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# Soluble β-glucan from *Grifola frondosa* induces tumor regression in synergy with TLR9 agonist via dendritic cell-mediated immunity

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## ABSTRACT

**The maturation of dendritic cells into more-immunostimulatory dendritic cells by stimulation with different combinations of immunologic agents is expected to provide efficient, adoptive immunotherapy against cancer. Soluble β-glucan maitake D-fraction, extracted from the maitake mushroom *Grifola frondosa*, acts as a potent immunotherapeutic agent, eliciting innate and adoptive immune responses, thereby contributing to its antitumor activity. Here, we evaluated the efficacy of maitake D-fraction, in combination with a Toll-like receptor agonist, to treat tumors in a murine model. Our results showed that maitake D-fraction, in combination with the Toll-like receptor 9 agonist, cytosine–phosphate–guanine oligodeoxynucleotide, synergistically increased the expression of dendritic cell maturation markers and interleukin-12 production in dendritic cells, but it did not increase interleukin-10 production, generating strong effector dendritic cells with an augmented capacity for efficiently priming an antigen-specific, T helper 1–type T cell response. Maitake D-fraction enhances cytosine–phosphate–guanine oligodeoxynucleotide-induced dendritic cell maturation and cytokine responses in a dectin-1–dependent pathway. We further showed that a combination therapy using cytosine–phosphate–guanine oligodeoxynucleotide and maitake D-fraction was highly effective, either as adjuvants for dendritic cell vaccination or by direct administration against murine tumor. Therapeutic responses to direct administration were associated with increased CD11c$^+$ dendritic cells in the tumor site and the induction of interferon-γ–producing CD4$^+$ and CD8$^+$ T cells. Our results indicate that maitake D-fraction and cytosine–phosphate–guanine oligodeoxynucleotide synergistically activated dendritic cells, resulting in tumor regression via an antitumor T helper cell 1–type response. Our findings provide the basis for a potent antitumor therapy using a novel combination of immunologic agents for future clinical immunotherapy studies in patients. *J. Leukoc. Biol.* 98: 1015–1025; 2015.**

Abbreviations: DC = dendritic cell, KLH = keyhole limpet hemocyanin, MD-fraction = maitake D-fraction, ODN = oligodeoxynucleotide, PAMPs = pathogen-associated molecular patterns, PRRs = pattern recognition receptors, qPCR = quantitative polymerase chain reaction, TIL = tumor-infiltrating lymphocyte

*The online version of this paper, found at www.jleukbio.org, contains supplemental information.*

## Introduction

DCs are potent antigen-presenting cells that provide an essential link between the innate and adaptive immune responses against various pathogens [1]. DCs express PRRs that recognize PAMPs. PAMPs are broadly shared by pathogens and induce innate immune signaling that leads to fully mature immunostimulatory DCs. Mature immunostimulatory DCs are able to drive T cell clonal expansion and prime adaptive immune responses. PRRs include TLRs, nod-like receptors, RIG-I-like receptor, and C-type lectin receptors, and each receptor detects distinct PAMPs, derived from bacteria, viruses, mycobacteria, and fungi [2].

Antitumor immunity is initiated by DCs that capture tumor antigens from tumor cells and induce the CD4$^+$ Th-1 cell/CD8$^+$ cytotoxic T cell response. In contrast, impaired cytokine production by DCs is linked to cancer progression [1, 3, 4]. Therefore, inducing the maturation of DCs into more-immunostimulatory DCs may offer a useful strategy for tumor immunotherapy. Various TLR agonists are currently under investigation in clinical trials. Antitumor responses to TLR agonists are largely attributable to the stimulation of DCs, which, in turn, activate tumor-specific T cell responses [5–8]. However, treatment with TLR agonists have met with limited success in treating human cancers [9, 10]. Because the combination of PRRs orchestrates pathogen-specific host immune responses to fight infections, a combination therapy using appropriate PAMPs may induce a strong antitumor immune response. Dectin-1, the C-type lectin receptor for β-(1,3)-glucans, has a crucial role in recognizing live pathogenic fungi, such as *Candida albicans* and *Aspergillus fumigatus* [11, 12], and particulate β-glucans, such as zymosan and curdlan [13, 14]. Dectin-1 can collaborate with

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multiple TLRs to induce many cytokines synergistically. Zymosan, a crude yeast cell-wall extract composed primarily of β-glucan, is recognized by both dectin-1 and TLR2, and induces TNF-α and IL-12 production through dectin-1/TLR2 signaling in murine DCs [14, 15].

Maitake mushroom (*Grifola frondosa*) is a popular, edible mushroom in Japan, especially because its fruiting body can be artificially produced. We have reported previously that MD-fraction, a highly purified, soluble β-(1,3) (1,6)-glucan derived from *G. frondosa*, induces antitumor activity by activating the host immune system [16]. MD-fraction activates macrophages, DCs, and NK cells and promotes a Th-1-type immune response [17, 18]. A phase I/II trial in the United States using a polysaccharide extract from maitake mushrooms showed that administration of the extract affected the host immune system without a dose-limiting toxicity [19]. Recently, MD-fraction was shown to increase GM-CSF production in murine-resident macrophages, resulting in proliferation and dectin-1–mediated TNF-α production [20]. In addition, MD-fraction induces IL-12 production in murine bone marrow–derived DCs through a dectin-1–dependent pathway [21].

In this study, we assessed the ability of β-glucan MD-fraction, used in combination with TLR agonists, to induce DC-mediated antitumor immunity. We found that MD-fraction and a TLR9 agonist, unmethylated CpG ODN, synergistically induced IL-12 production by DCs in a dectin-1–dependent manner, but CpG ODN-induced IL-10 production was not affected by the addition of MD-fraction. Furthermore, we demonstrated that the antitumor effect of MD-fraction in combination with CpG ODN, either as adjuvants for DC therapy or via direct administration in the colon-26 carcinoma mouse model, was more effective than a single therapy involving either alone. This is the first demonstration, to our knowledge, of soluble β-glucan and a TLR agonist augmenting antitumor immunity in vivo.

## MATERIALS AND METHODS

### Mice and reagents

Male 5–8-wk-old DBA/2J and female BALB/c mice were purchased from CLEA Japan (Higashiyama, Japan) for this study. Animal care and processing were approved by the animal care committee at the Kobe Pharmaceutical University.

### Preparation of MD-fraction

MD-fraction was prepared from maitake fruiting bodies (Yukiguni Maitake, Niigata, Japan) as described previously [16, 20]. In brief, MD-fraction was isolated from a boiling-water extract of maitake fruiting bodies, followed by ethanol precipitation, alkaline extraction after acid precipitation, and protein depletion using the Sevag method. The water-soluble fraction, MD-fraction, showed a single peak with a molecular mass of 1200–2000 kDa, and it consisted of 98% polysaccharide and 2% peptide. In addition, we tested for LPS contamination using the Endospecy ES-24S set (Seikagaku Biobusiness, Tokyo, Japan) and did not detect LPS contamination in the prepared MD-fraction.

### Cell preparation and culturing

GM-CSF–induced bone marrow–derived DCs were grown in complete RPMI (RPMI-1640 medium supplemented with 10% FBS, 0.03 mg/ml L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin) supplemented with murine GM-CSF (20 ng/ml) and IL-4 (10 ng/ml), as described previously [22]. GM-CSF–generated DCs were harvested and analyzed on d 7–8, and >85% of the cells expressing CD11c were used.

