Neoagarohexaose activates dendritic cells via Toll-like receptor 4, leading to stimulation of natural killer cells and enhancement of antitumor immunity

Moon Hee Lee[1,*], Jong-Hwa Jang[2,*], Gun Young Yun[1,*], Min-Goo Lee[3], Tae Heung Kang[1], Hee Dong Han[1], Seung Jun Lee[1], Hyuk Soon Kim[1], Wahn Soo Choi[1], Won Sun Park[4], Yeong-Min Park[1,**] and In Duk Jung[1,**]

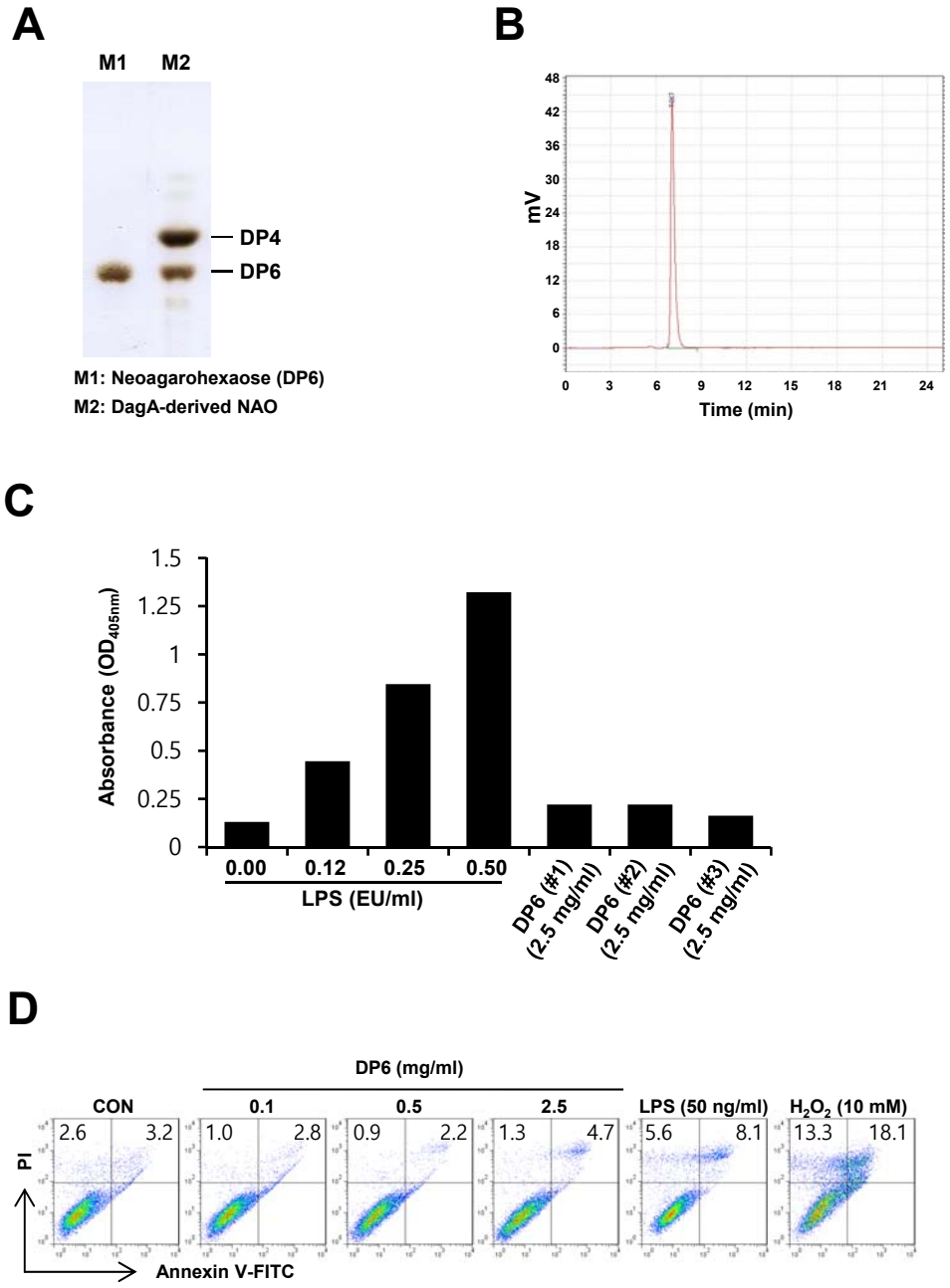
[1]*Department of Immunology, Laboratory of Dendritic Cell Differentiation and Regulation, School of Medicine, Konkuk University, Chungju 380-701, South Korea.*

[2]*Department of Dental Hygiene, Hanseo University, Seosan 356-706, South Korea.*

[3]*Department of Physiology, Korea University, College of Medicine, Anam-dong, Sungbuk-Gu, Seoul 136-705, South Korea.*

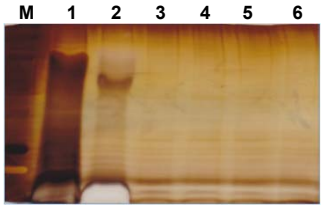
[4]*Institute of Medical Sciences, Department of Physiology, Kangwon National University School of Medicine, Chuncheon, 200-701, South Korea.*

Supplementary Figure 1



Supplementary Figure . Identification and cytotoxicity of DP6. (A) Thin-layer chromatography (TLC) of DP4 and DP6 produced by b-agarase DagA. (B) The purity of DP6 was analyzed by high performance liquid chromatography (HPLC). (C) The endotoxin assay (LAL assay) was performed to check for LPS contamination in three different batches of purified DP6. (D) DCs were treated with indicated concentration of DP6, LPS (50 ng/mL), or H_20_2 (10mM) for 24 h and stained with AnnexinV and PI, and analyzed by flow cytometry. The results of one representative experiment out of three experiments with similar results are shown.

