extracted from *A. formosanus* (AGAF) exerts several prebiotic effects on gut microbiota, prevents bone loss, and reduces leukopenia during cancer therapy and immunotherapy (Yang et al. 2012, 2013a, 2013b, 2014a). Previous studies have indicated that polysaccharides could trigger the activation of immune effectors such as dendritic cells (DCs), natural killer (NK) cells, and T and B cells (Wang et al. 2014a; Zhang et al. 2014).

DCs are essential in innate and adaptive immunity and their numerous functions are closely related to maturation status. Morphological changes and the upregulation of costimulatory molecules (CD86, CD83, CD80, and CD40) during DC maturation are essential for the activation and recruitment of T cells or other immune effectors. Moreover, mature DCs initiate adaptive immune responses to present antigens through MHC class I and II to prime $CD8^+$ and $CD4^+$ T cells, respectively. Because of the critical role of DC maturation in inducting host immunity, determining the factors regulating these processes is necessary.

DC maturation can be induced by pathogen stimulation through pattern recognition receptors, such as toll-like receptors (TLRs) (Rhule et al. 2008). On TLR engagement, DCs migrate to lymphoid organs, where they prime T-cell activation. Although several TLRs have been identified in DCs, TLR2 and TLR4 appear to be particularly critical for DC maturation. Polysaccharides from the roots of *Cyathulae officinalis* Kuan enhance cellular and humoral immune responses by upregulating DC maturation via TLR2 and TLR4 signaling pathways (Feng et al. 2013). *Lycium barbarum* polysaccharide stimulation induces DC maturation through TLR2 and/or TLR4 signaling

Abbreviations: AGAF, type II arabinogalactan of *Anoectochilus formosanus*; DC, dendritic cell; MHC, major histocompatibility complex; CTLs, cytotoxic T lymphocytes; TLR, toll-like receptor; CFSE, carboxyfluorescein diacetate succinimidyl ester.

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(Zhu et al. 2013). However, the molecular mechanism by which AGAF induces DC maturation has not been studied. This study investigated whether AGAF induces DC maturation to prime T-cell activation and examined TLR signaling involvement.

Materials and methods

Preparing AGAF

A. formosanus was purchased from Yu-Jung Farm (Puli, Taiwan). This orchid species is not subject to international protection regulations. AGAF was prepared as described previously (Yang et al. 2012). In brief, the entire plant was homogenized in distilled water and then partitioned with ethyl acetate to obtain aqueous extracts. Ethanol was added to the aqueous extracts to precipitate crude polysaccharides, which were then treated with α-amylase, protease, and amyloglucosidase (Megazyme, Wicklow, Ireland) to remove starch and proteins. The indigestible polysaccharide was fractionated using anion-exchange chromatography on a DEAE-650 M column and eluted with a 20 mM Tris buffer followed by a sodium chloride gradient (0–0.3 M) to collect the neutral polysaccharide fraction, AGAF. The AGAF used in this study was pharmaceutical-grade (with a purity of $>$ 99%, according to analysis using the phenol-sulfuric acid method), protein, and the nuclear acid contamination in the AGAF was negligible (absorbance at 280 nm and 260 nm wavelengths was near zero). The monosaccharide composition of AGAF comprised arabinose, galactose, glucose, and mannose at a ratio of 22.4:56.5:15.4:5.4 (Yang et al. 2012). High-performance size exclusion chromatography (HPSEC) was used to determine the molecular weight (Mr) and purity of fractional collected AGAF. The molecular weight distribution of AGAF was a symmetrical peak with an average Mr of 29 kDa. The endotoxin content of AGAF, determined using the Toxin Sensor™ chromogenic LAL endotoxin assay kit (Gen-Script, NJ, USA), was less than 0.01 EU/ml, and there was no possibility of lipopolysaccharide contamination in the preparation process.

Cultivating DC2.4 cells

The murine DC line, DC2.4, adheres to the C57BL/6 bone marrow-derived DC line (Shen et al. 1997) and was provided by Dr. Hsieh (Department of Animal Science and Biotechnology, Tunghai University, Taichung, Taiwan). The DC2.4 cell line was cultured in a Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, 2 mM glutamine, and 10% FBS (Gibco).

