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Maturation of dendritic cells by maitake α-glucan enhances anti-cancer effect of dendritic cell vaccination

Yuki Masuda[a], Yoshiaki Nakayama[a], Takehiro Mukae[a], Akihiro Tanaka[b], Kenta Naito[b], Morichika Konishi[a,*]

[a] Department of Microbial Chemistry, Kobe Pharmaceutical University, Kobe, Japan
[b] Research and Development Department, Yukiguni Maitake Co., Ltd., Niigata, Japan

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ABSTRACT

Dendritic cells (DCs) play a primary role in antigen presentation to $CD4^+$ and $CD8^+$ T cells and induce acquired immune response against cancer cells. Therefore, determining positive modulators of DC activation to improve therapeutic approaches for cancer immunotherapy is greatly needed. In this study, we investigated the effect of maitake α-glucan YM-2A, isolated from *Grifola frondosa*, on the maturation and function of DCs and its adjuvant effect on a tumor-associated antigen (TAA)-loaded DC vaccine against murine tumor. We showed that YM-2A induced morphological changes and increased cell-surface maturation markers and cytokine production in DCs. In a mixed lymphocyte reactions assay, YM-2A-treated DCs increased proliferation and production of IFN-γ by allogeneic $CD4^+$ and $CD8^+$ T cells. These results indicate that YM-2A phenotypically and functionally activates DCs. Furthermore, YM-2A-treated TAA-loaded DC vaccine significantly reduced tumor growth and improved survival in two murine tumor models, CT-26 tumor-bearing BALB/c mice and B16 melanoma-bearing C57BL/6 mice. YM-2A-treated TAA-loaded DC vaccine increased splenic IFN-γ producing $CD4^+$ and $CD8^+$ T cells in CT-26 tumor-bearing BALB/c mice. Antibody neutralization studies indicated that YM-2A-induced DC maturation is mediated, in part, by the Dectin-1-dependent pathway. Overall, YM-2A-treatment with a TAA-loaded DC vaccine could be an excellent candidate for immunotherapy against cancer.

1. Introduction

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that can capture, process and present tumor antigens to naïve, non-primed T cells to activate an antigen-specific immune response [1,2]. DC subsets show functional heterogeneity and may be immunogenic or tolerogenic depending on their inflammatory cytokines or pathogen-associated molecular patterns. Immunogenic DCs can induce both proliferation and activation of tumor-specific T cells, such as Type I helper T (Th1) cells and cytotoxic T lymphocytes (CTLs). In contrast, immature or tolerogenic DCs induce immune tolerance and are linked to cancer progression. Clinical trials of DC-based vaccines carried out in tumor patients have proved their safety and capacity for the induction of tumor antigen-specific immune responses [3]. Adjuvants can be used to enhance the immunogenicity of DC vaccines through different mechanisms, such as induction of cytokines and chemokines, increase of antigen uptake, improvement of DC maturation, and antigen presentation [4]. However, due to the limited effect of DC vaccines with adjuvants currently used in clinical trials, the development of more effective adjuvants is urgently needed [5].

Many studies have reported on the immunomodulatory effects of polysaccharides including glucans isolated from mushrooms, fungi, yeast, algae, lichens, and plants. β-Glucan is crucial component of the fungal cell wall, and recognized by β-glucan receptor dectin-1 on APCs [6]. Some natural medicines containing β-glucan have immunomodulatory effects and are used clinically for tumor immunotherapy in several countries [7,8]. Some α-glucans isolated from fungi also have potent immune stimulatory activity [9–12]. Although it has been reported that these α-glucans are recognized by several receptors such as TLRs and DC-SIGN, correlation between structure and activity has not been fully elucidated. The maitake mushroom (*Grifola frondosa*) is a popular, edible mushroom in Japan, especially because its fruiting body can be artificially produced. We have previously reported that soluble β-glucan MD-Fraction, derived from maitake mushrooms, induces antitumor immunity via dectin-1 dependent pathway [13,14]. Recently, the novel bioactive α-glucan YM-2A was isolated from the

* Corresponding author at: Department of Microbial Chemistry, Kobe Pharmaceutical University, 4-19-1, Motoyama-kitamachi, Higashinada-ku, Kobe 658-8558, Japan.
E-mail address: mkonishi@kobepharma-u.ac.jp (M. Konishi).

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maitake mushroom and characterized as a glycogen-like polysaccharide consisting of linear 4-linked α-D-Glcp residues substituted at position 6 with -α-D-Glcp branches [15]. We demonstrated that orally administrated YM-2A significantly inhibited tumor growth by enhancing the immune system. We also found that YM-2A did not directly inhibit tumor cell growth, but increased APC proliferation and inflammatory cytokine production, such as IL-12 and TNF-α. As IL-12 mediates the expression of other proinflammatory cytokines, TNF-α, IL-1β, and IL-6 [16], IL-12 produced by APCs induces the production of IFN-γ by T cells and favor the Th1 response that bridges innate and adaptive immunity [17,18]. This study suggests that YM-2A can directly activate DCs and induce Th1/CTL immune responses to inhibit tumor cell growth. In this study, we investigated whether YM-2A can enhance an antigen-presentation by DCs and induce antigen-specific immune response. We also assessed the adjuvant effect of YM-2A on DC vaccines against tumors.

2. Materials and methods

2.1. Preparation of polysaccharides from maitake mushroom

YM-2A was prepared from maitake fruiting bodies (Yukiguni Maitake, Niigata, Japan) as previously described [15]. In brief, the hot water extract of maitake fruiting bodies was collected by centrifugation and the macromolecules were precipitated by the addition of ethanol to a concentration of 50%. The precipitate was dissolved in distilled water and the supernatant was then collected by the addition of ethanol (25% final concentration). The precipitate (YM-2A) was collected by the addition of ethanol (42% final concentration), dissolved in distilled water and freeze-dried. The weight-average molecular weight of YM-2A was calculated to be 8.51×10^6 Da by high-performance size-exclusion chromatography (HPSEC) analysis with a multi-angle laser light scattering detector (MALS; DAWN HELEOS, Wyatt Technology, Santa Barbara, CA). ^{1}H and ^{13}C NMR and methylation analysis revealed that YM-2A is a glycogen-like polysaccharide consisting of linear 4-linked α-D-Glc*p* residues substituted at position 6 with -α-D-Glc*p* branches. The peak associated with β-glucan was not observed, suggesting that YM-2A sample used in this study has almost no contamination of maitake β-glucan MD-Fraction [15]. Maitake β-glucan was prepared from maitake fruiting bodies as described previously [13,19]. Maitake β-glucan was eluted as a single peak with a molecular mass of $1.2–2.0 \times 10^6$ Da by high-pressure liquid chromatography. We tested for endotoxin contamination using the Endospecy ES-24S set (Seikagaku Biobusiness, Tokyo, Japan) but did not detect any contamination in neither YM-2A nor maitake β-glucan. In addition, polymixin B treatment did not affect YM-2A and maitake β-glucan-induced cytokine production from peritoneal macrophages from DBA/2 mice, indicating that there was no endotoxin contamination in YM-2A or maitake β-glucan (data not shown).

2.2. Mice

Female BALB/c, C57BL/6 and DBA/2 mice were purchased from CLEA Japan (Higashiyama, Japan), and 5–8-week-old mice were used in the study. The study was carried out according to the guidelines of the Animal Care and Use Committee at the Kobe Pharmaceutical University. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Kobe Pharmaceutical University (Permit Number: 2017-051). At the end of the experiments, mice were sacrificed by cervical dislocation under isoflurane anesthesia and all efforts were made to minimize suffering.

