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

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Running Title: Neoagarohexose induces antitumor immunity

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ABSTRACT

β-agarase cleaves β-1,4 linkages of agar to produce neoagarooligosaccharides (NAO), which are associated with various physiological functions. However, the immunological functions of NAO are still unclear. In this study, we demonstrated that β-agarase DagA-produced neoagarohexaose (DP6), one among the many NAO products, promotes the maturation of dendritic cells (DCs) by Toll-like receptor 4 (TLR4). DP6 directly and indirectly enhanced the activation of natural killer (NK) cells in a TLR4-dependent manner *in vitro* and *in vivo*. Finally, the antitumor activity of DP6 against B16F1 melanoma cells was inhibited in NK cell-depletion systems by using NK-cell depleting antibodies *in vivo*. Taken together, the results indicate that DP6 augments antitumor immunity against B16F1 melanoma cells via the activation of DC-mediated NK cell activity in a TLR4-dependent manner. These results suggest that DP6 might be a promising candidate adjuvant that acts as an immune cell modulator for the treatment of melanoma.

INTRODUCTION

Agar, composed of β-D-galactose linked by β-1,4 linkages and 3,6-anhydro-α-L-galactose linked by α-1,3 linkages, can be hydrolyzed by α-agarase and β-agarase, respectively [1]. β-agarase cleaves the β-1,4 linkages of agar, producing neoagarooligosaccharides (NAO) including neoagarobiose (DP2), neoagarotetraose (DP4), and neoagarohexaose (DP6) [2]. While several reports suggest that NAO inhibit bacterial growth [3], and stimulate macrophages [4], the functional analysis of DP2, DP4, or DP6, which are single compounds of NAO, has not been undertaken. In this study, we used β-agarase (DagA) purified from *Streptomyces coelicolor* A3(2), which is a non-toxic, gram-positive soil bacterium that is well known for its use in the production of approximately 85% of all antibiotics [1]. However, the immunological functions of β-agarase DagA-produced NAO, especially DP6, have not been studied yet. Therefore, we focused on the physiological functions of β-agarase DagA-produced DP6, especially, its role in the activation of dendritic cell (DC)-mediated cancer immunotherapy to verify whether DP6 might be a promising immunomodulatory agent.

The communication between NK cells and DCs influences both innate and adaptive immunity and enhances Th1 and CTL-mediated antitumor efficacy [5]. Mature DCs (MHC II ^{high}CD86^{high}CD11c^{+}) stimulate NK cells via soluble factors (IL-2, IL-12, IL-15, IL-18, IFN-α, and IFN-β) as well as direct cell-to-cell contact (ligation of NKp46, NKp30, NKG2D, 2B4, and CD27 as well as IL-15 in trans), leading to cytotoxicity, cytokine secretion (IFN-γ and TNF-α), and proliferation of NK cells [11]. On the other hand, IFN-γ-producing NK cells (CD69^{+}NK1.1^{+}) induce the maturation of DCs and type-1 polarized DCs producing pro-inflammatory cytokines [6]. In addition, NK cell-derived IFN-γ up-regulates Th1 transcription factor GATA-3 [6]. The bidirectional interaction between NK cells and DCs is

believed to influence both, NK and T-cell responses against target cells [7].

Our study aimed to identify the immunological actions of the natural polysaccharide DP6. DP6 activates DCs by activating mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB) signaling via Toll-like receptor 4 (TLR4). In addition, the administration of DP6 was shown to have TLR4-dependent antitumor effects against B16F1 melanoma *in vivo*. Further, stimulation of NK cells in response to DP6 was demonstrated *in vitro* and *in vivo*. In addition, DP6-induced maturation of DCs was shown to trigger stimulation of $NK1.1^{+}$ cells *in vitro*. Lastly, NK cell-mediated antitumor immunity in response to DP6 was investigated *in vivo*. Together, the results from this study provide new insights into the immunomodulatory activities of DP6. To our knowledge, this is the first study to demonstrate that DP6 induces DC maturation, leading to the stimulation of NK cells and the enhancement of antitumor immunity.

RESULTS

DP6 induces the activation of DCs via TLR4

To verify the products of agar hydrolysis, reaction products including DP6 were separated and identified by thin layer chromatography (TLC). As shown in Supplementary Fig. 1A, our purified single compounds, DP6 (M1), were observed on a TLC plate, compared to DagA-derived NAO, which was used as a positive marker (M2). The purity of DP6 after gel permeation chromatography was quantified by HPLC Evaporative Light Scattering Detector (HPLC ELSD) and confirmed to be approximately 95% (Supplementary Fig. 1B). To exclude the possibility of contamination with endotoxin, the purified DP6 was subjected to LAL endotoxin detection assay (Supplementary Fig. 1C) and silver staining (Supplementary Fig. 2), followed by confirmation to ensure that the purified DP6 sample was not contaminated with endotoxins. In addition, to check whether DP6 had cytotoxic effects on dendritic cells (DCs), the rate of apoptosis or necrosis was analyzed by flow cytometry (Supplementary Fig. 1D) in DP6-treated DCs. While treatment with H_2O_2, as an apoptosis inducer, markedly increased the apoptosis, DP6 had no effects on cytotoxicity towards DCs.