DCs ($1 \times 10^6$ cells/ml) were stimulated with TLR agonists CpG1826, $Pam_3CSK_4$ (InvivoGen, San Diego, CA, USA), *Escherichia coli* serotype 0111:B4 LPS (Sigma-Aldrich, St. Louis, MO, USA), and/or MD-fraction at various concentrations for 24 h. For blocking antibody assays, DCs were preincubated with 1 μg/ml anti-dectin-1 (2A11; AbD Serotec, Kidlington, United Kingdom) for 1 h, followed by stimulation with CpG ODN, MD-fraction, or both for 24 h.

### Quantitation of cytokine production

IL-12p70 (R&D Systems, Abingdon, United Kingdom), IL-12p40, and IL-10 (PeproTech, Rocky Hill, NJ, USA) secretion was determined using ELISA kits according to the manufacturer's protocols. IFN-γ levels were determined by a sandwich ELISA, as described previously [22].

Real-time qPCR was used to examine mRNA expression levels of various cytokines. RNA was purified using RNeasy (Qiagen, Hilden, Germany) before first-strand cDNA synthesis using the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). qPCR using Thunderbird SYBR qPCR mix (Toyobo) was performed using primers for 18S, IL-12p40, TNF-α, IL-6, and IL-10, as described previously [20]. Other prime sequences were as follows: IL-12p35, forward primer 5′-CTAGACAAGGGCATGCTGGT-3′; IL-12p35 reverse primer, 5′-GCTTCTCCCACAGGAGGTTT-3′; IL-23p19 forward primer, 5′-GACCCAC-AAGGACTCAAGGA-3′; IL-23p19 reverse primer, 5′-GCTCCCCTTTGAAGAT-GTCA-3′; IL-1β forward primer, 5′-TGTGAAATGCCACCTTTTGA-3′; IL-1β reverse primer, 5′-GGTCAAAGGTTTGGAAGCAG-3′.

### Flow cytometry

DCs were incubated with mAb, including anti-CD11c, anti-I-A/I-E, anti-CD86, and anti-CD80, for 20 min at 4°C. Cells thus labeled were analyzed using FACSCalibur (BD Biosciences, San Jose, CA, USA).

Tumor tissues were minced and then digested in RPMI medium containing collagenase (70 units/ml; Wako Pure Chemical Industries, Osaka, Japan) for 45 min at 37°C. Tumor cells were filtered through 70-μm nylon strainers (BD Biosciences). TILs were enriched on Lympholyte-M (a Ficoll-Paque preparation; Cedarlane Laboratories, West Ontario, Canada), and they were labeled with anti-CD4, anti-CD8, anti-CD86, and anti-CD11c for 20 min at 4°C, washed, and analyzed using FACSCalibur. Intracellular staining of IFN-γ and IL-17 was performed using the Cytofix/Cytoperm kit (BD Pharmingen, BD Biosystems). All antibodies were purchased from BD Bioscience.

### Antigen presentation assay

KLH (Calbiochem, Cambridge, MA, USA) was absorbed to aluminum hydroxide adjuvant (LSL Co. Ltd., Tokyo, Japan), and 100 μg of KLH was injected into the footpads of DBA/2 mice, as described previously [22]. Seven days after injection, draining axial, popliteal, and inguinal lymph nodes were harvested from the mice, and $CD4^+$ T cells were isolated by using a MACS $CD4^+$ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) as responder T cells. DCs were cultured for 24 h with MD-fraction (10 μg/ml), CpG ODN (0.01 μM), or both in the presence or absence of KLH (100 μg/ml). DCs ($1 \times 10^4$ cells/well) were washed and cocultured with KLH-primed T cells ($1 \times 10^5$ cells/well) in U-bottom 96-well plates for 72 h. The levels of IL-12p40, IFN-γ, and IL-10 in the culture supernatants were then measured by ELISA.

### Tumor antigen pulsing of DCs for in vivo tumor studies

Tumor cell lysates were prepared by 4 freeze–thaw cycles of colon-26 cells ($1 \times 10^7$ cells/ml in PBS), as described previously [22]. The synergistic effect of MD-fraction and CpG ODN on IL-12 production was observed on DCs derived from BALB/c mice. DCs from BALB/c mice were incubated with tumor lysates at a ratio of 3:1 tumor cell equivalents, that is, DCs in the presence or absence of CpG ODN (0.1 μM), MD-fraction (100 μg/ml), or both overnight at 37°C in 5% $CO_2$. The DCs were harvested and resuspended in PBS at specific vaccine concentrations for use in further studies.

### In vivo tumor studies

Colon-26 carcinoma cells ($1 \times 10^5$ cells/mouse) were inoculated subcutaneously into BALB/c mice on d 0. For DC vaccination, the mice

were treated by subcutaneous injections of tumor-lysate–pulsed DCs ($5 \times 10^5$ cells/mouse) stimulated with CpG ODN, MD-fraction, or both on d 3, 6, and 9. For direct injections, the tumor-bearing mice received intratumoral injection of CpG ODN (10 μg/mouse) on d 9 and 13 after tumor challenge or intraperitoneal injection of MD-fraction (8 mg/kg) for 10 consecutive days, beginning 5 d after the tumor challenge. The tumor volume was calculated using the formula Tumor volume ($cm^3$) = (longest diameter × shortest diameter$^2$)/2.

### In vitro cytotoxic T lymphocyte assay

TILs were isolated from tumor-bearing mice and incubated with mitomycin C–treated colon-26 cells in the presence of recombinant IL-2 (50 ng/ml) for 72 h. After incubation, $CD8^+$ TILs were purified using CD8 magnetic beads (Miltenyi Biotec). Purified $CD8^+$ TILs were tested for cytotoxicity using colon-26 tumor cells as the target cells, as described previously [23]. The cytotoxic T lymphocyte (CTL) activity was determined using calcein AM–labeled colon-26 cells ($1 \times 10^5$ cells/ml) as a target at different E:T ratios. After incubation for 4 h, supernatants were harvested, and the fluorescence was read at 485/538 nm (excitation/emission).

### Statistical analysis

Data were analyzed using Prism software (GraphPad Software, La Jolla, CA, USA). The presented data are expressed as means ± SE. One-way ANOVA with the Tukey's post hoc test was used for analysis of multiple groups, and Student's *t* test was used to compare the 2 groups. Tumor growth data were analyzed using a 2-way ANOVA. Kaplan-Meier survival curves of mice in the tumor studies were analyzed by the log rank-survival test. $P < 0.05$ was considered statistically significant.

### Analysis of combination data

Normalized (percentage of maximum) IL-12 production was calculated by setting the minimum and maximum value to 0 and 100%, respectively. MacSynergy II software [24] was used to calculate additive/synergistic effects. A 3-dimensional plot demonstrated synergy as peaks above a theoretical additive plane and antagonism as depressions below it. Synergy volume ($\mu M^2\%$) was statistically evaluated at the 95% confidence level to categorize the degree of synergy: strong (>100 $\mu M^2\%$), moderate (50–100 $\mu M^2\%$), minor (25–50 $\mu M^2\%$), and additive (<25 $\mu M^2\%$).