2.3. Cell preparation and culturing

GM-CSF-induced bone marrow-derived DCs were produced by culture of bone marrow cells (1×10^6 cells/ml) in complete RPMI (RPMI-1640 medium supplemented with 10% FBS, 0.03 mg/ml L-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin) supplemented with murine GM-CSF (20 ng/ml) and IL-4 (10 ng/ml), as described previously [20]. FMS-like tyrosine kinase 3 ligand (FLT3L)-induced bone marrow-derived DCs were produced by culture of bone marrow cells (2×10^6 cells/ml) in complete RPMI supplemented with 100 ng/ml recombinant murine Flt3L (Miltenyi Biotec, Auburn, CA, USA) in 6-well plates. Half of the medium was removed and fresh cytokine-supplemented medium was added at days 4–5. Bone marrow-derived DCs were harvested and analyzed on days 7–8, and > 85% of the cells expressing CD11c were used. Spleen single-cell suspensions were prepared by filtration through 70 μm nylon strainers (BD Biosciences), and $CD11c^+$ cells were separated by using CD11c MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany).

DCs (1×10^6 cells/ml) were stimulated with YM-2A at various concentrations (0–500 μg/ml), TLR2 agonist Pam_3CSK_4 (0.5 μg/ml; InvivoGen, San Diego, CA, USA), TLR4 agonist lipopolysaccharide (0.1 μg/ml; *Escherichia coli* serotype 0111:B4, Sigma-Aldrich, St. Louis, MO, USA), maitake β-glucan MD-Fraction (100 μg/ml) for 24 h. After incubation, cytokine levels in the supernatant were determined using ELISA, according to the manufacturer's protocol (IL-12p40, TNF-α, IL-6, and IL-10; PeproTech, Rocky Hill, NJ, USA, IL-12p70; Invitrogen, Carlsbad, CA, USA). For the blocking antibody assays, DCs were pre-incubated with 1 μg/ml anti-dectin-1 (2A11; AbD Serotec, Kidlington, United Kingdom), anti-TLR2 (T2.5; HyCult Biotechnology, Uden, The Netherlands), anti-TLR4/MD-2 (MTS510; HyCult Biotechnology), anti-DC-SIGN/CD209 (MMD3; R&D Systems, Inc., Minneapolis, MN, USA), anti-Dectin-2/CLEC6A (D2.11E4; Abcam, Cambridge, MA, USA) or anti-Mincle (4A9; MBL Ltd., Nagoya, Japan) for 1 h, followed by stimulation with YM-2A, TLR agonists, or maitake β-glucan for 24 h.

2.4. Mixed lymphocyte reaction (MLR)

Responder T cells used for allogenic T cell reaction were isolated with a MACS $CD4^+$ or $CD8^+$ T cell isolation kit (Miltenyi Biotech, Auburn, CA, USA) from the whole spleen cells of C57BL/6 mice. Unstimulated, YM-2A- or LPS-stimulated DCs of DBA/2 mice were treated with 50 μg/ml mitomycin C (Kyowa Co. Ltd., Tokyo, Japan) for 1 h and cultured with allogenic T cells at a density of 1×10^5 cells/well in U-bottom 96-well microtiter plates for 72 h. Cell proliferation was measured with WST-8 reagent using Cell Count Reagent SF (Nakarai Tesque Inc., Kyoto, Japan) according to the manufacturer's instructions. IFN-γ levels in the supernatants were determined using ELISA, according to the manufacturer's protocol (PeproTech, Rocky Hill, NJ, USA).

2.5. In vivo tumor studies

Tumor cell lysates (tumor associated antigen; TAA) were prepared by 4 freeze–thaw cycles of colon-26 (CT-26) cells or B16 melanoma cells (1×10^7 cells/ml in PBS), as described previously [21]. DCs from BALB/c or C57BL/6 mice were incubated with CT-26 cell lysate or B16 cell lysate, respectively, at a ratio of 3:1 tumor cell equivalents, that is, DCs in the presence or absence of YM-2A (100 μg/ml) for 24 h. The DCs were harvested and resuspended in PBS at specific vaccine concentrations for use in further studies.

BALB/c or C57BL/6 mice were inoculated with CT-26 carcinoma cells (1×10^5 cells/mouse) or B16 melanoma cells (2×10^5 cells/mouse), respectively, by subcutaneous (sc) injection on day 0. For DC vaccination, the mice were treated by peritumoral injections of DCs (1×10^6 cells/mouse) stimulated with either YM-2A, TAA prepared from tumor, or both on days 3, 8, and 14. The tumor volume was calculated using the following formula: tumor volume (cm^3) = (longest diameter × shortest diameter^2) / 2. For survival studies, mice were monitored for survival and euthanized if they became moribund or if tumor reached 2 cm in maximum diameter.

2.6. 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 labeled were analyzed using FACSAria III (BD Biosciences, San Jose, CA, USA). Spleen single-cell suspensions were prepared by filtration through 70 μm nylon strainers (BD Biosciences), and were labeled with anti-CD4, anti-CD8, anti-CD86, and anti-CD11c for 20 min at 4 °C, washed, and analyzed using FACSAria III. For intracellular staining of IFN-γ, cells were cultured for 4 h with PMA (25 ng/ml) and ionomycin (1 μg/ml), and cytokine release was prevented by treatment with Golgi-stop (BD PharMingen). Following surface staining of CD4 or CD8, cells were fixed using the Cytofix/Cytoperm kit (BD PharMingen) and stained with mAb including IFN-γ. All antibodies were purchased from BD Bioscience.

2.7. Statistical analysis

Data were analyzed using Prism software (GraphPad Software, Inc.). Data presented are expressed as the mean ± standard error (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 two groups. Tumor growth data were analyzed using a two-way ANOVA. Kaplan-Meier survival curves of mice in the tumor studies were analyzed by the log rank-survival test. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. YM-2A induces maturation of DCs in dose-dependent manner

DC is functionally divided into conventional DCs (cDCs) and plasmacytoid DCs (pDCs). GM-CSF-induced bone marrow-derived DC belongs to cDCs, which originally shows a dendritic morphology where the dendrites further extend after activation [22,23]. As shown in Fig. 1A, treatment with YM-2A dose-dependently induced marked extension of dendrites of DCs indicative of cellular activation. The percentage of activated DCs to total cells, an index reflecting the extent of cell extending, was dose dependently increased by YM-2A (Fig. 1A). Similar morphological changes occurred by Pam_3CSK_4 and maitake β-glucan, whereas LPS induced cellular atrophy probably due to excessive activation. Consistent with our previous report [15], YM-2A increased the relative values of the number of viable DCs measured by WST-8 assay, indicating that YM-2A increases cell proliferation of DCs (data not shown). These results demonstrate that no cytotoxicity was observed in DCs for YM-2A concentrations as high as 500 μg/ml, but that cell proliferation was increased.

