Research Paper

Therapeutic potential of various β-glucan sources in conjunction with anti-tumor monoclonal antibody in cancer therapy

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Combined β-glucan with anti-tumor mAb therapy has demonstrated therapeutic efficacy in murine tumor models. The current study was designed to compare the therapeutic efficacy of various sources of β-glucans. Our studies demonstrated that yeast β-glucan, in combination with anti-tumor mAb, resulted in significantly smaller tumor burdens and achieved enhanced long-term survival compared to mAb alone or β-glucan extracts from mushrooms. Further studies indicated that yeast β-glucan particle was superior to mushroom extracts in inducing cytokine secretion, particularly IL-12 production in dendritic cells (DCs). In addition, results showed that cytokine production was markedly decreased in MyD88-deficient macrophages and DCs but not in complement receptor 3 (CR3)-deficient mice. Our data suggest that yeast β-glucan demonstrates much stronger adjuvant activity compared to mushroom β-glucan extracts in tumor therapy. This effect of yeast β-glucan may be in part ascribed to the cytokine secretion by DCs and macrophages and bioavailability of active β-glucan moiety.

Introduction

β-Glucans belong to a family of biological response modifiers (BRMs) that were initially reported in the literature over 40 years ago to help stimulate tumor rejection.[1] Produced by fungi, yeast and grains but not mammalian cells, they are comprised of D-glucose polymers.[2,3] The basic structure of these polymers differs from source to source, but mainly consists of a linear glucose polymer with β(1,3), β(1,4) or β(1,6) linkages. Oat and barley β-glucans are primarily linear with large regions of β(1,4) linkages separating shorter stretches of β(1,3) structures, whereas yeast β-glucans have a β(1,3) backbone with β(1,6)-linked β(1,3) branches.[4] Mushroom β-glucans are similar to yeast except that they are comprised of short β(1,6) branches coming off of a β(1,3) backbone, thereby lacking the extra β(1,3) branch extending from the β(1,6) branch point.[5] The yeast β-glucan is significant in that it's structure allows

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these polymers to form strong triple helices in solution and large aggregates, making this layer of the yeast cell wall very resistant to treatments directed at removing the yeast cell wall components.[3,6] Removal of these components results in a hollow glucan sphere or "ghost". Preparation of purified yeast β-glucan sphere is commercially available in the form of whole glucan particles (WGPs) as a food supplement. Further efforts resulted in the development of a water-soluble, pharmaceutical grade yeast β-glucan whose biological effects have been extensively studied in vivo.[7-12]

Our previous studies demonstrated that soluble, small molecular weight yeast-derived β-glucans exert their function through neutrophils for priming complement receptor 3 (CR3, CD11b/CD 18, $\alpha_m\beta_2$ integrin, Mac-1) to kill iC3b-opsonized yeast or tumor cells.[13-17] Further study showed that orally ingested WGP is taken up by M-cells in the gut and then phagocytosed by intestinal macrophages trafficking into spleen and bone marrow.[18] A smaller active moiety of β-glucan released from macrophages subsequently primes neutrophil CR3, by way of binding a lectin domain within the COOH-terminal region of CD11b subunit of CR3.[19] β-Glucan-primed neutrophils are then subject to C5a-dependent chemotaxis via leukotriene B_4 (LTB_4) released from tumor endothelial cells.[20] The activated neutrophils migrate into the tumor milieu, engage iC3b-opsonized tumor cells mediated by complement-activating anti-tumor monoclonal antibodies (mAbs) and exert their cytotoxic effect. Therefore, yeast-derived β-glucan-mediated tumor immunotherapy employs a novel mechanism by which to prime the innate immune neutrophils, thereby making mAb therapy significantly more efficacious.