To investigate whether DP6 affects the maturation of DCs, the expression of surface molecules (CD80, CD86, MHC class I, and MHC class II) were measured. Expression levels of the surface molecules in DP6-treated DCs was increased as well as LPS-treated DCs (Fig. 1A-left and right panel). In addition, the secretion of pro- and anti-inflammatory cytokines (TNF-α, IL-6, IL-1β, IL-12p70, and IL-10) was significantly elevated in DP6-treated DCs (Fig. 1B). Furthermore, DP6 significantly diminished the percentage of dextran$^+$CD11c$^+$ cells (Fig. 1C), as compared to untreated DCs, indicating that DP6 enhances phenotypic and functional maturation of DCs

Toll-like receptors (TLRs) have been suggested to play an important role in the

activation of DCs [8], and TLR4 is necessary for the activation of immune cells by several natural polysaccharides [9]. Therefore, to examine whether TLR signaling is involved in DP6-mediated DC activation, the expression of surface molecules and the production of cytokines were measured in DP6-treated DCs derived from WT, TLR2$^{-/-}$, TLR4$^{-/-}$, and TLR9$^{-/-}$ mice. DP6 induced the expression of surface molecules, and the production of cytokines in DCs from TLR4$^{-/-}$ mice was significantly decreased as compared to that in DCs from WT, TLR2$^{-/-}$, and TLR9$^{-/-}$ mice (Fig. 2A and 2B).

Next, to investigate whether DP6 stimulates the activation of MAPKs, AKT, and NF-κB, which are crucial for TLR4-mediated DC activation [10], the phosphorylation levels of MAPKs and AKT and the degradation levels of p65 in response to DP6 were identified in DCs from WT and TLR4$^{-/-}$ mice (Fig. 2C). As shown in Fig. 2C, DP6-induced phosphorylation of ERK, p38MAPKs, JNK, and AKT was elevated in DCs from WT mice, however, those was not induced in DP6-treated DCs from TLR4$^{-/-}$ mice. In addition, DP6 decreased the level of the p65 subunit of NF-κB in the cytosolic fraction of DCs from WT mice but not in the cytosolic fraction of DCs from TLR4$^{-/-}$ mice. These results indicate that TLR4-mediated activation of MAPKs, AKT, and NF-κB might be involved in DP6-mediated DC activation.

DP6 augments TLR4-dependent antitumor immunity *in vivo*

Since DCs are known to induce antitumor activity by modulating immune responses [8], the possible antitumor effect of DP6 against B16F1 melanoma cells *in vivo* was investigated. Briefly, C57BL/6 mice were intraperitoneally (i.p.) administered PBS or DP6 (100 or 200 mg/kg), every other day and subcutaneously (s.c.) inoculated with B16F1 melanoma cells during the course of PBS or DP6 administration (Fig. 3A). As shown in Fig.

3B, administration of 100 and 500 mg/kg DP6 showed significant inhibition of tumor growth compared to that observed in the group administered PBS (Fig. 3B). Therapeutic effect of DP6 against B16F1 melanoma cells was shown in images depicting two groups of mice at 18 days, one treated with PBS and the other with 500 mg/kg DP6 (Fig. 3C).

TLR4 is known to be involved in DP6-induced DC activation (Fig. 2); hence, the role of TLR4 in DP6-mediated antitumor effects in WT and $TLR4^{-/-}$ mice was investigated. Mice were i.p. administered 500 mg/kg DP6 every other day and s.c. inoculated with B16F1 melanoma cells during the course of DP6 administration. Administration of DP6 significantly diminished the tumor mass in WT mice compared to that in $TLR4^{-/-}$ mice administered DP6 (Fig. 3D). DP6 exerted TLR4-dependent antitumor effects on B16F1 melanoma cells *in vivo*.

DP6 directly and indirectly enhances the activation of NK cells in a TLR4-dependent manner and augments NK cell-mediated antitumor immunity *in vitro* and *in vivo*

It has been previously reported that NK cells are essential for killing MHC class I-negative tumor cells such as B16F1 melanoma cells [11]. As DP6 showed an antitumor effect on B16F1 melanoma cells *in vivo* in this study (Fig. 3), it was expected that DP6 would induce NK cell stimulation *in vitro* and *in vivo*. To investigate whether DP6 induces the activation of NK cells *in vivo*, the activated $CD69^{+}NK1.1^{+}$ cells among the peritoneal exudate cells (PECs) derived from C57BL/6 mice administrated 500 mg/kg DP6 were identified by flow cytometry analysis. As shown in Fig. 4A, mice administrated DP6 generated a higher percentage of $CD69^{+}$ $NK1.1^{+}$ cells in PECs as compared to that observed in mice administrated PBS, which was used as a negative control. Next, to verify whether DP6 activates NK cells *in vitro*, $NK1.1^{+}$ cells were isolated from C57BL/6 mice by the MACS bead method and then treated with 2.5 mg/ml of DP6. The percentage of activated

$CD69^{+}NK1.1^{+}$ cells in response to DP6 was evaluated by flow cytometry analysis (Fig. 4B). The secretions of IFN-γ and TNF-α were significantly elevated in culture supernatants from NK cells treated with 2.5 mg/ml of DP6 (Fig. 4C). Taken together, these results demonstrated that DP6 contributed to NK cell activation *in vitro* and *in vivo*.

Since we previously found that DP6 can activate both DCs (Fig. 1) and NK cells (Fig. 4A-C), we wondered whether DP6 controlled the crosstalk between DCs and NK cells, which is a critical factor in anti-cancer immunity [12]. To investigate whether DP6 modulates the crosstalk between DCs and NK cells, $NK1.1^{+}$ cells were treated with the culture supernatant of DP6-treated DCs from WT and $TLR4^{-/-}$ mice. The percentage of $CD69^{+}$ $NK1.1^{+}$ cells treated with DC supernatant was determined by flow cytometry. While the activation of $CD69^{+}$ $NK1.1^{+}$ cells and secretion of IFN-γ were enhanced in cells cultured with the supernatant from WT-DCs activated by DP6, these parameters were significantly diminished in cells cultured with the supernatant from $TLR4^{-/-}$-DCs activated by DP6 (Fig. 4D and 4E). The data demonstrated that DP6 might induce the activation of NK cells by activating DCs via TLR4.