The process of DC maturation, in general, involves not only morphological changes (e.g. formation of dendrites), but also a redistribution of MHC molecules from intracellular endocytic compartments to the DC surface, an increase in the surface expression of costimulatory molecules (e.g. CD80 and CD86), and the secretion of cytokines (e.g. IL-12). To evaluate whether YM-2A influences DC phenotypic maturation, YM-2A stimulated DCs were analyzed for the expression of DC maturation markers. Fig. 1B and C shows that YM-2A upregulated the expression of MHC class II molecules, CD80 and CD86, on DCs in dose-dependent manner. In addition, YM-2A significantly increased IL-12p40, TNF-α, and IL-6 production by DCs, but not IL-10 (Fig. 1D). 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 [24]. As Fig. 1D, YM-2A significantly increased IL-12p70 production from DCs. This made it clear that YM-2A enhances IL-12 production in DCs.

Culturing of bone marrow cells with GM-CSF is the model of migratory DCs and of monocyte-derived inflammatory DCs. We therefore investigated whether YM-2A induces maturation of other DC subsets. As shown in Fig. 2A, YM-2A enhanced DC activation markers on primary splenic DCs as well as GM-CSF-induced bone marrow-derived DCs. FLT3L-induced bone marrow-derived DC is one such model for studying the origin of pDCs [25]. Fig. 2B shows that YM-2A also increased DC activation markers on $CD11c^+B220^+$ pDC generated by Flt3L. These results suggest that YM-2A can induce maturation of the various types of DCs, such as primary DCs, bone marrow-derived GM-CSF-induced cDC and Flt3L-induced pDCs.

3.2. YM-2A-stimulated DCs induce allogenic T cell responses

YM-2A increased the expression of DC maturation markers involved in the presentation of antigens to T cells (Fig. 1B and C). Therefore, we investigated the ability of YM-2A-treated DC to stimulate T-cell proliferation and cytokine production in an allogeneic mixed lymphocyte reaction (MLR). Splenic $CD4^+$ or $CD8^+$ T cells were isolated from C57BL/6 (H-2b) mice and cultured with YM-2A or LPS-treated or untreated DCs from DBA/2 (H-2d) mice for 72 h. YM-2A-treated DCs significantly induced the proliferation of allogeneic $CD4^+$ and $CD8^+$ T cells (Fig. 3, left). In addition, IFN-γ levels produced by allogeneic $CD4^+$ and $CD8^+$ T cells were more significantly increased by YM-2A-treated DCs than by untreated DCs (Fig. 3, right). These results suggest that YM-2A-treated DCs have the ability to prime and activate allogeneic T cells.

3.3. YM-2A significantly enhances the antitumor efficacy of tumor-associated antigens (TAA) loading DC vaccines against CT-26 tumor and B16 melanoma in vivo

Numerous studies have evaluated the effectiveness of ex vivo-generated DC vaccines in inducing T cell immunity and in preventing tumor relapses. The most-common type of DCs used for clinical studies were monocyte-derived DCs, cultured in GM-CSF and IL-4 [5]. They were loaded with tumor-associated antigens (TAA) in a variety of ways and used to promote tumor Ag-specific antitumor T cell immunity [5]. We examined whether YM-2A could enhance the therapeutic efficacy of DC-based tumor immunotherapy using two murine tumor models, CT-26 tumor-bearing BALB/c mice and B16 melanoma-bearing C57BL/6 mice. DC vaccines were injected to peritumoral areas on days 3, 8, and 14 after tumor inoculation. A control group received PBS, the DC group received immature DCs without TAA and YM-2A, the YM-2A DC group received YM-2A treated DCs, the TAA DC group received DCs pulsed with TAA, and the YM-2A/TAA DC group received YM-2A treated DCs pulsed with TAA. In both tumor models, the tumor growth was significantly inhibited by therapeutic administration of YM-2A/TAA DCs, compared with PBS, DC and TAA DC group ($*P < 0.05$ on day 27; Fig. 4A, $*P < 0.05$ on day 24; Fig. 4B). Administration of YM-2A DCs significantly inhibited tumor growth compared with PBS and DC group ($*P < 0.05$ on day 27; Fig. 4A, $*P < 0.05$ on day 24; Fig. 4B), but not significantly different from TAA DCs. YM-2A/TAA DCs significantly inhibited tumor growth compared with YM-2A DCs in CT-26 model, but not defferent in B16 model. YM-2A DCs and YM-2A/TAA DCs prolonged survival compared with PBS, DC and TAA DC group in both tumor models. Survivals of YM-2A DC and YM-2A/TAA DC group were not significantly different in both tumor-bearing mice (Fig. 4C and D). These results indicate that YM-2A converts immature DCs into antitumor immunostimulatory DCs, enhancing the therapeutic efficacy of the DC vaccine against tumor.

3.4. YM-2A-treated TAA-loaded DC vaccine induces Th1 immunity in CT-26 tumor-bearing mice

YM-2A was demonstrated to induce DC maturation and facilitated T cell priming in vitro (Figs. 1–3). To investigate whether the antitumor effect is correlated with the T-cell responses induced by YM-2A/TAA DC vaccines, spleens were collected from the CT-26 tumor-bearing BALB/c