Supplementary Figure 2



M: marker
1: *E.coli* LPS (10 mg/ml)
2: *Pseudomonas aeruginosa* LPS (10 mg/ml)
3: PBS
4. DP6 (1 mg/ml)
5. DP6 (10 mg/ml)
6. DP6 (100 mg/ml)

Supplementary Figure 2. Silver staining assay to check the LPS contamination on purified DP6. PBS (lane 3), *E. coli* LPS (10 mg/ml, lane 1), *P. aeruginosa* LPS (10 mg/ml, lane 2) and DP6 (1, 10, and 100 mg/ml, lane 4-6) were applied to each well on SDS-PAGE and stained by the conventional silver staining method as described in Materials and Methods section.

Reagents and antibodies

Recombinant, mouse granulocyte-macrophage colony-stimulating factor (rmGM-CSF) was purchased from JW Creagene (Gyeonggi-do, Korea). Dextran-fluorescein isothiocyanate (FITC; 40,000 Da) and lipopolysaccharide (LPS, from *Escherichia coli* O111:B4 and *Pseudomonas aeruginosa* 10) were purchased from Sigma-Aldrich (St. Louis, MO, USA). CD16/CD32 (Clone: 93) was purchased from BioLegend (San Diego, CA, USA). The following FITC- or phycoerythrin (PE)-conjugated monoclonal antibodies (Abs) were purchased from eBioscience (San Diego, CA, USA): CD11c (HL3), CD80 (16-10A1), CD86 (GL1), I-A^b β-chain (AF-120.1), H-$2K^b$ (AF6-88.5), and NK1.1 (PK136).

Cell lines

B16F1 cells, purchased from the Korea Cell Line Bank (Seoul, Korea), were cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM L-glutamine (all purchased from Biowest) at 37°C in an atmosphere containing 5% CO_2.

Preparation of DP6

DP6 was obtained from DyneBio Inc. (Gyeonggi-do, Korea). After dissolving 10 g of agarose in 1 L water at 100°C for 10 min to produce 1% (w/w) agarose solution, it was cooled to 40°C by shaking on a shaking incubator, following which 250,000 units DagA was added and the solution was incubated with shaking at 100 rpm for 24 h at 40°C. The non-degraded material was removed by centrifugation (6,000 rpm for 15 min at 4°C), and the supernatant was collected as NAO. The components of NAO included DP4 and DP6, as visualized by thin layer chromatography (TLC); it was ultra-filtrated through a 5-kDa membrane (Millipore, MA, USA). The composition of NAO was determined on Bio-Gel P-2 gel (Bio-Rad

Laboratories, CA, USA) by gel permeation chromatography (GPC) and quantified by high performance liquid chromatography (HPLC).

Silver Staining

Escherichia coli LPS, *Pseudomonas aeruginosa* LPS and DP6 were fractionated by the SDS-PAGE. The separated SDS-PAGE gels were stained with the conventional method of silver staining kit (Biosesang, Seongnam, Korea).

Generation and culture of DCs

Bone marrow, flushed from the tibiae and femurs of 6–8-week-old female C57BL/6 mice, was depleted of red blood cells (RBC) by treatment with RBC-lysing buffer (Sigma-Aldrich). The cells were plated in 6-well culture plates (1×10^6 cells/ml; 2 ml/well) in RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 20 ng/ml rmGM-CSF at 37°C in an atmosphere containing 5% CO_2. On Days 3 and 5, the floating cells were carefully removed and fresh medium was added. On Day 6, non-adherent cells and loosely adherent, proliferating DC aggregates were harvested for analysis or stimulation. On Day 7, 80% or more of the non-adherent cells expressed CD11c.

Quantification of antigen uptake

An aliquot of 2×10^5 DCs was equilibrated at 37°C or 4°C for 30 min and pulsed with 1 mg/mL FITC-conjugated dextran for 45 min, after which the reaction was stopped with cold staining buffer. Cells were washed twice, stained with PE-conjugated anti-CD11c, and analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Cytokine ELISA

The quantities of TNF-α, IL-1β, IL-6, IL-10, IL-12p70, and IFN-γ in the culture supernatant were determined using sandwich ELISA, according to manufacturer instructions (eBioscience).

Western blot

Western bot analysis was experimented according to the procedure of Lee *et al.* [18].

Isolation of NK cells by magnetic activated cell sorting (MACS)

NK cells were purified from the spleen of C57BL/6 mice by using MACS-negative selection beads (Miltenyi Biotec, Germany), according to manufacturer instructions. A single-cell suspension of splenocytes was prepared and resuspended in RPMI 1640 (Biowest, France) medium supplemented with 10% FBS, 10 mM HEPES, 2 mM l-glutamine, 100 units/mL penicillin-streptomycin, and 5 mM 2-mercaptoethanol.

Isolation of peritoneal exudate cells

Peritoneal exudate cells (PECs) were harvested 24 h after i.p. DP6 administration. Their abdomens were swabbed with 70% alcohol and 10 ml cold, sterile PBS was injected into the peritoneal cavity. PECs were harvested using a syringe, with 2×10^5 cells being collected for each sample, and washed twice with cold PBS. PECs were pre-stained with anti-mouse CD16/32 for 10 min before staining with FITC-conjugated NK1.1 and PE-conjugated CD69.