Given the observed effect of DC-mediated NK cell stimulation by DP6, the role of NK cells in DP6-mediated antitumor effects was investigated. The NK cell-depleted mice did not generate a significantly different tumor mass and weight as compared to that observed in the group administered PBS (Fig. 4F and 4G). Therefore, these data demonstrated that NK cells are essential for DP6-mediated antitumor immunotherapy *in vivo*.

DISCUSSION

Several natural polysaccharides with immunostimulatory properties have been investigated to date. In addition, natural polysaccharides with immunostimulatory properties such as β-glucans have been considered as a promising adjuvant to cancer immunotherapy [13]. While immunological functions of β-agarase-produced NAO have been studied [19], those of NAO produced by nontoxic β-agarase DagA from *Streptomyces coelicolor* A3(2) have not been studied yet. In this study, β-agarase DagA-produced DP6: 1) induced DC activation in a TLR4-dependant manner; 2) triggered the stimulation of NK cells *in vitro* and *in vivo* and DP6-activated DCs indirectly induced the stimulation of NK cells *in vitro*; and 3) induced DC- or NK cell-mediated antitumor immunity via TLR4.

The results of this study demonstrated that DP6 caused the activation of DCs via TLR4 by activating MAPKs, AKT, and NF-κB (Fig. 2C). Whereas TLR4 acts as a main receptor of DP6 in DC activation, other candidate receptors still need to be considered because both TLR4 and dectin-1 have been reported as main receptors of β-glucans, which is well-known natural polysaccharides, in DCs [14]. Even though dectin-1 is a well-known receptor of polysaccharides in DCs, it is not involved in DP6-mediated DC activation (data not shown). Our study suggested that TLR4 mainly acts as a receptor of DP6; however, it is still unclear whether DP6 might be a ligand of other receptors. Therefore, the involvement of other receptors and related intracellular signaling pathways in response to DP6 remain to be explored in further studies.

Owing to the observation in Figure 1 that DP6 induced the activation of DCs, it was speculated that DP6 might be effective in cancer immunotherapy. In our experiments, we have used B16F1 melanoma cell line instead of B16F10 for cancer immunotherapy *in vivo*, because of the B16F1 was found to generate the drug-resistant variants at lower rate than the

B16F10 [15]. Accordingly, we observed that DP6 administration led to a significant anti-tumor response against B16F1 melanoma cells *in vivo*. In general, other natural polysaccharides such as β-glucan do not directly act on cancer cells but enhance antitumor immunity via activation of neutrophils, B cells, T cells, NK cells, dendritic cells, and macrophage-mediated immune responses [16]. Since NK cells are essential for the antitumor response against tumors with down-regulated expression of MHC class I molecules such as B16F1 and TC-1 P3 (A15) [17], it is hypothesized that NK cells play a role in DP6-mediated anti-cancer activity against B16F1. The data obtained in this study demonstrate that DP6 enhances NK cell-mediated antitumor immunity, implying that DP6 administration results in antitumor effects against MHC class I down-regulated tumor cells via NK cell-mediated cytotoxicity.

The reciprocal interaction between DCs and NK cells is crucial to enhance the efficacy of anticancer immunotherapy [6]. DCs trigger NK cell functions by increasing cytotoxicity, cytokine secretion (IFN-γ, TNF-α), and proliferation [6]. This study demonstrated DP6-mediated DC activation; therefore, it is plausible that DP6-activated DCs might trigger the stimulation of NK cells. As expected, soluble factors led to an increase in the percentage of $CD69^{+}NK1.1^{+}$ cells and the level of IFN-γ secreted by $NK1.1^{+}$ cells *in vitro*. This study thus proved that DP6-activated DCs stimulate NK cells. In future studies, it will be interesting to verify whether DP6-stimulated NK cells activate DCs in reverse.

Taken together, our results suggest that DP6 exhibits antitumor activity against B16F1 melanoma cells by controlling the crosstalk between DCs and NK cells via TLR4, indicating that DP6 could be considered a promising adjuvant for cancer immunotherapy.

MATERIALS AND METHODS

Animal

Female C57BL/6 mice aged 6–8 weeks were purchased from Orient (Daejeon, Korea) and Female C57BL/6J TLR2 knockout mice (TLR2$^{-/-}$; B6.129-*Tlr2*^{tm1Kir}/J), C57BL/10 TLR4 knockout mice (TLR4$^{-/-}$; C57BL/10ScNJ), and C57BL/10 TLR9 knockout mice (TLR9$^{-/-}$; C57BL/6J-*Tlr9*^{M7Btlr}/Mmjax) were purchased from the Jackson Laboratory (USA) and maintained in filter-top cages under standard conditions (12-hour light/dark cycle), with food and water provided *ad libitum* and in specific pathogen-free animal facility at the Korea Research Institute of Bioscience and Biotechnology. Animals were sacrificed in the CO_2 chamber and organs were harvested instantly.

Cytotoxicity Analysis

Bone marrow-derived dendritic cells (BMDCs) from C57BL/6 mice were treated with 0.5, 1.0, 2.5 mg/ml DP6 , 2.5 mg/ml LPS and 10mM H_2O_2 for 30 min and Add a volume of CellTiter-Glo® Reagent (Promega). And then BMDCs were analyzed viability through luminescence.

Flow cytometry analysis

The stained cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA), and data were processed using the Cell Quest Pro software (BD Biosciences).

In vivo Ab-mediated depletion of NK cells

C57BL/6 (five mice/group) were injected intraperitoneally (i.p.) with the mAb anti-NK1.1

(clone#:PK136, BioXcell) on days −2, 0, +2, +4, +6, +8, +10, and +12 (0.2 mg·mouse^{-1}·d^{-1}) for NK cell depletion. Mice were injected i.p. with 500 mg/kg DP6 or phosphate-buffered saline (PBS; negative control) on days 2, 5, 8, 11, 14, and 17. On day 0, B16F1 melanoma cells (1×10^5 cells/mouse) were injected subcutaneously (s.c.) into the left inguinal region.