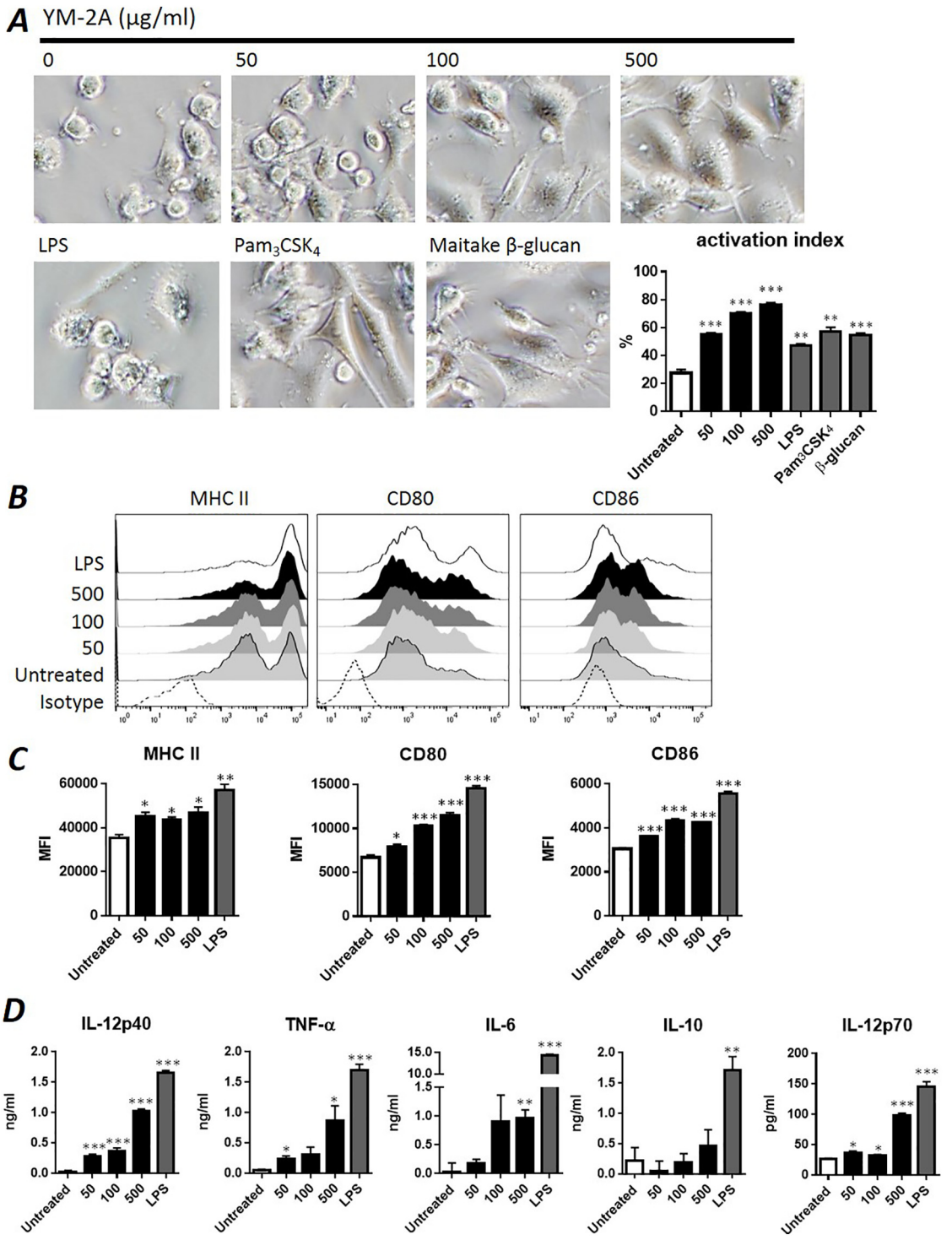

Fig. 1. YM-2A induces GM-CSF-induced bone marrow-derived DC maturation and cytokine production.
GM-CSF-induced bone marrow-derived DCs (1×10^6 cells/ml) were stimulated with YM-2A (0–500 μg/ml), LPS (0.1 μg/ml), Pam_3CSK_4 (0.5 μg/ml) or maitake β-glucan (100 μg/ml) for 24 h. (A) Morphology of DCs visualized by optical microscopy (×200) after stimulation. The activation index percentage was expressed as the number of cells with activated morphology relative to the total number of cells. Expression levels of MHC II, CD80, and CD86 in $CD11c^+$ cells as representative histogram profiles (B), and summary of MFI (C). (D) IL-12p40, TNF-α, IL-6, IL-10 and IL-12p70 in the culture supernatants were measured by ELISA. The data shown are representative (n = 3) of three experiments. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with untreated control.

mice to detect the frequencies of $CD4^+$ and $CD8^+$ T cells. Although YM-2A/TAA DC vaccines did not affect spleen weight (data not shown) or the percentages of $CD4^+$ cells, they significantly increased the percentages of $CD8^+$ cells in spleen cells compared with all other groups (Fig. 5A, B and C).

Antitumor immunity is initiated by APCs, such as DCs, which capture tumor antigens from tumor cells and induce the $CD4^+$ Th-1 cell/$CD8^+$ CTL response, producing the antitumor cytokine IFN-γ [3,26]. Next, we assessed whether YM-2A/TAA DC vaccines can induce IFN-γ expression by $CD4^+$ and $CD8^+$ T cells in tumor-bearing mice. Intracellular cytokine staining showed that YM-2A/TAA DC vaccines significantly increased the percentage of IFN-γ-expressing cells in

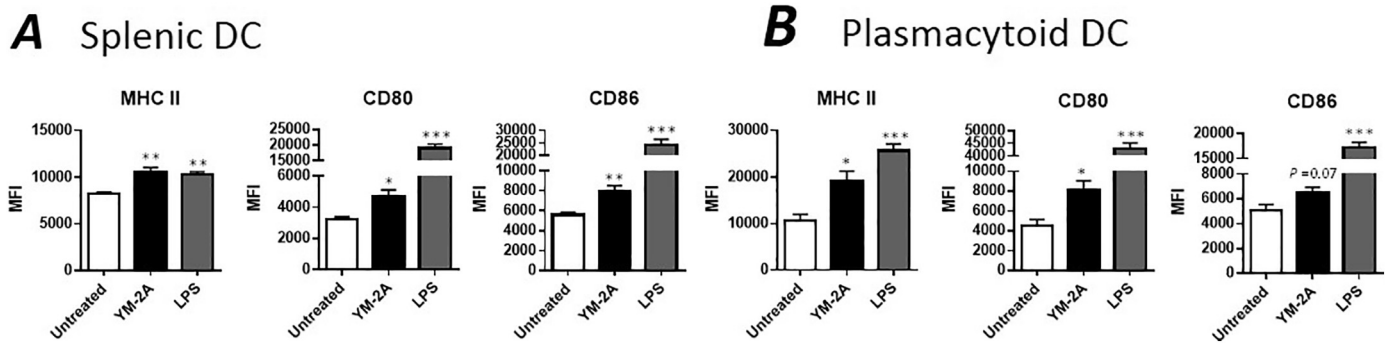

Fig. 2. YM-2A induces both primary splenic DC and Flt3L-induced bone marrow-derived pDC maturation.
(A) MACS-enriched splenic CD11c$^+$cells were incubated with YM-2A (500 μg/ml) or LPS (0.1 μg/ml) for 24 h. (B) Flt3L-induced bone marrow-derived DCs (1×10^6 cells/ml) were stimulated with YM-2A (500 μg/ml), LPS (0.1 μg/ml) for 24 h. Expression levels of MHC II, CD80, and CD86 gated in CD11c$^+$ cells (primary DC) and CD11c$^+$B220$^+$ cells (pDCs) as summary of MFI. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, compared with untreated control.

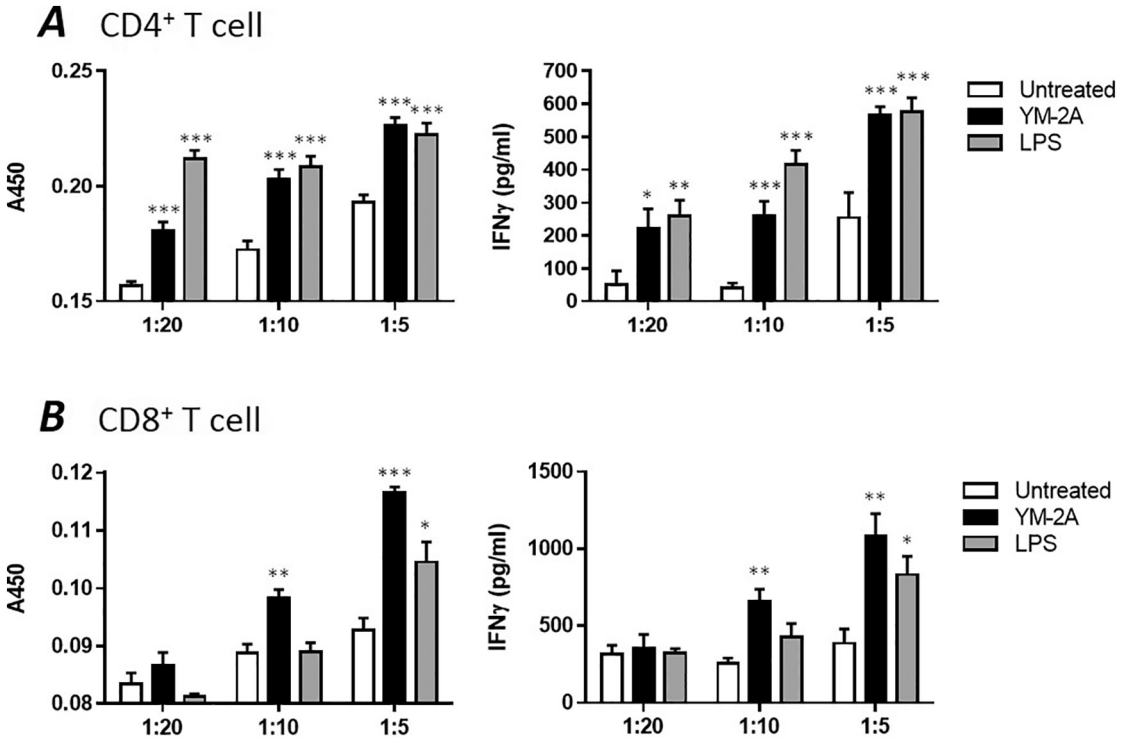

Fig. 3. Allostimulatory activity of YM-2A-treated DCs.
Unstimulated or YM-2A- or LPS-stimulated DCs were treated with 50 μg/ml mitomycin C for 1 h and cultured with allogeneic CD4$^+$ and CD8$^+$ T cells (1×10^5 cells/well) at a ratio of stimulators to responders (1:5 to 1:20) for 72 h. (A) Cell proliferation was measured with WST-8 reagent. (B) IFN-γ levels in supernatants were analyzed by ELISA. Data represent the mean ± SE (n = 4–6) of two separate experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with untreated control.