## RESULTS

### MD-fraction, in combination with TLR agonists, enhances IL-12 production in DCs but not IL-10 production

During a fungal infection, β-glucans combine with other PAMPs, including TLR agonists, to stimulate immune cells [25]. To investigate the collaborative effect of MD-fraction on TLR agonist–induced cytokine production in DCs, we treated DCs with the TLR9 agonist CpG ODN, the TLR2 agonist $Pam_3CSK_4$, or the TLR4 agonist LPS alone or in combination with MD-fraction. As shown in **Fig. 1A**, each TLR agonist increased IL-12p40 and IL-10 production from the DCs in a dose-dependent manner. The addition of MD-fraction (10 μg/ml) significantly increased IL-12p40 production in DCs in combination with CpG ODN and $Pam_3CSK_4$; a similar but less-dramatic effect was observed in combination with LPS (Fig. 1A). As shown in Fig. 1B, MD-fraction alone significantly increased IL-12p40 production in a dose-dependent manner, and the IL-12p40 production with a combination of MD-fraction and the TLR agonists was greater than the sum of the IL-12p40 levels produced after exposure to the 2 stimuli individually, indicating that the combination of MD-fraction and TLR agonists increases IL-12p40 synergistically in DCs. On the other hand, TLR agonists–induced IL-10 production was not affected by the addition of MD-fraction (Fig. 1A and B). Because TLR9 agonists have been evaluated in clinical trials as cancer therapeutics [7], and the combined MD-fraction and CpG ODN resulted in an increase in IL-12 production, CpG ODN was chosen for further investigation, with a view to potentially using it in cancer immunotherapy. To further assess the synergistic effects, IL-12p40 production in DCs with the combination of CpG ODN and MD-fraction at various concentrations was tested and analyzed using MacSynergy II software, according to the Bliss independence model [24] (Fig. 1C and D). In the 3-dimensional representations (Fig. 1D), the combination of MD-fraction and CpG ODN, because of a strong synergy, resulted in an increase in IL-12p40 production, as indicated by the high peak in synergy above the theoretical additive plane and the overall synergy volume of 304.05 $\mu M^2\%$. No antagonistic effect was observed with this combination, with an antagonism volume of 0 $\mu M^2\%$. The effect of the combination of MD-fraction and CpG ODN on IL-10 production was not significantly different from the theoretical additive effect (data not shown).

IL-12p70 is a heterodimer consisting of the p35 and p40 chains encoded on separate chromosomes. A common p40 chain is shared by IL-12 and IL-23, yet they comprise unique p35 and p19 chains, respectively [26]. Therefore, we tested IL-12p70 production from DCs stimulated with CpG ODN, MD-fraction, or both. Although MD-fraction alone (10 μg/ml) did not induce IL-12p70, MD-fraction dramatically increased IL-12p70 production in combination with CpG ODN (Fig. 1E). This made it clear that MD-fraction and CpG ODN enhance IL-12 production synergistically in DCs.

### MD-fraction enhances CpG ODN-induced IL-12 production via dectin-1

We have demonstrated previously that MD-fraction induces IL-12 production in DCs through the dectin-1–dependent pathway [21]. To investigate whether the collaborative induction of IL-12 by MD-fraction and CpG ODN is associated with dectin-1 signaling, DCs were pretreated with anti-dectin-1 neutralizing antibody. Consistent with previous reports, we saw that the enhanced IL-12 production by MD-fraction alone was significantly inhibited by anti-dectin-1 antibody, whereas IL-12 production by CpG ODN alone was not inhibited (**Fig. 2A**). Synergistic induction of IL-12 by MD-fraction and CpG ODN was significantly reduced to almost the same level as CpG ODN alone, by the anti-dectin-1 antibody. On the other hand, no significant difference was found in IL-10 production between the treatment with anti-dectin-1 antibody and isotype antibody (Fig. 2B). These results suggest that MD-fraction enhances CpG ODN-induced IL-12 production in DCs through the dectin-1–dependent pathway.

### MD-fraction and CpG ODN synergistically enhance the expression of maturation markers on DCs

The maturation of DCs is closely linked to up-regulation of the expression of MHC molecules CD80 and CD86. Because

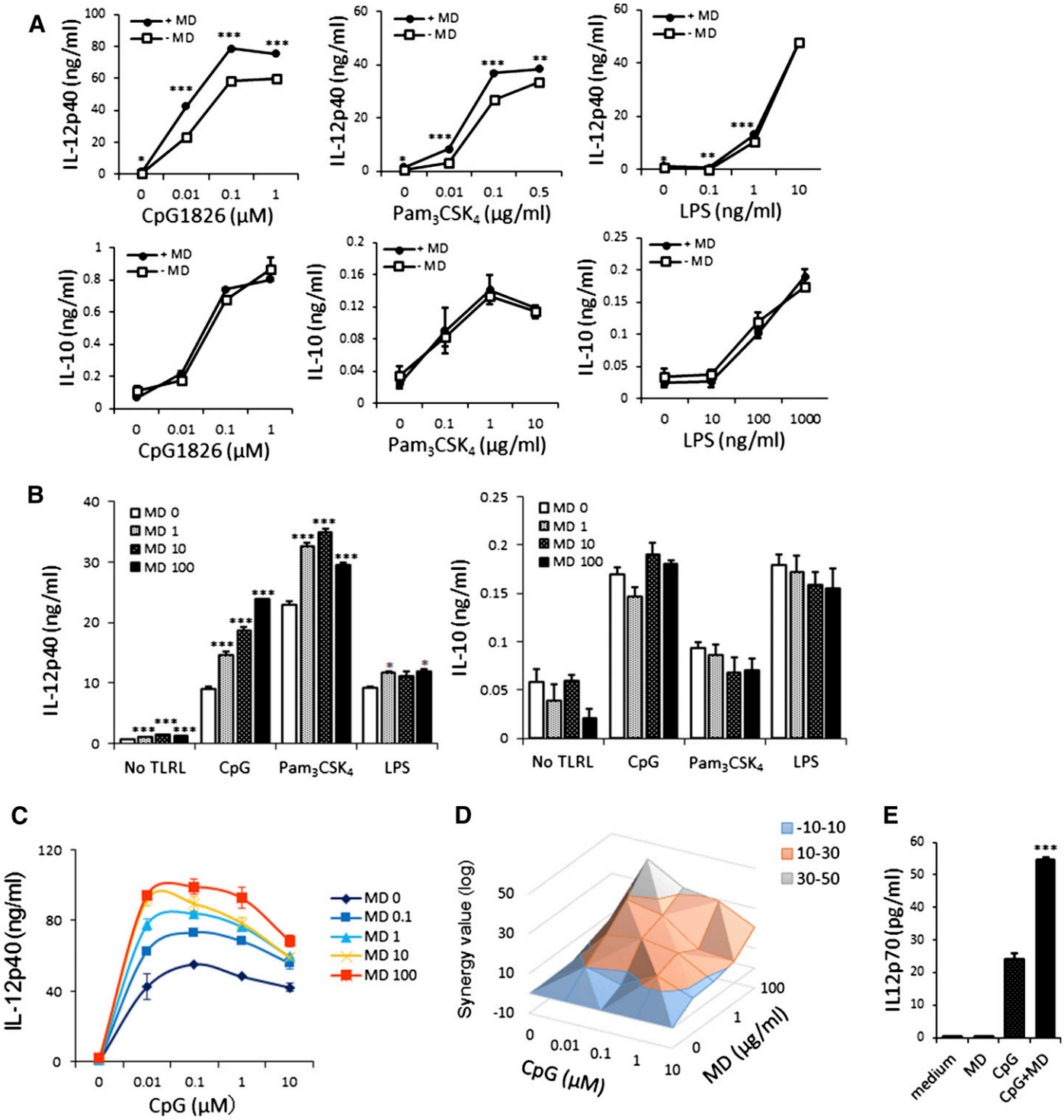


**Figure 1. IL-12 and IL-10 production in DCs after stimulation with a combination of MD-fraction and TLR agonists.** (A) DCs were incubated with the indicated TLR agonists and/or MD-fraction (10 μg/ml). (B) DCs were incubated with MD-fraction (1, 10, or 100 μg/ml) and/or TLR agonists, including CpG ODN (0.01 μM), $Pam_3CSK_4$ (0.1 μg/ml), or LPS (1 ng/ml). Supernatants were collected 24 h later, and the cytokine levels were determined by ELISA. (C) DCs were incubated with MD-fraction (0–100 μg/ml), CpG ODN (0-10 μM), or both for 24 h; after which, the IL-12p40 level in the supernatants was measured. (D) MacSynergy plot of the MD-fraction and CpG ODN at the 95% confidence level. MacSynergy II analysis of the data from Fig. 1C is shown. (E) DCs were incubated with MD-fraction (10 μg/ml), CpG ODN (0.01 μM), or both. The data presented are representative of 3 independent experiments. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ compared with MD-fraction 0 μg/ml (A and B) or all other groups (E).