Statistical analysis

All experiments were repeated at least three times. The levels of significance for comparison between samples were determined by Turkey's multiple comparison tests by using GraphPad InStat software (Ver 3.1, GraphPad). The data in the graphs are expressed as the mean ± SEM.

We presented the information of another Material and Methods in a Supplementary Methods.

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FIGURE LEGENDS

Figure 1 DP6 induces the phenotypical and functional maturation of DCs. (A-B) Immature DCs were treated with 0.1, 0.5, and 2.5 mg/ml DP6 or 50 ng/ml LPS for 24 h. (A) Flow cytometry was used to analyze the expression of surface molecules on $CD11c^+$ cells (left panel). The percentage of positive cells is shown in each panel and bar graph (right panel). The results of one representative experiment out of three experiments are shown. The data are presented as the means and standard error of the mean (SEM). $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to untreated DCs. (B) ELISA was performed to test TNF-α, IL-6, IL-1β, IL-12p70, and IL-10 production in DP6- or LPS-treated DCs. The data are presented as the means and standard error of the mean (SEM, $n = 3$). $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to untreated DCs. (C) Endocytic activity of DP6-treated DCs. Endocytic activity of dextran-FITC uptake by DCs treated with medium, LPS, or DP6 was assessed at 37°C or 4°C (as a control) by flow cytometry analysis. The percentages of dextran-$CD11c^+$ cells are indicated. The results of one representative experiment out of three experiments with similar results are shown.

Figure 2. DP6 induces Toll-like receptor 4 (TLR4)-mediated DC activation. (A-B) Immature DCs from WT, $TLR2^{-/-}$, $TLR4^{-/-}$, and $TLR9^{-/-}$ mice were treated with 0.5 or 2.5 mg/ml DP6 or 50 ng/ml LPS for 24 h. (A) Histogram showing CD80, CD86, MHC class I, or MHC class II expression on $CD11c^+$ cells. The percentage of positive cells is shown in each panel. The results of one representative experiment out of three experiments are shown. (B) ELISA was performed to test IL-1β, IL-12p70, and IL-10 production in DP6- or LPS-treated DCs. The data are presented as the means and standard error of the mean (SEM, $n = 3$). $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to 2.5 mg/ml DP6-treated WT DCs. (C) Immature DCs

from WT and TLR4$^{-/-}$ mice were treated with 1 mg/ml DP6 at the indicated time points. The cells were harvested, and the cell lysates were detected by immunoblot with anti-p-ERK, anti-ERK, anti-p-p38, anti-p38, anti-p-JNK, anti-JNK, anti-p-JNK, anti-p-AKT, anti-AKT, anti-p65, or anti-α-tubulin Abs (upper panel). The bar graph illustrates the relative intensity of upper panel (lower panel).

Figure 3. DP6 augments antitumor immunity in a B16F1 melanoma tumor model in TLR4-dependent manner. (A-C) Mice (six mice per group) were injected i.p. with PBS or DP6 on days -8, -6, -4, -2, 0, 2, 4, and 8 and were injected s.c. with B16F1 melanoma cells. (A) Schematic representation depicting *in vivo* tumor challenge. (B) Tumor growth was monitored by measuring the diameter of the tumor three times a week. ***, $p < 0.001$ compared to PBS-injected group. (C) Images of PBS-injected and 500 mg/ml DP6-injected groups of mice depicting *in vivo* tumor challenge on day 18. (D) A linear graph summarizing data on the tumor mass obtained. WT and TLR4$^{-/-}$ mice (six mice per group) were injected i.p. with 500 mg/ml DP6 on days -8, -6, -4, -2, 0, 2, 4, and 8 and were injected *s.c.* with B16F1 melanoma cells. Tumor growth was monitored by measuring the diameter of the tumor every other day. Data shown are the mean ± SEM. *, $p < 0.05$ compared to DP6-injected WT mice.

Figure 4. DP6 directly and indirectly enhanced the activation of NK cells in TLR4-dependent manner and augmented NK cell-mediated antitumor immunity *in vitro* and *in vivo*. (A) Histogram (left) and bar graph (right) depicting the percentage of CD69^{+} NK1.1^{+} cells isolated from PECs obtained from PBS- and DP6-injected mice. C57BL/6 mice (five mice per group) were injected i.p. with PBS or DP6, and PECs were then isolated 18 h later and stained and analyzed by flow cytometry analysis. The results of one representative

experiment out of three experiments are shown. **$P < 0.01$ compared to PBS-treated PECs. (B-C) Flow cytometry and ELISA analysis were used to determine the activation of DP6-treated NK1.1$^+$ cells *in vitro*. (B) The percentage of CD69$^+$ cells among NK1.1$^+$ cells was analyzed by flow cytometry and the data are presented as a histogram (left) and bar graph (right). The results of one representative experiment out of three experiments are shown. *, $p < 0.05$ compared to untreated NK cells. (C) ELISA was performed to test IFN-γ and TNF-α production in NK1.1$^+$ cells. The data are presented as the means and standard error of the mean (SEM, $n = 3$). **, $p < 0.01$; ***, $p < 0.001$ compared to untreated NK1.1$^+$ cells. (D-E) Flow cytometry and ELISA analysis were used to determine the activation of NK1.1$^+$ CD3$^-$ cells treated with the supernatant from PBS- or DP6-treated WT or TLR4$^{-/-}$ DCs. (D) The percentage of CD69$^+$ cells among NK1.1$^+$ cells was analyzed by flow cytometry and the data are presented as a histogram (left) and bar graph (right). The results of one representative experiment out of three experiments are shown. **, $p < 0.01$ compared to untreated NK cells. (E) ELISA was performed to test IFN-γ production in NK1.1$^+$ cells. The data are presented as the means and standard error of the mean (SEM, $n = 3$). ***, $p < 0.001$ compared to NK1.1$^+$ cells treated with the supernatant from DP6-treated WT DCs. (F-G) *In vivo* antibody depletion experiments. (F) Linear graph depicting the tumor mass of tumor-bearing mice (five mice per group) treated with DP6 with or without NK1.1 depletion. Tumor growth was monitored by measuring the diameter of the tumor every other day. **, $p < 0.01$ compared to PBS-injected mice. (G) Bar graph depicting the tumor weight in tumor-bearing mice (five mice per group) treated with DP6 with or without NK1.1 depletion. *, $p < 0.05$ compared to PBS-injected mice.