splenic CD4$^+$ and CD8$^+$ T cells (Fig. 5D, E, F and G). We also found that YM-2A/TAA DC vaccines enhanced CD86 expression of splenic DCs compared with all other groups (Fig. 5H). These data indicate that the YM-2A/TAA DC vaccine enhances Th1 polarization and induces anti-tumor CD8$^+$ T cell development.

3.5. YM-2A induces maturation of DCs via C-type lectin Dectin-1

Various polysaccharides were reported to activate APCs, such as DCs and macrophages. λ-Carrageenan, which contains β-1,4 and α-1,3 glycoside linkages, promotes DC maturation through the TLR4-mediated signaling pathway [27]. β-Glucans, including maitake β-glucan, are recognized by Dectin-1 on macrophages and DCs [13,21,28]. On the other hand, YM-2A is characterized as a glycogen-like polysaccharide consisting of α-1,4 and α-1,6 glycoside linkages [15]. Some glycogens have been reported to be recognized by TLR2 on macrophages [11,29]. To investigate which receptors are associated with DC maturation by YM-2A, DCs were pretreated with either anti-TLR2, -TLR4, or Dectin-1 neutralizing antibodies. As shown in Fig. 6A–D, DC maturation induced by TLR4 ligand LPS, TLR2 ligand Pam_3CSK_4, or maitake β-glucan was significantly inhibited by anti-TLR4, -TLR2, or Dectin-1 antibodies, respectively. Unexpectedly, YM-2A-induced DC maturation was not inhibited by anti-TLR4 or -TLR2 antibodies, but almost completely abolished by anti-Dectin-1 antibody (Fig. 6A–D). Dectin-1 is a member of the C-type lectin-like receptors (CLRs) [30,31]. We therefore investigate the role of the representative CLRs, such as Dectin-2, Mincle and DC-SIGN, on YM-2A-induced DC maturation. Neutralizing antibodies against these receptors did not inhibit YM-2A-induced DC maturation (Supplemental Fig. S1). These results suggest that YM-2A induces DC maturation through the Dectin-1 pathway.

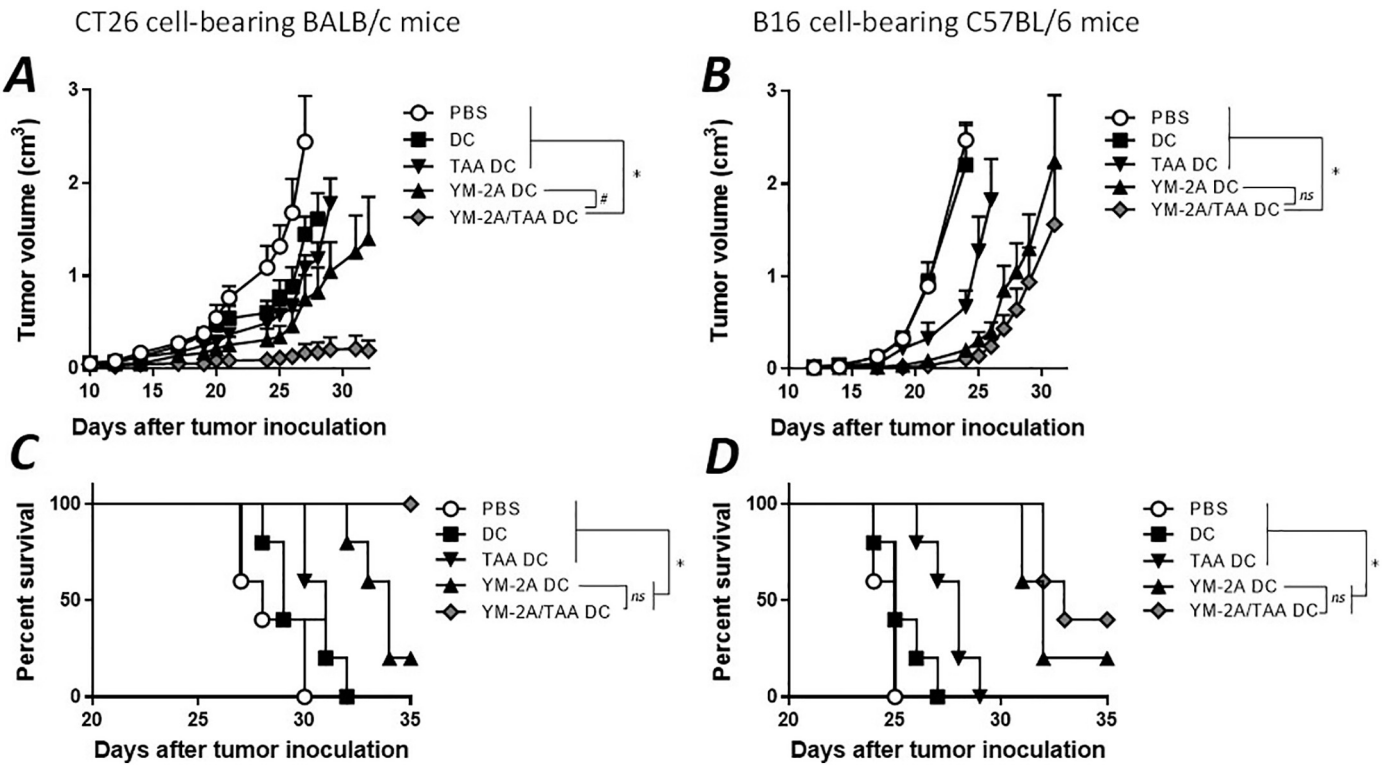

Fig. 4. Adjuvant effect of YM-2A in DC-based tumor immunotherapy.
BALB/c and C57BL/6 mice were subcutaneously inoculated with colon-26 cells (1×10^5 cells/mouse) or B16 melanoma cells (2×10^5 cells/mouse), respectively, on day 0. For DC vaccination, the mice were treated by peritumoral injections of DCs (1×10^6 cells/mouse) stimulated with either YM-2A, TAA prepared from tumor, or both on days 3, 8, and 14. (A and B) Tumor cell growth are shown. Data represent the mean ± SE (n = 5). *$P < 0.05$ on day 29–32 (A) or day 24(B). (C and D) Kaplan-Meier curves and Log-rank test for overall survival (*$P < 0.005$).