Despite the elucidation of the mechanisms involved in the in vivo priming of CR3 by yeast-derived β-glucan, it is unknown whether β-glucans have any biological activity on antigen presenting cells (APCs) such as dendritic cells (DCs) and macrophages, thus stimulating adaptive immune responses. In addition, it is unknown whether β-glucans from different sources, such as mushroom β-glucans, have similar function to yeast-derived β-glucan. Our previous studies have shown that yeast-derived WGP β-glucans stimulate resident peritoneal macrophages to secrete cytokines such as TNFα and IL-6.[21] These pro-inflammatory cytokines can potentially enhance the activation of adaptive immunity, such as antigen presentation and T cell activation. Therefore, the administration of β-glucan could link the activation of both innate and adaptive immunity.

In the current study, we determined therapeutic efficacy of varying sources of β-glucans with anti-tumor mAb in tumor regression and

tumor free survival. To explore the mechanism of action of β-glucan food supplements, surface marker expression levels and cytokine production by macrophages and DCs were analyzed upon β-glucan extract stimulation. Our data suggest that yeast-derived β-glucan WGP demonstrates much stronger adjuvant activity compared to mushroom β-glucans. This effect may be due to the levels of proinflammatory cytokine production and bioavailability of active β-glucans.

Results

Tumor regression and tumor-free survival mediated by combining β-glucan with anti-tumor mAb therapy. The current study was designed to compare the therapeutic efficacy of β-glucans obtained from various sources in conjunction with anti-tumor mAb in a murine tumor model. To this end, mice were implanted with lymphoma RMAS-MUC1 cells. When the tumors reached palpable size, groups of mice were