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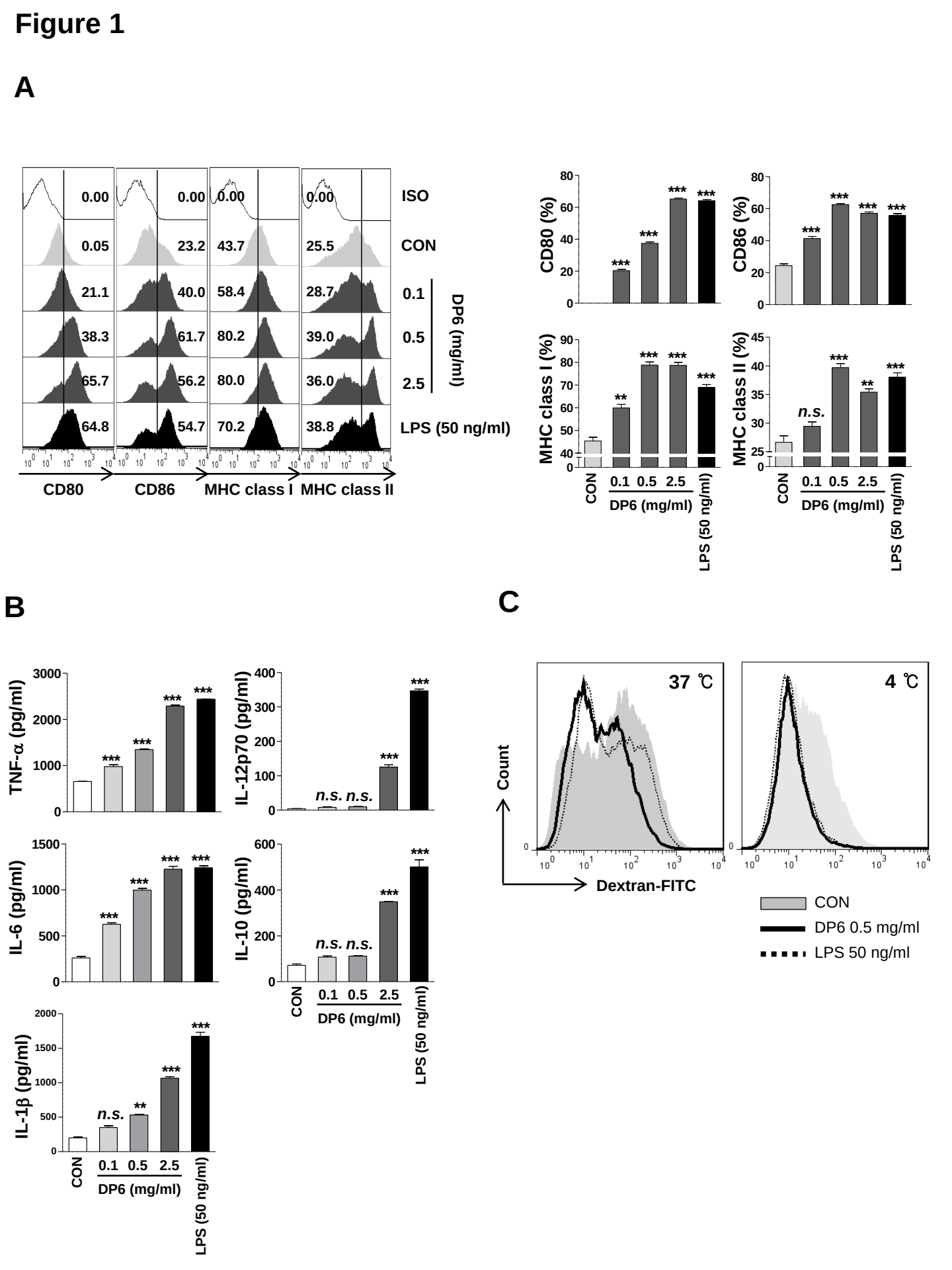
Figure 1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IL-6 (pg/ml)
IL-10 (pg/ml)
IL-1β (pg/ml)
C
37 ℃
4 ℃
Count
Dextran-FITC
CON
DP6 0.5 mg/ml
LPS 50 ng/ml