4. Discussion

In this study, we demonstrated that YM-2A induced morphological changes and increased cell-surface maturation markers and cytokine production in DCs. In MLR assays, YM-2A-treated DCs increased proliferation and production of IFN-γ by allogeneic $CD4^+$ and $CD8^+$ T cells. These results indicate that YM-2A phenotypically and functionally activates DCs. Furthermore, the YM-2A-treated TAA-loaded DC vaccine increased splenic IFN-γ producing $CD4^+$ and $CD8^+$ T cells, resulting in reduced tumor growth.

Our previous studies demonstrated that YM-2A not only increased cell proliferation, but also up-regulated mRNA expression of IL-12p40, IL-12p35, IL-1β, IL-6 and TNF-α in DCs [15]. This study extends beyond previous studies that have suggested that YM-2A treatment induces a strong inflammatory phenotype in DCs. Here, we showed that YM-2A increased the expression levels of maturation markers, such as CD80, CD86, and MHC II (Fig. 1B and C). In addition, YM-2A increased the production of various cytokines, such as IL-12 (p40 and p70), TNF-α, and IL-6, but not IL-10 (Fig. 1D). IL-12 can promote Th1 immune responses, which induce the production of large amounts of IFN-γ from activated T and NK cells [3,26]. On the other hand, DCs secrete IL-10, which acts in an autocrine manner to suppress APC function [32]. Our results showed that YM-2A induced DC to secrete high levels of IL-12, and that YM-2A-treated DCs significantly strengthened the proliferation of $CD4^+$ and $CD8^+$ T cells and produced elevated levels of IFN-γ (Fig. 3), suggesting that YM-2A-treated DCs enhanced T cell activation in the Th1/CTL immune response. These results suggest that YM-2A has the potential to be used as adjuvant for a therapeutic cancer vaccine.

In recent decades, many researchers have attempted to identify adjuvants to induce DC maturation for DC vaccine immunotherapy [2,3,18,33]. In our study, TAA derived from dying tumor cells were used to load DCs to generate tumor-specific CTLs in two murine tumor models, CT-26 tumor-bearing BALB/c mice and B16 melanoma-bearing C57BL/6 mice. Our data showed that administration of YM-2A-treated TAA-loaded DCs resulted in a dramatic decrease in both tumor growth (Fig. 4A and B) and improved survival rate (Fig. 4C and D). Administration of YM-2A DCs also strongly inhibited tumor growth (Fig. 4 and B) and improved survival (Fig. 4C and D). This is because that peritumoraly injected YM-2A DCs might be pulsed with antigen from tumor tissue. The ability of YM-2A to induce antitumor efficacy of the DC vaccine may be due its ability to enhance the proportion of IFN-γ-secreting lymphocytes in tumor-bearing mice (Fig. 5). It is well known that β-glucan is a potent activator of DC [34,35]. We previously showed that maitake β-glucan-treated TAA-loaded DCs inhibited tumor growth by 50% in CT-26-bearing BALB/c mice [21]. In this study, YM-2A-treated TAA-loaded DCs inhibited tumor growth by up to 94% in CT-26-bearing BALB/c mice (Fig. 4A). Even considering that injected DC number and schedule were different between the experiment of YM-2A (Fig. 4A) and maitake β-glucan [21], YM-2A enhanced the effect of DC vaccine at least as much as maitake β-glucan.

DCs express PRRs, such as TLRs, CLRs, NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs), all of which recognize various microbial PAMPs [6,12,22,36]. Some glycogens (e.g. enzymatically synthesized glycogen, α-glucan from *Pseudallescheria boydii*, α-glucan from *Lentinula edodes*, and mycobacterial α-glucan) were reported to activate macrophages through TLR 2, TLR 4, or DC-SIGN [9,11,12,29]. YM-2A has been characterized as a glycogen-like polysaccharide consisting of α-1,4 and α-1,6 glycoside linkages [15]. Antibody neutralization studies indicated that YM-2A-induced DC maturation is mediated, at least in part, by the Dectin-1-dependent pathway, not by TLR2, TLR4, Dectin-2, DC-SIGN, and Mincle. However, our results do not formally exclude a possible role of other receptors in YM-2A recognition by DCs. Future analyses are required to define the receptor involved in YM-2A recognition.