treated with yeast-derive WGP or extracts from different mushrooms (Reishii, Shitake, Maitake and AHCC) in combination with anti-MUC1 mAb. Treatment with mAb alone, β-glucan alone, or saline served as controls. As shown in Figure 1A, all β-glucan sources when used in combination with mAb showed significantly greater tumor regression compared to mAb therapy alone or saline-treated animals (p < 0.05). In combination with anti-tumor mAb, yeast-derived β-glucan WGP and mushroom extracts from Shitake and AHCC produced significantly smaller tumor burdens as compared other mushroom extracts (Reishi vs. WGP, p < 0.01; Maitake vs. WGP, p < 0.01; Shitake vs. WGP, p > 0.05; AHCC vs. WGP, p > 0.05). Rapid tumor growth was observed in all subjects from Day 17 through Day 20, but at Day 20 subjects receiving WGP, AHCC, Shitake and Maitake therapy demonstrated tumor size regression, whereas saline-treated or mAb only treated subjects demonstrated tumor size progression. From Day 20 until the end of the study period, WGP, Shitake and AHCC showed the greatest degree of tumor regression. In addition, yeast β-glucan WGP conferred the highest degree of tumor regression as compared to all sources of β-glucan, with the lowest tumor size progression from baseline. Although all β-glucan extracts when used in combination with mAb exhibited significantly short-term tumor regression, tumor-bearing animals receiving WGP in combination with anti-tumor mAb treatment demonstrated 80% long-term survival whereas mice with Shitake therapy showed 40% survival and Reishi, AHCC or Maitake therapy demonstrated 30% survival (Fig. 1B). These results show that long-term survival is significantly enhanced by addition of yeast β-glucan WGP to mAb therapy as compared to other sources of β-glucan. Taken together, these
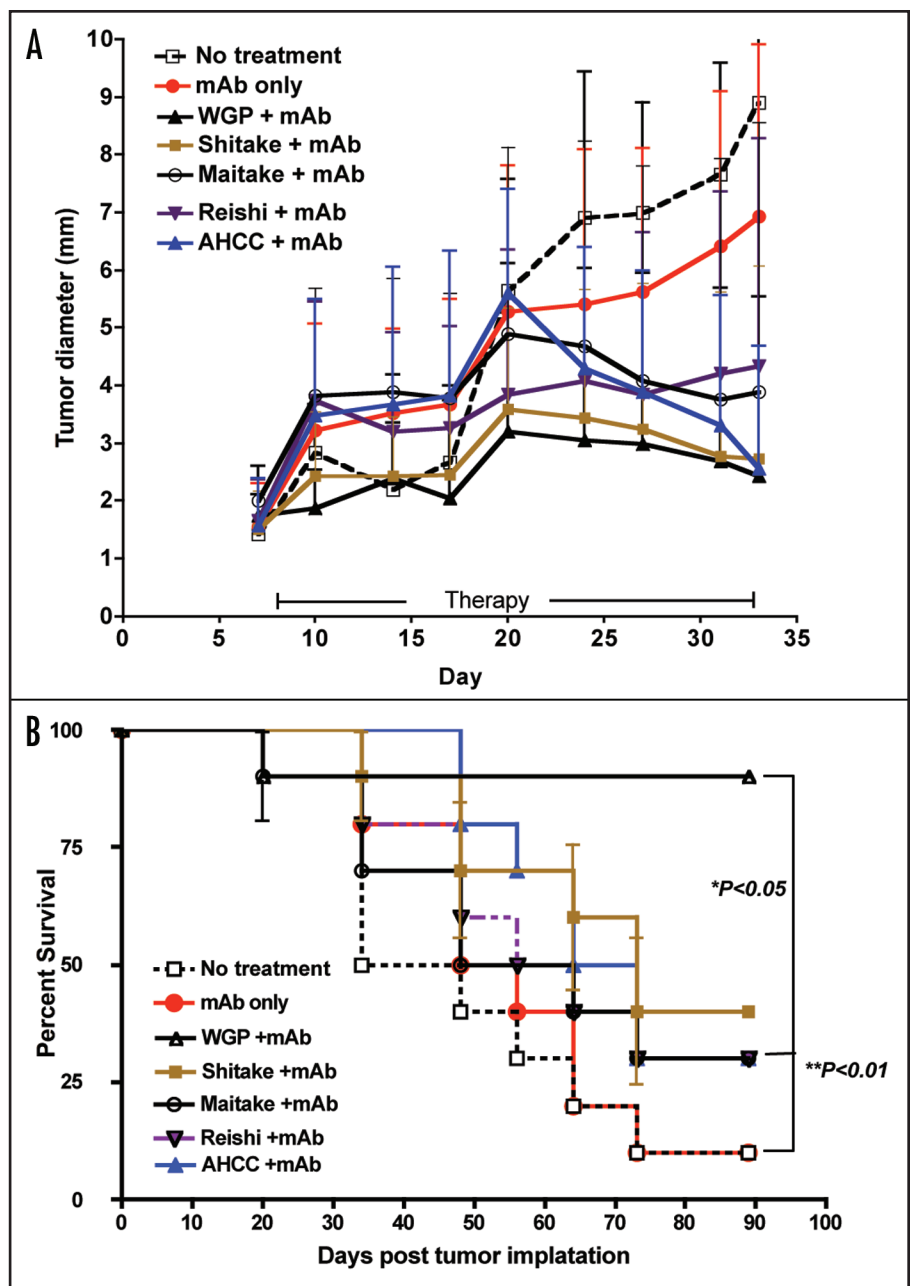