Figure 2

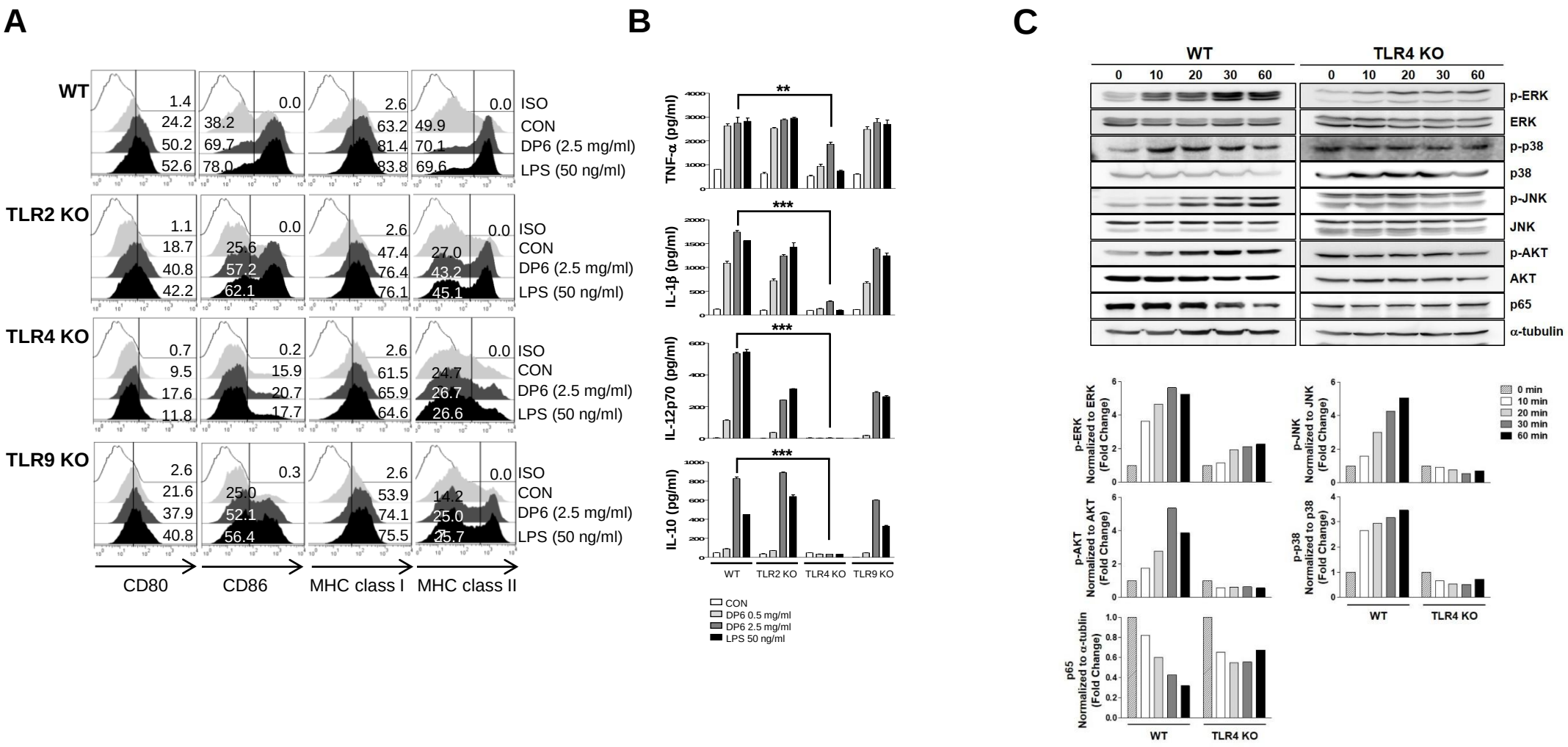

A
WT
TLR2 KO
TLR4 KO
TLR9 KO
ISO
CON
DP6 (2.5 mg/ml)
LPS (50 ng/ml)
CD80
CD86
MHC class I
MHC class II
B
TNF-α (pg/ml)
IL-1β (pg/ml)
IL-12p70 (pg/ml)
IL-10 (pg/ml)
**
***
WT
TLR2 KO
TLR4 KO
TLR9 KO
CON
DP6 0.5 mg/ml
DP6 2.5 mg/ml
LPS 50 ng/ml
C
WT
TLR4 KO
0
10
20
30
60
p-ERK
ERK
p-p38
p38
p-JNK
JNK
p-AKT
AKT
p65
α-tubulin
p-ERK Normalized to ERK (Fold Change)
p-JNK Normalized to JNK (Fold Change)
p-AKT Normalized to AKT (Fold Change)
p-p38 Normalized to p38 (Fold Change)
p65 Normalized to α-tublin (Fold Change)
0 min
10 min
20 min
30 min
60 min
WT
TLR4 KO