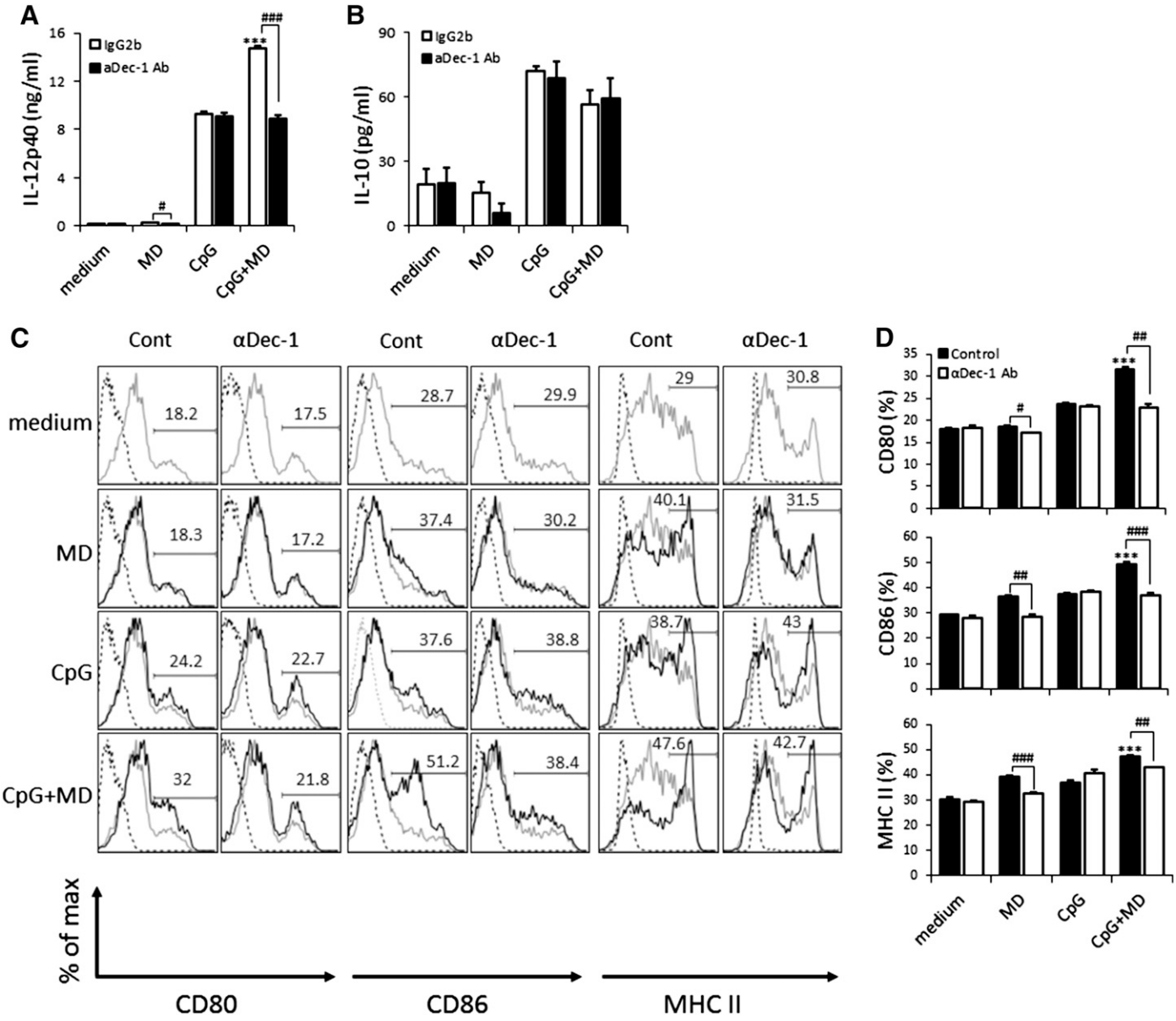


**Figure 2. A combination of MD-fraction and CpG ODN synergistically induces maturation and boosts secretion of IL-12 in DCs through dectin-1 signaling.** DCs were preincubated with anti-dectin-1 antibody (1 μg/ml) for 1 h, followed by stimulation with MD-fraction (10 μg/ml), CpG ODN (0.01 μM), or both for 24 h. (A and B) Cytokine levels were determined by ELISA. (C and D) Expression of surface molecules was analyzed by flow cytometry. The histograms show the expression of each molecule on $CD11c^+$-gated cells. The percentages of positive cells are shown for each histogram. The dot-histogram profile indicates the isotype control, the gray histogram indicates the medium control, and the black, solid histogram indicates the stimulation with MD-fraction, CpG ODN, or both. The data presented are representative of 3 independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate a significant difference between the combination treatment group and all other groups. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ compared with the groups linked by the bracket.

expression of these molecules often correlates with T cell–priming ability, it is generally assumed that DCs expressing high cell-surface levels of these molecules are functionally mature [27]. Therefore, we examined whether MD-fraction combined with CpG ODN increased the cell-surface expression of these molecules on DCs. MD-fraction or CpG ODN alone significantly increased the levels of CD80, CD86, and MHC class II, compared with the medium control, and the combining of MD-fraction with CpG ODN resulted in further increases in these molecules (Fig. 2C and D). To examine the ability of MD-fraction to enhance DC maturation in combination with CpG ODN through dectin-1, we measured the expression of these molecules on DCs in the presence of anti-dectin-1 antibody. The expression of CD80, CD86, and MHC class II on DCs treated with MD-fraction alone or the combination of MD-fraction and CpG ODN was decreased significantly when blocked by the anti-dectin-1 antibody. These results show that MD-fraction enhances CpG ODN-induced DC maturation in

a dectin-1–dependent manner, causing increased expression of maturation markers.