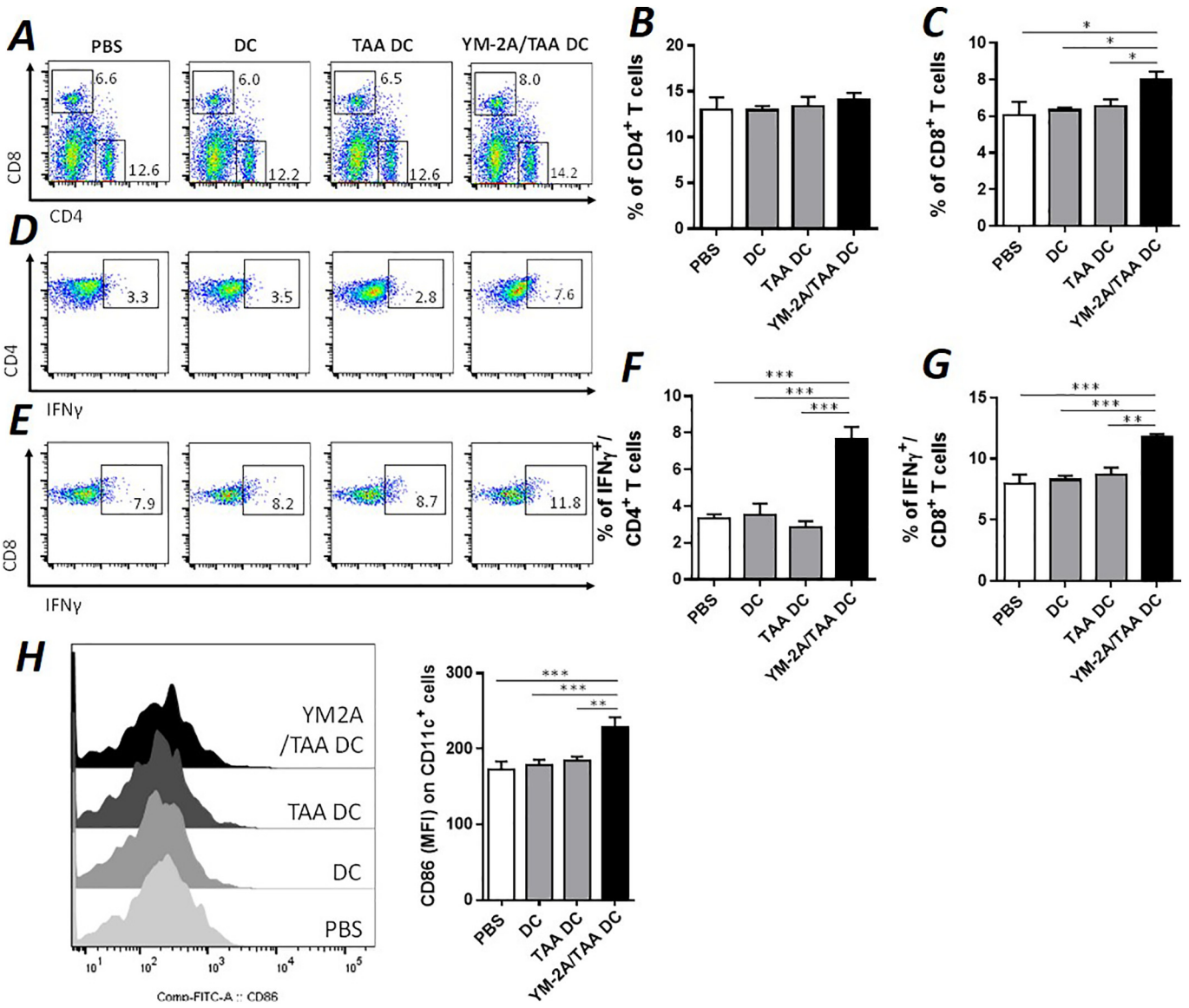

Fig. 5. YM-2A/TAA DC vaccine enhances antitumor immunity in the spleens of CT-26-tumor bearing mice.
On day 28 after colon-26 inoculation in BALB/c mice, splenocytes were stained with specific antibodies and analyzed by flow cytometry. Representative plots of percentages of $CD4^+$ and $CD8^+$ T cells (A) in a whole spleen cell suspension, and the expression of IFN-γ in gated $CD4^+$ or $CD8^+$ T cells in the spleen (D and E) are shown. Summarized data is shown (B, C, F and G). (H) The expression of CD86 on splenic DCs; representative histograms (left) and summarized data (right). Data represent the mean ± SE (n = 4–5) of two separate experiments. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the groups linked by the bracket.

Because of their low toxicity, natural products have recently emerged as promising therapeutic reagents for cancer and infectious diseases [3,37]. To our knowledge, this is the first study showing that α-glucan induces DC maturation and enhances DC vaccine as an adjuvant. In this study, we demonstrated that YM-2A-treated bone marrow-derived DCs induced a Th1 immune response and inhibited tumor growth. Since oral administration of YM-2A could inhibit tumor growth and improve survival rate in mouse models [15], and YM-2A induces maturation of the various types of DCs, such as primary DCs, bone marrow-derived GM-CSF-induced cDC and Flt3L-induced pDCs, future studies should focus on determining whether oral administration of YM-2A also enhances a DC vaccine against tumor growth. Overall, YM-2A-treatment of a TAA-loaded DC vaccine could be an excellent candidate for immunotherapy against cancer.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2018.12.039.

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Declaration of interest

The authors declare no conflicts of interest.

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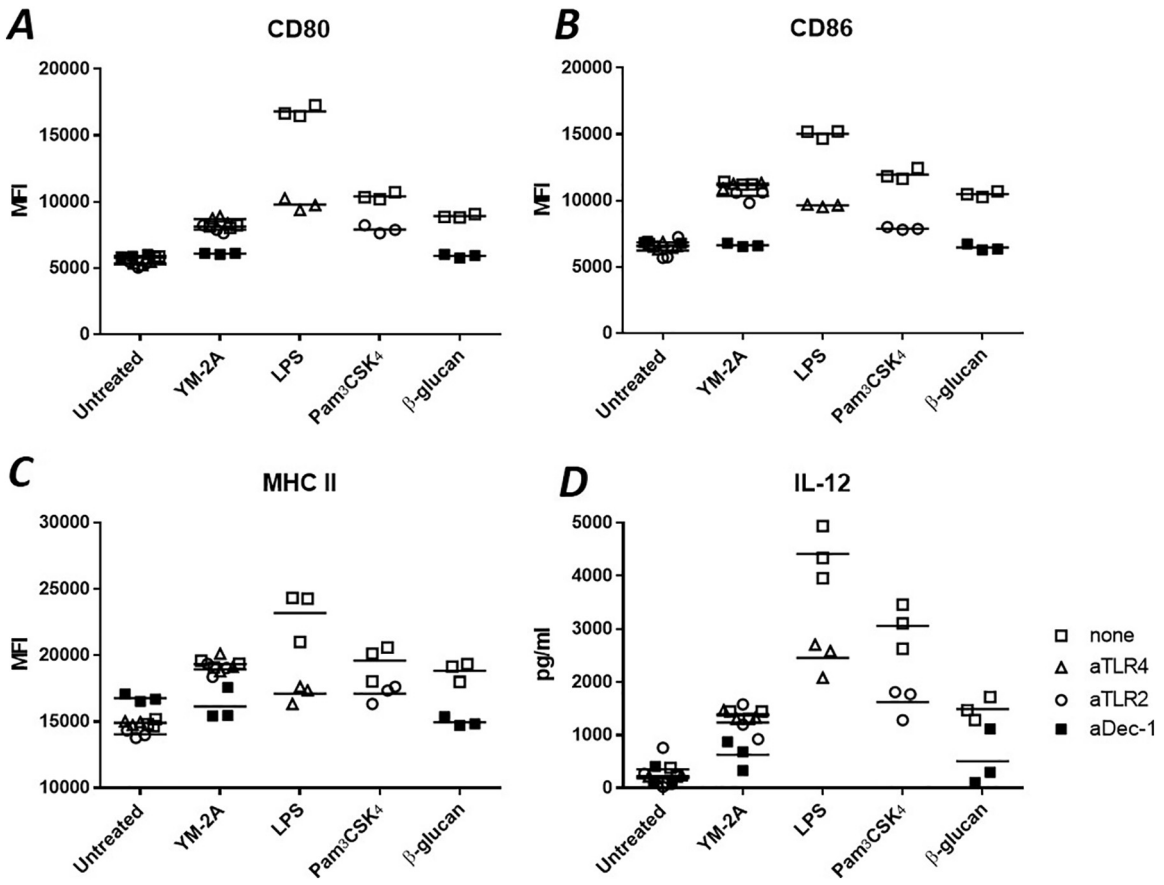

Fig. 6. YM-2A induces maturation and boosts secretion of IL-12 in DCs through Dectin-1.
DCs were pre-incubated with anti-TLR4, −TLR2 or Dectin-1 antibodies for 1 h, followed by stimulation with YM-2A (100 μg/ml), LPS (0.1 μg/ml), Pam_3CSK_4 (0.5 μg/ml), or maitake β-glucan (100 μg/ml) for 24 h. Expression of surface molecules (A–C) was analyzed by flow cytometry and IL-12p40 (D) level was determined by ELISA. Data represent the mean ± SE (n = 3) of two separate experiments.