Figure 1. The tumoricidal activity of immunotherapy with β-glucan extracts from yeast and mushrooms. C57Bl/6 mice (n = 10) were implanted s.c. with RMA-S-MUC1 cells and tumors were allowed to form over five-days before therapy. Mice received PBS, anti-MUC1 mAb (200 μg twice a week) with or without β-glucan extracts (dosages were indicated in the materials and methods) for four weeks. Both tumor growth (A) and survival (B) were monitored. Tumor measurements were made at the indicated time. Mice were sacrificed when the tumors reached 12 mm in diameter. *Points*, mean; *bars*, SE.

data suggest that yeast-derived β-glucan WGP demonstrates much stronger adjuvant activity compared to mushroom β-glucans with respect to anti-tumor therapy.

Yeast-derived β-glucan WGP stimulates macrophages and DCs to release strong proinflammatory cytokines. To further explore the mechanism of action of β-glucan-mediated immune stimulation, we examined production of cytokines by macrophages and DCs. As indicated in Figure 2, all sources of β-glucans triggered macrophages to produce large amounts of proinflammatory cytokines such as
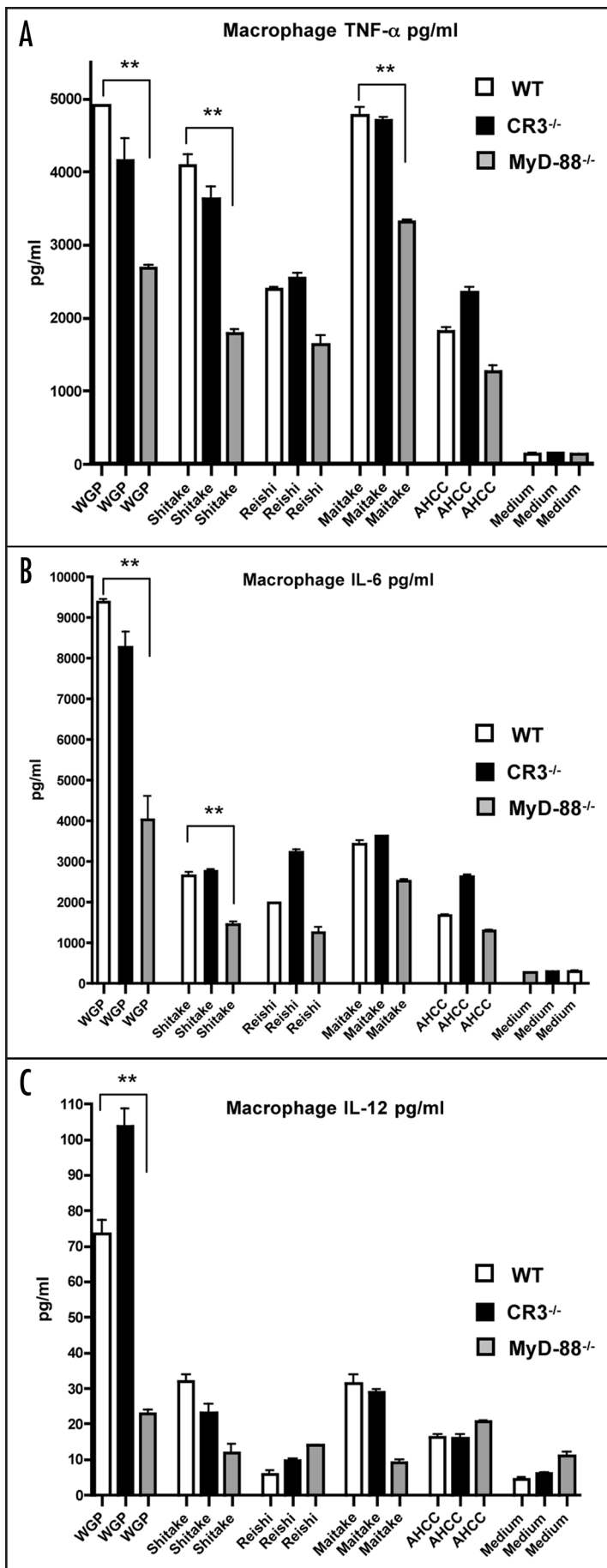
Figure 2. Cytokine release in murine peritoneal macrophages upon β-glucan extract stimulation. Resident peritoneal macrophages harvested from C57Bl/6 WT, CR3-deficient or MyD-88-deficient mice were stimulated with yeast-derived β-glucan WGP or mushroom β-glucan extracts (100 µg/ml) for overnight. The supernatants were collected and detected for cytokines by ELISA. Mean ± SE of duplicates is shown. Data represent one representative of two independent experiments. **p < 0.01.

TNFα and IL-6. Among mushroom β-glucans, Shitake and Maitake produced more TNFα as compared to AHCC and Reishi. Similar levels of TNFα production were elicited by yeast-derived β-glucan WGP, Shitake and Maitake. In contrast, yeast-derived β-glucan WGP stimulated profound IL-6 production as compared to mushroom β-glucan extracts. All mushroom β-glucans triggered similar levels of IL-6 secretion in murine macrophages. Interestingly, only yeast-derived β-glucan WGP stimulated macrophages to produce significant amount of IL-12. Mushroom β-glucans did not induce appreciable levels of IL-12 production. These data suggest that yeast-derived β-glucan WGP are superior to stimulate proinflammatory cytokine secretion as compared to mushroom β-glucans, particularly with regard to the production of IL-12 and IL-6.