### MD-fraction modulates cytokine expression in DCs stimulated with CpG ODN

Dectin-1 collaborates with multiple TLRs to modulate TLR-induced various cytokine responses, including IL-12, TNF-α, IL-10, and IL-23 [13, 28–31]. We next examined whether mRNA expressions of various cytokines in DCs could be influenced by the combination of MD-fraction and CpG ODN. Consistent with Fig. 1, MD-fraction in combination with CpG ODN increased IL-12p35 and IL-12p40 expression synergistically, and this collaborative effect continued for 3~24 h (**Fig. 3**). It is noteworthy that MD-fraction (10 μg/ml), CpG ODN (0.01 μM), or both did not affect IL-23p19 expression at these concentrations. We confirmed that particulate β-glucan zymosan (100 μg/ml) significantly increased IL-23p19 expression in DCs, but MD-fraction (10 or 100 μg/ml) did not (data not shown). CpG ODN, but not MD-fraction, dramatically increased IL-10 expression after 24 h. No collaborative effect of MD-fraction and CpG ODN after 3 or 24 h stimulation was observed in IL-10 expression. The expressions of inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, was synergistically increased by the combination of MD-fraction and CpG ODN after 3 h stimulation, and the collaborative effect of MD-fraction and CpG ODN on IL-1β and IL-6 continued for 24 h after stimulation. Taken together, these results show that MD-fraction in combination with CpG ODN synergistically induces the expression of IL-12p35, IL-12p40, TNF-α, IL-1β, and IL-6, but not IL-23p19 or IL-10, indicating that MD-fraction and CpG ODN can synergistically induce inflammatory DCs, which converts immature DCs to potent effector DCs.

### MD-fraction in combination with CpG ODN increases DC ability to induce antigen-specific T cell responses

Stimulation of DCs with CpG ODN in the presence of MD-fraction induced a high inflammatory cytokine profile, such as IL-12, TNF-α, IL-1β and IL-6, but not IL-10 and IL-23. These DCs may promote the induction of Th1 response. We tested this hypothesis using a DC antigen-presenting assay. KLH (100 μg in alum) was injected into the footpads of naïve mice. After 7 d, $CD4^+$ T cells were isolated from lymph nodes and were cocultured with KLH-pulsed and CpG ODN/MD-stimulated DCs for 72 h. $CD4^+$ T cells cocultured with KLH-pulsed and CpG ODN-stimulated DCs had increased IL-12 and IL-10 (**Fig. 4**). In contrast, the response induced by coculture of DCs with KLH, CpG ODN, and MD-fraction was more Th1-polarized, with high levels of IL-12 and IFN-γ and an unchanged IL-10. Furthermore, KLH-pulsed and CpG ODN/MD-stimulated DC-induced IFN-γ production was strongly reduced in coculture with the anti-dectin-1 antibody. Enhanced production of IL-12 was also observed at the single-cell level by intracellular staining, using

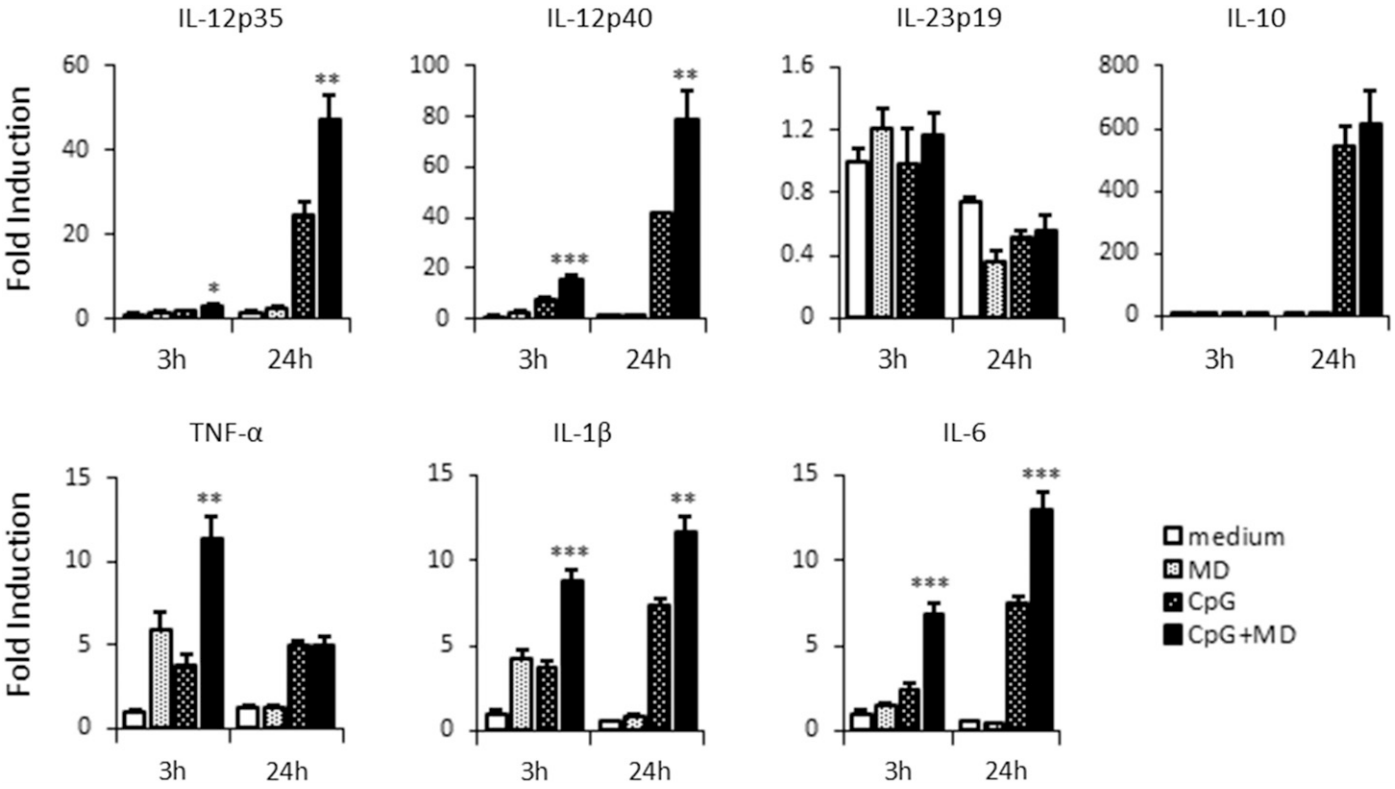


**Figure 3. Cytokine inductions by stimulation of MD-fraction and CpG ODN in DCs.** DCs were incubated with MD-fraction (10 μg/ml), CpG ODN (0.01 μM), or both. Cells were collected 3 or 24 h later, and mRNA expression levels of cytokines were determined by real-time PCR. The relative expression level was normalized to the expression level with the medium control at 3 h. The data presented are representative of 2 independent experiments. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ indicate a significant difference between the combination treatment (CpG ODN + MD-fraction) group and all other groups.

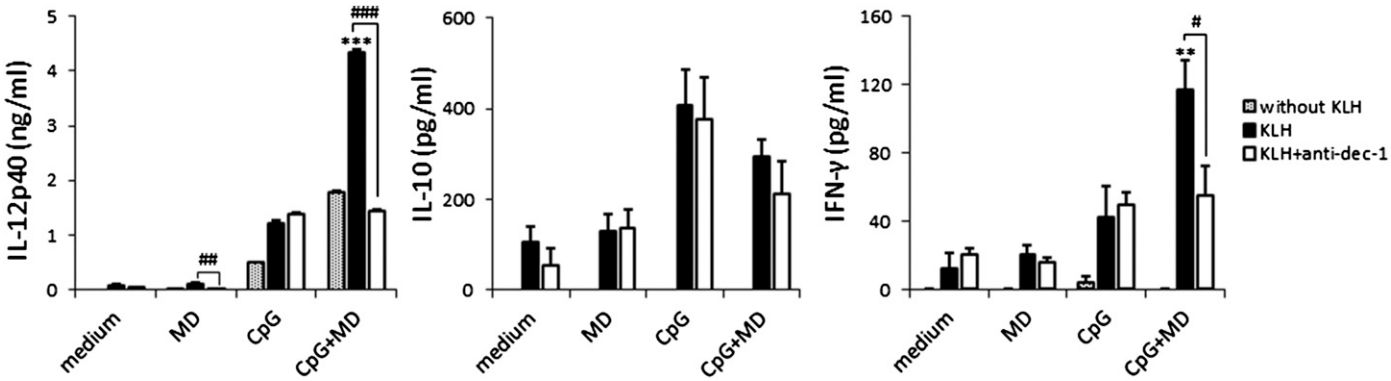


**Figure 4. MD-fraction increases the antigen-presenting activity of DCs.** DCs were pretreated with or without anti-dectin-1 antibody (1 μg/ml) for 1 h, followed by stimulation with MD-fraction (10 μg/ml) and/or CpG ODN (0.01 μM) for 24 h in the presence or absence of KLH (100 μg/ml). DCs were washed and cultured with KLH-primed T cells ($1 \times 10^5$ cells/well) for 72 h (DC:T cell ratio, 0.1:1). The levels of IL-12, IL-10, and IFN-γ in the culture supernatants were then measured by ELISA. Data represents the means ± SE of 2 separate experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate a significant difference between the combination treatment group and all other groups. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ compared with the groups linked by the bracket.