Figure 3

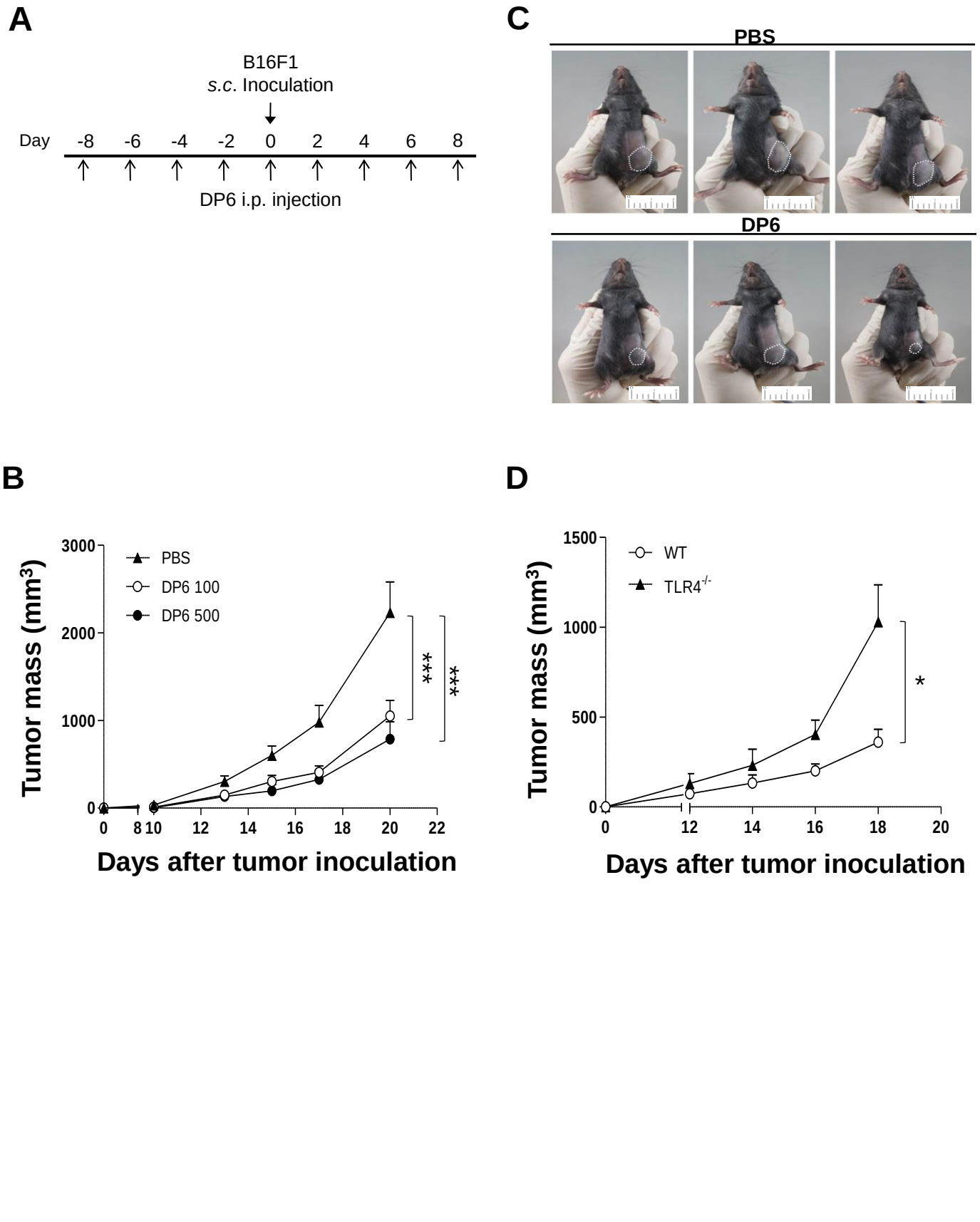
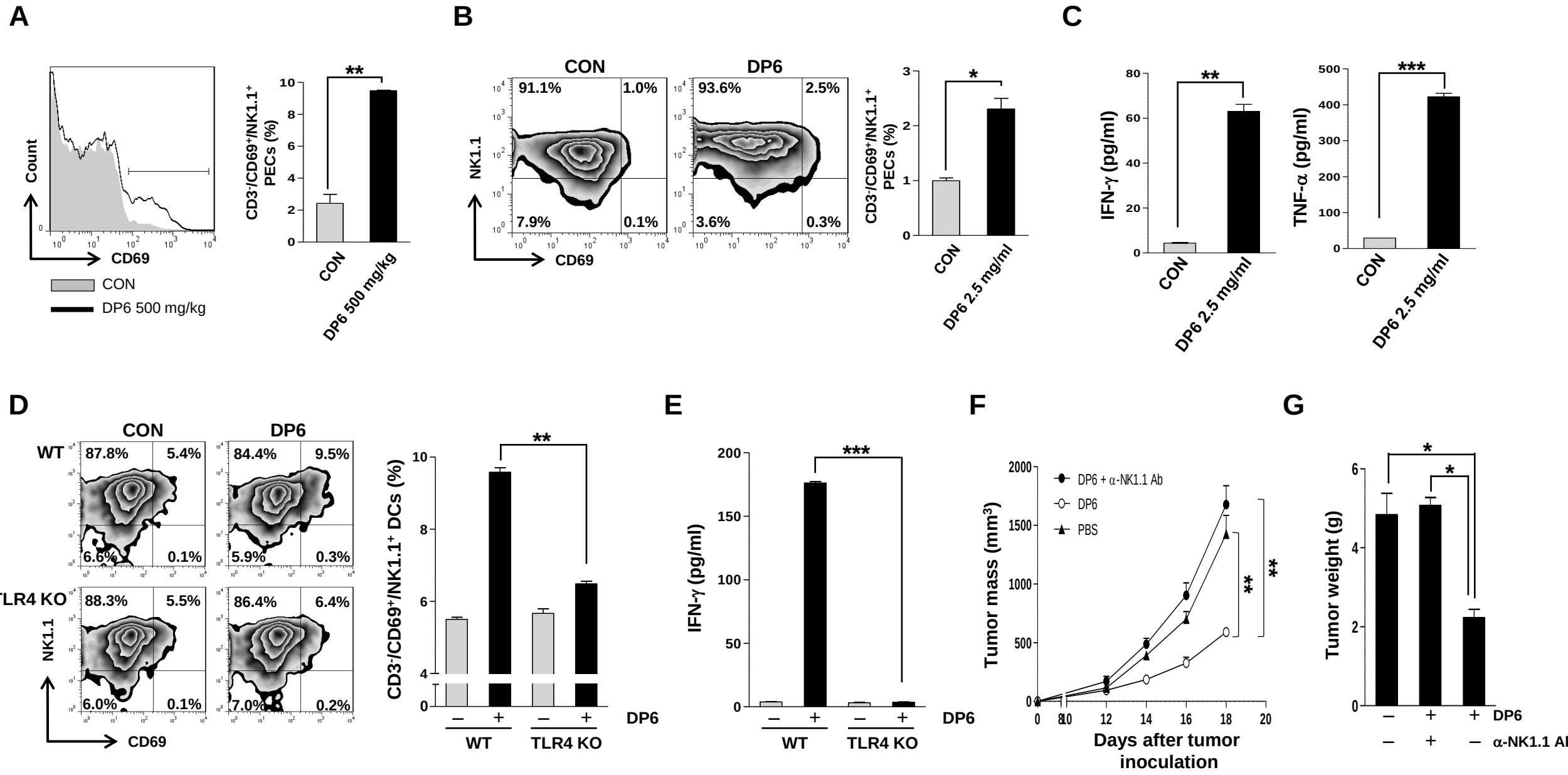
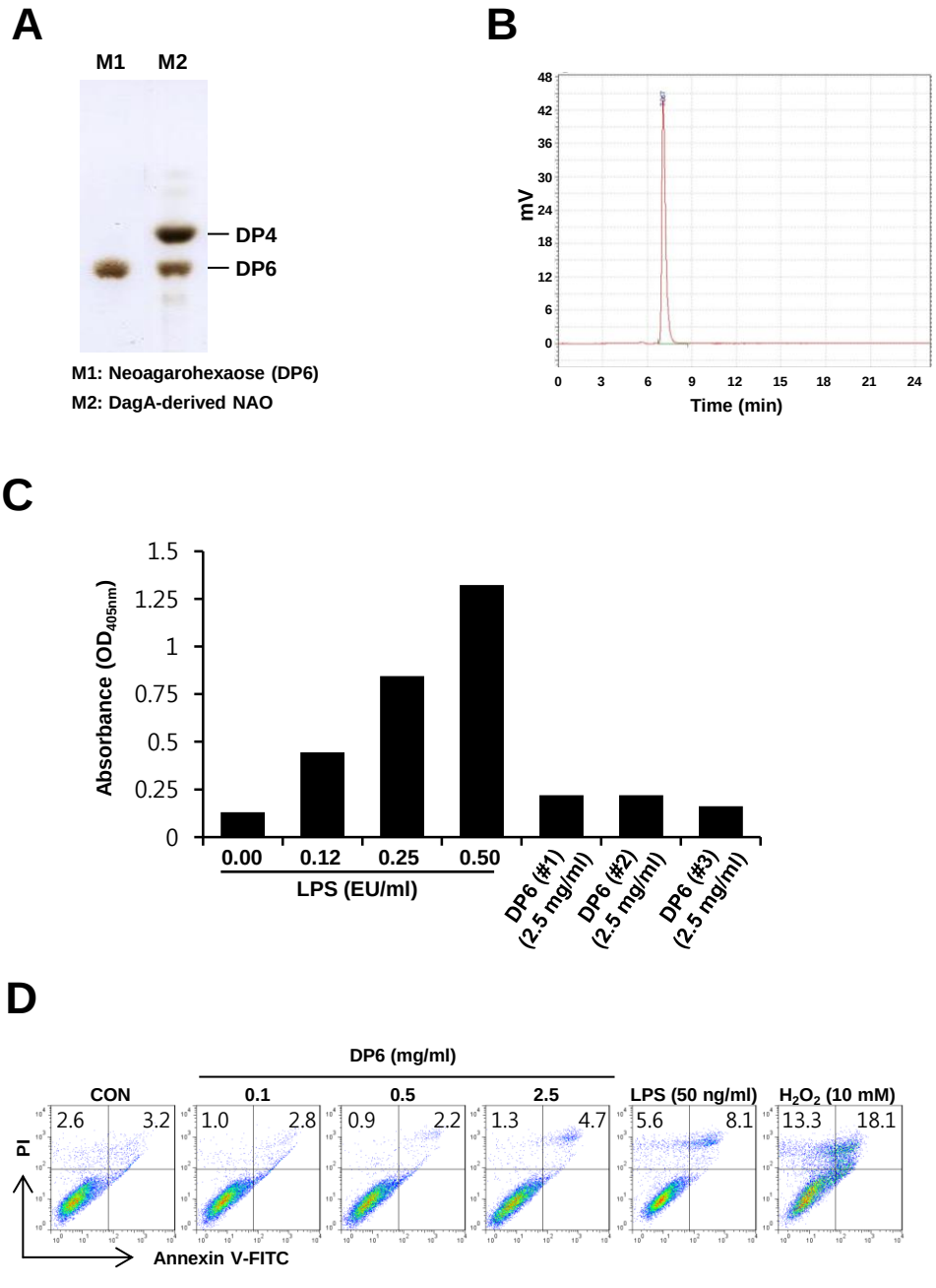