Cultivating mouse primary immature dendritic cells

Bone marrow-derived DCs (BMDCs) from BALB/c mice were prepared as described by Inaba et al. (Inaba et al. 1992). The immature bone marrow DCs were cultured in a complete RPMI 1640 medium containing 10% FBS, 2 ml of penicillin–streptomycin, 20 ng/ml of recombination GM-CSF, and IL-4 (ProSpec). BMDCs were cultured and differentiated in a humidified atmosphere of 5% CO_2 at 37 °C for 5–6 days. Monocyte-derived DCs (MODCs) from BALB/c mice were generated from whole blood and adherent peripheral blood mononuclear cells were cultured in the presence of GM-CSF and IL-4, as described previously (Schreurs et al. 1999). The MODCs were grown and differentiated in a complete RPMI 1640 medium and a humidified atmosphere of 5% CO_2 at 37 °C for 4 days. Cells were analyzed by Flow cytometry with CD11c antibody and cell purity >90% was prepared for use in the experiment.

Cell viability assay

The cell viability of AGAF against DCs was evaluated using an MTS assay. The cells were seeded into a 96-well plate at a density of 10^4 cells/well. The cells were cultured in a CO_2 incubator at 37 °C for 1 day. AGAF was added to the cells in triplicate to analyze the viability of the cells at the indicated concentration. The cells were incubated for 48 h after treatment. Subsequently, 20 μl of MTS reagent was added to each of the treated wells and incubated for 4 h. Finally, the absorbance at 490 nm was measured using a microplate reader.

T-cell isolation and proliferation

T-cell isolation from BALB/c mice was performed according to the Dynabeads mouse CD4 and CD8 isolation kit guideline (Life Technologies, Taiwan). This immunomagnetic cell isolation method uses Dynabeads magnetic particles coated with CD4 and CD8 antibodies to capture and isolate $CD4^+$ and $CD8^+$ T cells from whole blood. In brief, magnetic beads coated with anti-CD14 antibody (for monocyte depletion) were added to deplete monocytes by first putting down monocytes. Beads coated with anti-CD4 and anti-CD8 monoclonal antibodies were then added to capture and isolate $CD4^+$ and $CD8^+$ T cells from the monocyte-depleted residual buffer and to gain the high purity of $CD4^+$ and $CD8^+$ T cells (the purities of the $CD4^+$ and $CD8^+$ T cells were detected using flow cytometry as being approximately 94.5% and 92.7%, respectively). The isolated $CD4^+$ and $CD8^+$ T cells (1×10^5 cells/ml) were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, 1.2 μM; Invitrogen) for 10 min at 37 °C, washed twice with a complete RPMI medium, and cultured with DCs (1×10^5 cells/ml). The cells were harvested after 3 days. T-cell proliferation was measured according to CFSE fluorescence, which was analyzed using flow cytometry.

White blood cell and splenic cell preparation in BALB/c mice

The BALB/c male mice were injected intravenously with AGAF-triggered DC2.4 cells (2×10^6 cells/mice) for 3 days. Animals were divided into 4 groups (0, 1, 10, and 100 μg/ml of AGAF-treated DC2.4 cells), each with $n = 8$. Finally, the animals were euthanized using CO_2. Peripheral blood was collected for red blood cell (RBC) sedimentation with 3% (w/v) Dextran-500 (average molecular weight 200,000–500,000). RBCs were lysed with a lysis buffer (for 5 ml: 41.45 g NH_4Cl, 5 g $NaHCO_3$, and 1 ml of 0.5 M EDTA in H_2O) for 20 min at room temperature. After spinning down, the obtained cell pellet was prepared for flow cytometry analysis. The spleens were removed, weighed, and then used to prepare the splenic cells. The spleens were aseptically removed and placed in a cold RPMI 1640 medium (Gibco). The spleens were then teased apart and passed through nylon mesh (BD Falcon). The splenic cell suspensions were hemolyzed by an RBC lysis buffer and neutralized with 5 ml of an RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, 100 μM 2-2-mercaptoethanol, 1.0 mM sodium pyruvate, and gentamycin (Gibco). The single splenic cell suspensions were centrifuged at 4 °C, 300 $\times$ g for 10 min for collection.

Small interfering RNA (siRNA) transfection

At a density of 2×10^5 DC2.4 cells/well, cells were seeded onto a 6-well dish with a normal growth medium for 24 h before transfection. Subsequently, 5 μl of 10 μM siRNA and 5 μl of the Santa Cruz siRNA transfection reagent were mixed separately with 100 μl of the siRNA transfection medium. The reagent sets were combined and incubated at room temperature for 30 min. After the cells were washed with the siRNA transfection medium, transfection complexes were added to the cells in the presence of 800 μl/well of the siRNA transfection medium for 6 h, after which an equal volume of normal growth medium (2 $\times$) was added to each well. The media were replaced with normal growth medium (1 $\times$) after 24 h and the cells were then cultured with AGAF for 48 h for coculture with T cells and extracted for western blotting.