flow cytometry (Supplemental Fig. 1). These results suggest that MD-fraction increases IL-12 in CpG ODN-activated DCs and induces Ag-specific Th1 responses in a dectin-1–dependent pathway.

## MD-fraction significantly enhances the antitumor efficacy of CpG ODN-activated DC vaccines against tumors in vivo

Numerous studies have evaluated the effectiveness of DC vaccines in preventing tumor relapses and in extending patients' survival. The most-common type of DCs used for these human clinical studies were monocyte-derived DCs, generated from monocytes with GM-CSF and IL-4 [4]. We examined whether the combination MD-fraction and CpG ODN could improve the therapeutic efficacy of GM-CSF–generated, DC-based tumor immunotherapy using a murine tumor model. We inoculated mice with a subcutaneous administration of colon-26 cells ($1 \times 10^5$ cells/mouse). On d 3, 6, and 9 after tumor inoculation, tumor-lysate–pulsed DCs ($5 \times 10^5$ cells/mouse) stimulated with CpG ODN, MD-fraction, or both were given to the mice 3 times. Therapeutic administration of lysate-pulsed DCs stimulated with CpG ODN did not inhibit colon tumor growth, whereas lysate-pulsed DCs stimulated with MD-fraction significantly inhibited tumor growth, compared with the PBS control ($P < 0.05$ on d 22; **Fig. 5A**). Furthermore, mice treated with lysate-pulsed DCs with CpG ODN and MD-fraction had the slowest tumor growth (Fig. 5A) and the most survivors (Fig. 5B), and these were significantly greater than lysate-pulsed DCs with MD-fraction alone. Tumors in all mice at the end stage seemed to reach at least 1.0 $cm^3$ (data not shown). These results indicate that the combination treatment of CpG ODN and MD-fraction converts immature DCs to antitumor immunostimulatory DCs, enhancing the therapeutic efficacy of a tumor antigen-pulsed DC vaccine.

## Coadministration of MD-fraction with CpG ODN enhanced the antitumor effect in murine cancer model

We next tested the antitumor effect of direct administration of MD-fraction and CpG ODN into tumor-bearing mice. Mice were injected subcutaneously with $1 \times 10^5$ colon-26 carcinoma cells on d 0, and then received intraperitoneally (8 mg/kg) MD-fraction from d 5 to 15, intratumoral (10 μg) CpG ODN, or both, on d 9 and 13. Treatment with MD-fraction or CpG ODN alone induced a modest delay in tumor growth ($P < 0.001$ on d 18, compared with PBS control). In contrast, tumors in mice treated with the combination therapy of MD-fraction and CpG ODN grew markedly slower than tumors in mice treated with MD-fraction or CpG ODN alone (Fig. 5C). Tumors in all mice at the end stage seemed to reach at least 1.0 $cm^3$ (data not shown). Mice treated with this combined therapy survived significantly longer compared with all other groups (Fig. 5D). These findings suggested that the combination of MD-fraction and CpG ODN is a more-effective therapy for tumors than is MD-fraction or CpG ODN alone.

The synergistic antitumor effect of direct administration of MD-fraction and CpG ODN prompted us to study immunologic changes within the tumor. We investigated DC populations and function in the tumor on d 18 after tumor challenge. The number of DCs ($CD11c^+$) and activated DCs ($CD11c^+CD86^+$) in the tumor increased markedly in response to the combination treatment of MD-fraction and CpG ODN (**Fig. 6A and E**). Because previous reports have shown that dectin-1 signaling in DCs that recognize fungi can induce both Th1 and Th17 responses [32, 33], we examined Th1- and Th17-polarizing cytokines in tumor-infiltrating $CD4^+$ T cells by intracellular staining for IFN-γ and IL-17. The combination treatment of CpG ODN and MD-fraction did not change the total number of tumor-infiltrating $CD4^+$ T cells (Fig. 6F). It is noteworthy that the combination treatment increased the percentage of $IFN\gamma^+IL\text{-}17^-$ cells among the $CD4^+$ cells, rather than the $IFN\gamma^+IL\text{-}17^+$ and $IFN\gamma^-IL\text{-}17^+$ cells (Fig. 6B). The absolute number of $IFN\gamma^+IL\text{-}17^-CD4^+$ cells in the tumor was also significantly increased by the treatment of CpG ODN and MD-fraction compared with the individual treatments (Fig. 6F). On the other hand, the combination treatment of CpG ODN and MD-fraction increased neither the percentage of $IL\text{-}4^+CD4^+$ cells among TILs nor the absolute number of $IL\text{-}4^+CD4^+$ cells in the tumor, indicating that this combination treatment did not induce a Th2-mediated