A
B16F1
s.c. Inoculation
Day
-8
-6
-4
-2
0
2
4
6
8
DP6 i.p. injection
C
PBS
DP6
B
Tumor mass (mm3)
3000
2000
1000
0
PBS
DP6 100
DP6 500
***
***
0
8
10
12
14
16
18
20
22
Days after tumor inoculation
D
Tumor mass (mm3)
1500
1000
500
0
WT
TLR4-/-
*
0
12
14
16
18
20
Days after tumor inoculation

Figure 4

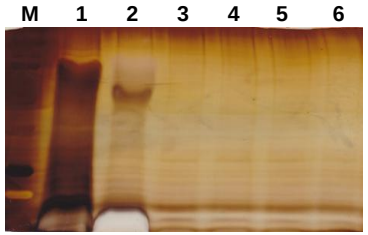

A
Count
CD69
CON
DP6 500 mg/kg
CD3-/CD69+/NK1.1+ PECs (%)
**
CON
DP6 500 mg/kg
B
CON
DP6
91.1%
1.0%
7.9%
0.1%
93.6%
2.5%
3.6%
0.3%
NK1.1
CD69
CD3-/CD69+/NK1.1+ PECs (%)
*
CON
DP6 2.5 mg/ml
C
IFN-γ (pg/ml)
**
CON
DP6 2.5 mg/ml
TNF-α (pg/ml)
***
CON
DP6 2.5 mg/ml
D
CON
DP6
WT
87.8%
5.4%
6.6%
0.1%
84.4%
9.5%
5.9%
0.3%
TLR4 KO
88.3%
5.5%
6.0%
0.1%
86.4%
6.4%
7.0%
0.2%
NK1.1
CD69
CD3-/CD69+/NK1.1+ DCs (%)
**
DP6
WT
TLR4 KO
E
IFN-γ (pg/ml)
***
DP6
WT
TLR4 KO
F
Tumor mass (mm3)
DP6 + α-NK1.1 Ab
DP6
PBS
**
**
Days after tumor inoculation
G
Tumor weight (g)
*
*
DP6
α-NK1.1 Ab

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.