References

[1] K. Palucka, J. Banchereau, Cancer immunotherapy via dendritic cells, Nat. Rev. Cancer 12 (2012) 265–277.
[2] A. Gardner, B. Ruffell, Dendritic cells and cancer immunity, Trends Immunol. 37 (2016) 855–865.
[3] P.M. Santos, L.H. Butterfield, Dendritic cell-based cancer vaccines, J. Immunol. 200 (2018) 443–449.
[4] W. Kastenmuller, K. Kastenmuller, C. Kurts, R.A. Seder, Dendritic cell-targeted vaccines—hope or hype? Nat. Rev. Immunol. 14 (2014) 705–711.
[5] A. Ballestrero, D. Boy, E. Moran, G. Cirmena, P. Brossart, A. Nencioni, Immunotherapy with dendritic cells for cancer, Adv. Drug Deliv. Rev. 60 (2008) 173–183.
[6] S.V. Tsoni, G.D. Brown, beta-Glucans and dectin-1, Ann. N. Y. Acad. Sci. 1143 (2008) 45–60.
[7] V. Vetvicka, Glucan-immunostimulant, adjuvant, potential drug, World J. Clin. Oncol. 2 (2011) 115–119.
[8] G. Chihara, J. Hamuro, Y. Maeda, Y. Arai, F. Fukuoka, Fractionation and purification of the polysaccharides with marked antitumor activity, especially lentinan, from *Lentinus edodes* (Berk.) Sing. (an edible mushroom), Cancer Res. 30 (1970) 2776–2781.
[9] T.C. Lo, F.M. Hsu, C.A. Chang, J.C. Cheng, Branched alpha-(1,4) glucans from *Lentinula edodes* (L10) in combination with radiation enhance cytotoxic effect on human lung adenocarcinoma through the Toll-like receptor 4 mediated induction of THP-1 differentiation/activation, J. Agric. Food Chem. 59 (2011) 11997–12005.
[10] W. Zhang, P. Li, D. Song, H. Niu, S. Shi, S. Wang, et al., Structural characterization and biological activities of two alpha-glucans from radix paeoniae alba, Glycoconj. J. 33 (2016) 147–157.
[11] V.C. Bittencourt, R.T. Figueiredo, R.B. da Silva, D.S. Mourao-Sa, P.L. Fernandez, G.L. Sassaki, et al., An alpha-glucan of *Pseudallescheria boydii* is involved in fungal phagocytosis and Toll-like receptor activation, J. Biol. Chem. 281 (2006) 22614–22623.
[12] J. Geurtsen, S. Chedammi, J. Mesters, M. Cot, N.N. Driessen, T. Sambou, et al., Identification of mycobacterial alpha-glucan as a novel ligand for DC-SIGN: involvement of mycobacterial capsular polysaccharides in host immune modulation, J. Immunol. 183 (2009) 5221–5231.
[13] Y. Masuda, H. Inoue, H. Ohta, A. Miyake, M. Konishi, H. Nanba, Oral administration of soluble beta-glucans extracted from *Grifola frondosa* induces systemic antitumor immune response and decreases immunosuppression in tumor-bearing mice, Int. J. Cancer 133 (2013) 108–119.
[14] Y. Masuda, M. Inoue, A. Miyata, S. Mizuno, H. Nanba, Maitake beta-glucan enhances therapeutic effect and reduces myelosupression and nephrotoxicity of cisplatin in mice, Int. Immunopharmacol. 9 (2009) 620–626.
[15] Y. Masuda, Y. Nakayama, A. Tanaka, K. Naito, M. Konishi, Antitumor activity of orally administered maitake alpha-glucan by stimulating antitumor immune response in murine tumor, PLoS One 12 (2017) e0173621.
[16] U. Grohmann, M.L. Belladonna, R. Bianchi, C. Orabona, E. Ayroldi, M.C. Fioretti, et al., IL-12 acts directly on DC to promote nuclear localization of NF-kappaB and primes DC for IL-12 production, Immunity 9 (1998) 315–323.
[17] K. Tsung, J.B. Meko, G.R. Peplinski, Y.L. Tsung, J.A. Norton, IL-12 induces T helper 1-directed antitumor response, J. Immunol. 158 (1997) 3359–3365.
[18] J. Banchereau, A.K. Palucka, Dendritic cells as therapeutic vaccines against cancer, Nat. Rev. Immunol. 5 (2005) 296–306.
[19] H. Nanba, A. Hamaguchi, H. Kuroda, The chemical structure of an antitumor polysaccharide in fruit bodies of *Grifola frondosa* (maitake), Chem. Pharm. Bull. (Tokyo) 35 (1987) 1162–1168.
[20] Y. Masuda, K. Ito, M. Konishi, H. Nanba, A polysaccharide extracted from *Grifola frondosa* enhances the anti-tumor activity of bone marrow-derived dendritic cell-based immunotherapy against murine colon cancer, Cancer Immunol. Immunother. 59 (2010) 1531–1541.
[21] Y. Masuda, D. Nawa, Y. Nakayama, M. Konishi, H. Nanba, Soluble beta-glucan from *Grifola frondosa* induces tumor regression in synergy with TLR9 agonist via dendritic cell-mediated immunity, J. Leukoc. Biol. 98 (2015) 1015–1025.
[22] J.A. Villadangos, P. Schnorrer, Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo, Nat. Rev. Immunol. 7 (2007) 543–555.
[23] J. Helft, J. Bottcher, P. Chakravarty, S. Zelenay, J. Huotari, B.U. Schraml, et al., GM-CSF mouse bone marrow cultures comprise a heterogeneous population of $CD11c^+MHCII^+$ macrophages and dendritic cells, Immunity 42 (2015) 1197–1211.
[24] B. Oppmann, R. Lesley, B. Blom, J.C. Timans, Y. Xu, B. Hunte, et al., Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12, Immunity 13 (2000) 715–725.
[25] Y. Xu, Y. Zhan, A.M. Lew, S.H. Naik, M.H. Kershaw, Differential development of murine dendritic cells by GM-CSF versus Flt3 ligand has implications for inflammation and trafficking, J. Immunol. 179 (2007) 7577–7584.

[26] G. Trinchieri, Interleukin-12 and the regulation of innate resistance and adaptive immunity, Nat. Rev. Immunol. 3 (2003) 133–146.
[27] J. Li, A. Aipire, J. Li, H. Zhu, Y. Wang, W. Guo, et al., λ-Carrageenan improves the antitumor effect of dendritic cellbased vaccine, Oncotarget 8 (2017) 29996–30007.
[28] Y. Masuda, T. Togo, S. Mizuno, M. Konishi, H. Nanba, Soluble beta-glucan from *Grifola frondosa* induces proliferation and Dectin-1/Syk signaling in resident macrophages via the GM-CSF autocrine pathway, J. Leukoc. Biol. 91 (2012) 547–556.
[29] R. Kakutani, Y. Adachi, H. Takata, T. Kuriki, N. Ohno, Essential role of Toll-like receptor 2 in macrophage activation by glycogen, Glycobiology 22 (2012) 146–159.
[30] C. Del Fresno, S. Iborra, P. Saz-Leal, M. Martinez-Lopez, D. Sancho, Flexible signaling of myeloid C-type lectin receptors in immunity and inflammation, Front. Immunol. 9 (2018) 804.
[31] I.M. Dambuza, G.D. Brown, C-type lectins in immunity: recent developments, Curr. Opin. Immunol. 32 (2015) 21–27.
[32] K.N. Couper, D.G. Blount, E.M. Riley, IL-10: the master regulator of immunity to infection, J. Immunol. 180 (2008) 5771–5777.
[33] G. Schuler, B. Schuler-Thurner, R.M. Steinman, The use of dendritic cells in cancer immunotherapy, Curr. Opin. Immunol. 15 (2003) 138–147.
[34] C. Qi, Y. Cai, L. Gunn, C. Ding, B. Li, G. Kloecker, et al., Differential pathways regulating innate and adaptive antitumor immune responses by particulate and soluble yeast-derived {beta}-glucans, Blood 117 (2011) 6825–6836.
[35] H.S. Goodridge, C.N. Reyes, C.A. Becker, T.R. Katsumoto, J. Ma, A.J. Wolf, et al., Activation of the innate immune receptor Dectin-1 upon formation of a 'phagocytic synapse', Nature 472 (2011) 471–475.
[36] D.M. Underhill, Collaboration between the innate immune receptors dectin-1, TLRs, and Nods, Immunol. Rev. 219 (2007) 75–87.
[37] R. Vengoji, M.A. Macha, S.K. Batra, N.A. Shonka, Natural products: a hope for glioblastoma patients, Oncotarget 9 (2018) 22194–22219.