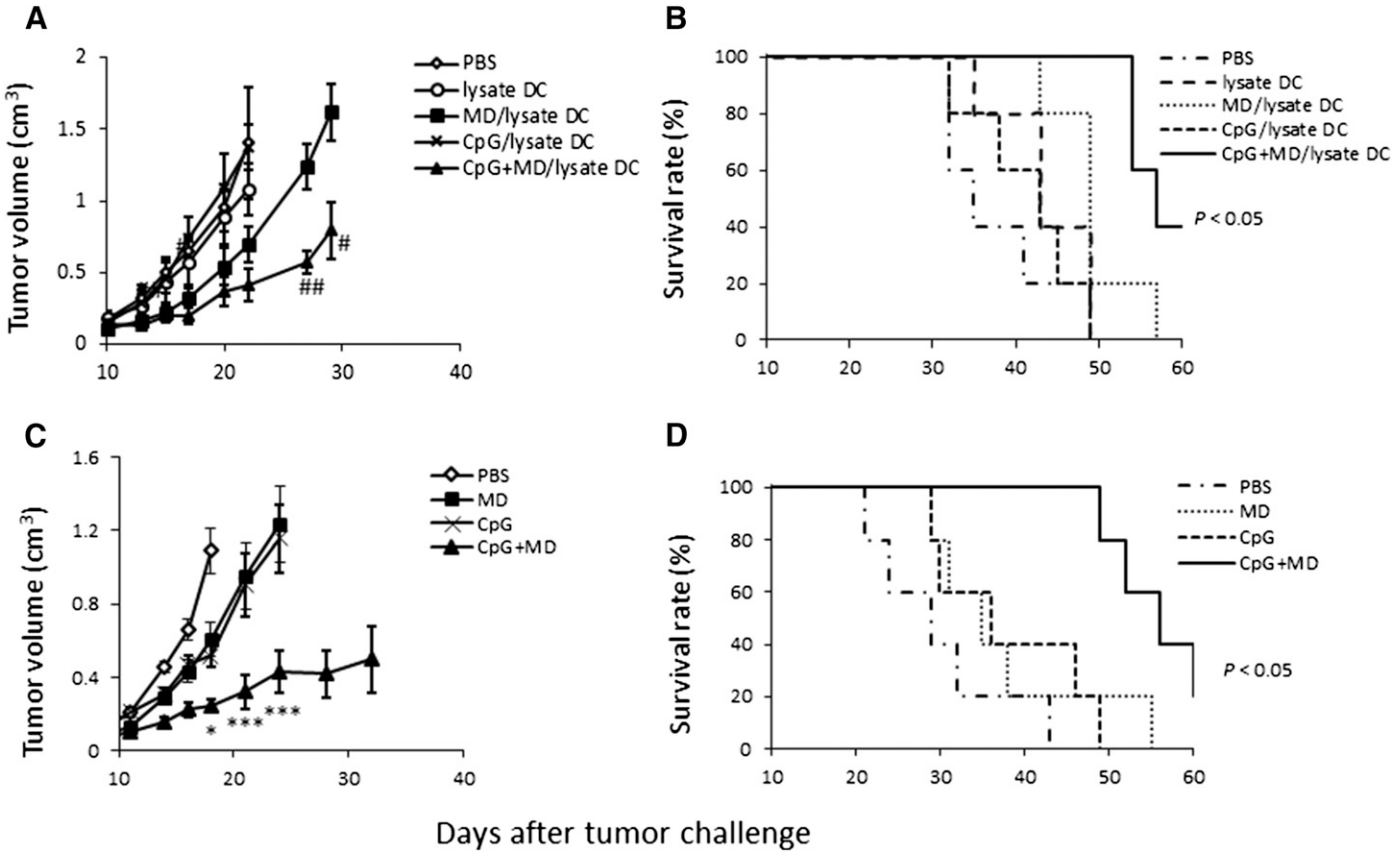


**Figure 5. MD-fraction enhances the antitumor efficacy of CpG ODN against murine tumors in vivo.** BALB/c mice were subcutaneously inoculated with colon-26 cells ($1 \times 10^5$ cells/mouse) on d 0. (A and B) For DC vaccination, the mice were treated by subcutaneous injections of tumor-lysate–pulsed DCs ($5 \times 10^5$ cells/mouse) stimulated with CpG ODN, MD-fraction, or both on d 3, 6, and 9. (C and D) For direct injections, the tumor-bearing mice received intratumor injections of CpG ODN on d 9 and 13 after tumor challenge, intraperitoneal injection of MD-fraction, or both for 10 consecutive d, beginning 5 d after the tumor challenge. Tumor cell growth (A and C) and survival rate (B and D) are shown. Data ($n = 5$) represent the means ± SE of 2 separate experiments. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. lysate-pulsed DCs with MD-fraction (A). $^{*}P < 0.05$ and $^{***}P < 0.001$ indicate a statistically significant difference between the combination therapy group and all other groups (C).

response (Fig. 6C and F). The total number of tumor-infiltrating $CD8^+$ T cells was significantly increased by the combination treatment (Fig. 6G). Both the percentage of $IFN\gamma^+CD8^+$ cells among TILs and the absolute number of $IFN\gamma^+CD8^+$ cells in the tumor were also significantly increased (Fig. 6D and G). These results indicate that the combination treatment of CpG ODN and MD-fraction leads to the infiltration of DCs into the tumor, causing a Th1-mediated antitumor T cell response.

Enhancement of tumor-infiltrating $CD8^+$ T cell function by treatment with CpG ODN and MD-fraction further demonstrated that when these cells, which were isolated from tumors in mice, were treated with a combination therapy of MD-fraction and CpG ODN, they exhibited significantly greater cytotoxicity compared with that observed in all other groups (Fig. 6H).

## DISCUSSION

In this study, we showed that MD-fraction combined with the TLR9 agonist CpG ODN stimulated DCs that support antitumor immunity. The combination of CpG ODN and MD-fraction is highly effective, either as an adjuvant for DC vaccination or by direct administration against a murine tumor. CpG ODN and MD-fraction synergistically increased IL-12 production and DC maturation markers on the DCs through a dectin-1–dependent pathway. However, MD-fraction did not affect IL-10 production in DCs after combined stimulation with CpG ODN. In addition, combined stimulation induced the mRNA expression of IL-12p35, IL-12p40, TNF-α, IL-1β, and IL-6, but not of IL-23p19 and IL-10, in the DCs. Collectively, the combined stimulation of MD-fraction and CpG ODN provided a strong inflammatory signature to DCs.

The dectin-1 ligand β-glucan has been reported to enhance TLR agonist-induced cytokine responses, including IL-12, IL-10, and IL-23, in human DCs [13, 29]. Other reports have demonstrated that dectin-1 signaling enhanced TNF-α, IL-23, IL-6, and IL-10 production, while down-regulating IL-12 relative to the levels produced by the TLR agonist alone in murine DCs and human DCs [30, 31]. Thus, the combined effects of β-glucan and TLR agonists on DCs are debatable. This contradiction may result from the use of different cell types and different β-glucan structures from various sources, as well as its purity. It has been reported that the soluble form of β-glucan does not activate dectin-1 signaling [34]. We found that the cytokine response of DCs induced by soluble β-glucan MD-fraction was less dramatic

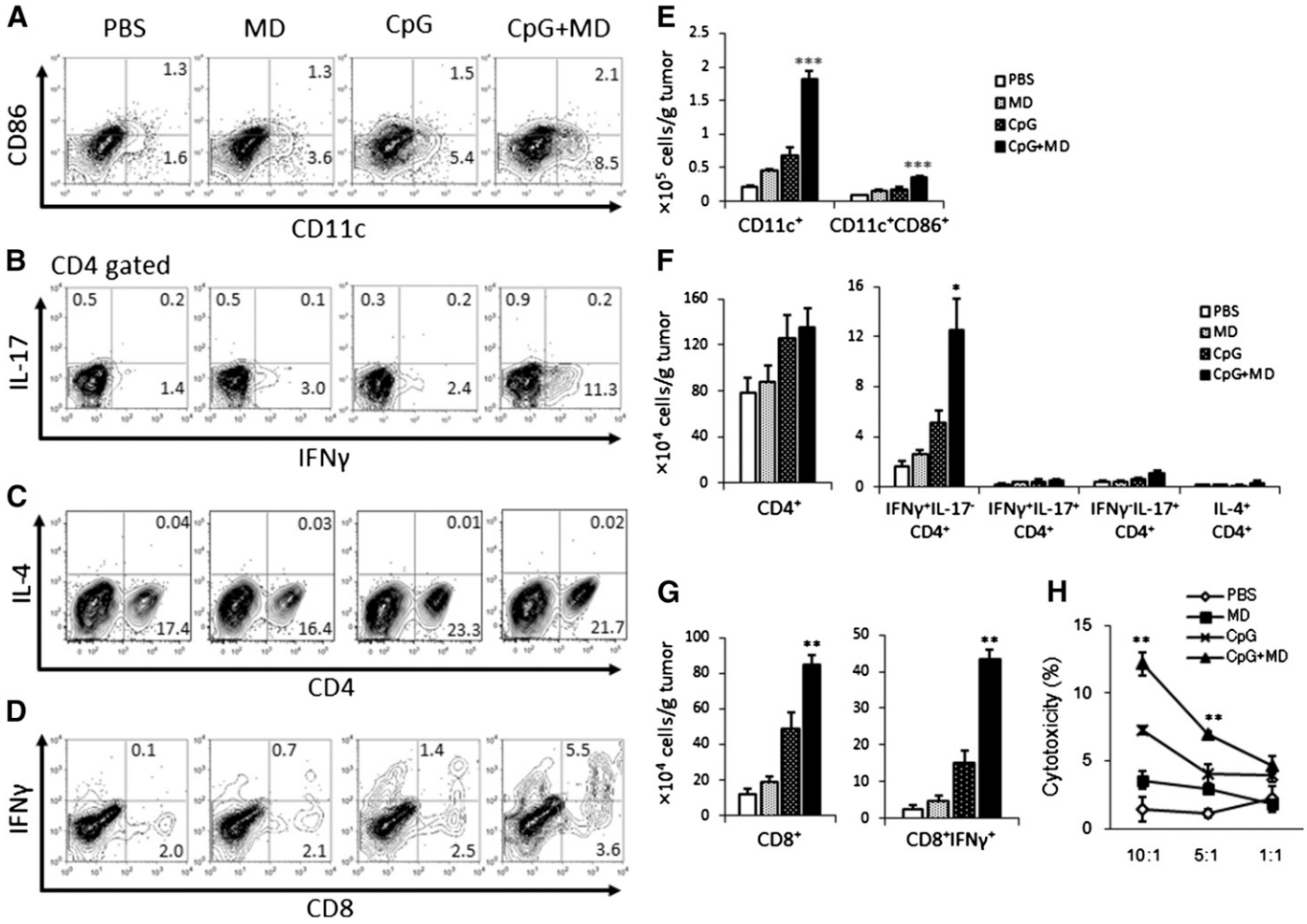


**Figure 6. Direct administration of MD-fraction and CpG ODN induces antitumor immunity.** (A–D) TILs were isolated and analyzed by flow cytometry. Representative flow plots showing the percentage of each subtype of TIL (A, C, and D) or $CD4^+$ cells (B), the number of each subtype in 1 g of tumor tissue (E, F, and G). (H) The data from the in vitro CTL assay are shown. $CD8^+$ T cells were mixed with colon-26 target cells at various E:T ratios in vitro. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate a significant difference between the combination treatment group and all other groups.

than that induced by particulate β-glucan curdlan (data not shown). However, MD-fraction synergized with CpG ODN to functionally induce DC maturation by augmenting the expression of cell-surface molecules and the production of proinflammatory cytokines, including IL-12, TNF-α, IL-1β, and IL-6 in DCs. Our study provides new combinations of immunomodulating stimuli, purified soluble β-glucan MD-fraction and the TLR9 agonist CpG ODN, for cancer immunotherapy.

Priming of macrophages or DCs with GM-CSF induces the dectin-1–mediated inflammatory response [35, 36]. We have demonstrated previously that MD-fraction directly induces GM-CSF production, resulting in dectin-1/Syk activation in resident macrophages [20]. Because the DCs used in this study were generated with GM-CSF, it raises the possibility that the DCs were ready to induce a dectin-1–mediated inflammatory response against a β-glucan, such as MD-fraction. Culturing of bone marrow cells with GM-CSF has led to the development of models of migratory DCs and of monocyte-derived inflammatory DCs. On the other hand, Flt3L is one such model for studying the origin of plasmacytoid DCs and lymphoid–resident DCs [37]. Therefore, we tested the effect of MD and CpG on different DC cell types generated by GM-CSF or Flt3L, respectively. We confirmed that MD-fraction synergized with CpG ODN enhanced IL-12 production in Flt3L-generated DCs, as well as in GM-CSF-generated DCs (data not shown). Overall, GM-CSF priming is not essential for the synergistic effect of CpG ODN and MD-fraction.

Immunogenic DCs produce high levels of IL-12, induce the production of IFN-γ, and favor the Th1 response that bridges innate and adaptive immunity [1]. In contrast, DCs secrete IL-10, which acts in an autocrine manner to develop the immunosuppressive response [38]. The activation of the immune system by CpG ODN, induces effective immunity via IL-12 but is counteracted by simultaneous induction of immunosuppressive cytokines, including IL-10 [39]. We saw that MD-fraction and CpG ODN-induced synergistic DC activation causes the generation of strong effector DCs with augmented capacity for efficiently priming antigen-specific Th1-type T cell responses. In addition, we showed the antitumor effectiveness of tumor-lysate–pulsed DC vaccines in vivo was not enhanced by

stimulation with CpG ODN alone but was significantly enhanced by stimulation with the combination of CpG ODN and MD-fraction. One reason for this combinatorial effect of MD-fraction and CpG ODN on DC vaccines may be that MD-fraction increased the expression of DC maturation markers and IL-12 production by CpG ODN-stimulated DCs, but it did not increase IL-10 production.

Furthermore, we showed that the use of MD-fraction and CpG ODN as a direct combination therapy induced effective inhibition of tumor growth and survival, as well as the adoptive transfer of DC primed with MD-fraction and CpG ODN. Direct administration has the advantage over DC vaccination of not requiring expensive and time-consuming preparation of cells from an individual patient. Successful cancer immunotherapy induces the tumor-specific T cell response to a type-1 cytokine profile, such as IL-12, and IFN-γ [40]. We found that MD-fraction in combination with CpG ODN synergistically increased IL-12 production by DCs, but not IL-10 in vitro, and this was consistent with the increase of $CD11c^+$ DCs at the tumor site and the induction of tumor-infiltrating IFN-γ producing $CD4^+$ and $CD8^+$ T cells and a tumor-specific CTL response.

Direct administration of MD-fraction and CpG ODN increased the number of DCs at the tumor site. DCs consist of heterogeneous subsets, many with unique phenotypic and functional characteristics. Mainly 3 distinct DC subsets have been identified: migratory DCs, lymphoid-resident DCs, and plasmacytoid DCs [41]. However, which DC subset is directly acted on by MD-fraction in vivo is unclear. Previously, we reported that intraperitoneal administration of MD-fraction induced various cytokines, including GM-CSF, causing more granulocyte and macrophage progenitors in bone marrow and splenic DCs [18]. Increasing GM-CSF secretion influences the composition of the DC pool, and this becomes more pronounced during inflammation [41]. These facts suggest that direct administration of MD-fraction in vivo induces GM-CSF, resulting in the development of inflammation-induced DCs. In addition, we confirmed that MD-fraction enhanced IL-12 production in both GM-CSF and Flt3L-generated DCs (data not shown). Taken together, MD-fraction in combination with CpG ODN may synergistically activate both inflammation-induced and lymphoid tissue-resident DCs in vivo.

TLR9 agonists have been evaluated in clinical trials as cancer immunotherapeutics and as vaccine adjuvants [7]; however, the objective outcomes of these treatment approaches fall well short of the desired cure for cancer [9, 10, 42]. Our findings demonstrate that MD-fraction and CpG ODN synergistically activate DCs to an immunostimulatory phenotype, resulting in tumor regression through the induction of antitumor T cell responses. Thus, the combination of CpG ODN and MD-fraction has considerable potential as an immunotherapeutic approach against cancer.

## AUTHORSHIP

Y.M. performed most of the experiments, analyzed data, and wrote the manuscript. D.N. performed a few select experiment. Y.N., M.K., and H.N. contributed critical analysis of data, ideas for experiments, and to the writing of the manuscript.

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### DISCLOSURES

The authors declare no competing financial interests.

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