Previous studies demonstrated that crude β-glucan preparations such as zymosan activate toll-like receptors such as TLR2/TLR6.[26,27] Since MyD88 is the common adaptor protein utilized by most TLR signaling pathways,[28] cytokine production was determined in MyD88-deficient macrophages. In addition, since CR3 was previously defined as soluble β-glucan receptor,[15] cytokine secretion by macrophages from CR3-deficient mice was also examined. As shown in Figure 2A and B, production of TNFα and IL-6 by yeast-derived β-glucan WGP and mushroom β-glucans was significantly decreased, but not completely abrogated, in MyD88-deficient macrophages. WGP-induced IL-12 production was also significantly decreased in MyD-88-deficient mice (Fig. 2C). However, TNFα and IL-6 production was not affected in CR3-deficient mice. Interestingly, production of IL-12 by yeast-derived β-glucan was significantly increased in CR3-deficient macrophages (Fig. 2C).

DCs are critical APCs to induce adaptive immune responses. As shown in Figure 3A, yeast-derived β-glucan WGP, Shitake and Maitake triggered DCs to produce significant, but much lower, amounts of TNFα with respect to macrophages. IL-6 production was only induced by yeast-derived β-glucan WGP, but not by mushroom β-glucans (Fig. 3B). In addition, IL-12 production was significantly higher in DCs with respect to macrophages and was mainly triggered by yeast-derived β-glucan WGP and Maitake (Fig. 3C). As with macrophages, production of TNFα and IL-6 mediated by β-glucans was significantly decreased in MyD88-deficient DCs but was not affected in CR3-deficient DCs. In addition, TNFα production mediated by Shitake, Reishi, Maitake and AHCC was significantly elevated in CR3-deficient mice.

Upregulation of surface accessory molecules and MHC class II on macrophages and DCs upon exposure to β-glucan stimulation. To evaluate the expression levels of activation markers and molecules critical for antigen presentation, membrane surface markers on resident peritoneal macrophages and BMDCs were analyzed before and after WGP or mushroom β-glucan extract stimulation. As shown in Figure 4, CD40, CD80, CD86 and MHC class II molecules were constitutively expressed on resident peritoneal macrophages and BMDCs. In response to WGP or mushroom β-glucan stimulation,

Figure 3. Cytokine release in bone-marrow-derived DCs upon β-glucan extract stimulation. BMDCs from C57Bl/6 WT, CR3-deficient or MyD-88-deficient mice were stimulated with yeast-derived β-glucan WGP or mushroom β-glucan extracts (100 µg/ml) for overnight. The supernatants were collected and detected for cytokines by ELISA. Mean ± SE of duplicates is shown. Data represent one representative of two independent experiments. **p < 0.01, *p < 0.05.

CD40, CD80, CD86 and MHC class II molecules were significantly upregulated. Interestingly, particulate yeast β-glucan WGP showed much higher stimulatory effect as compared to mushroom β-glucans, particularly CD86 and MHC class II (Fig. 4). Taken together, β-glucans could stimulate macrophages and BMDCs to upregulate critical accessory and MHC class II molecules, which are important for the induction of adaptive immune responses.

β-glucan-mediated CR3-dependent cellular cytotoxicity. Our previous study demonstrated that β-glucan particles via oral gavage feeding were predominately taken up and processed by macrophages to release active moiety of smaller molecular weight β-glucan that primes neutrophil CR3 to elicit cytotoxicity of iC3b-opsonized tumor cells.[18] To evaluate whether mushroom β-glucans function similar to yeast-derived β-glucans, active β-glucans were prepared from ex vivo macrophage culture. The same amounts of hexose were used to prime human neutrophil CR3, which were then cocultured with iC3b-opsonized human ovarian carcinoma SKOV-3 cells. This was accomplished using a real-time measure of the impedance of electrical current by viable target cells adhered to a conductor on the bottom of wells in a 16 well plate. As indicated in Figure 5, β-glucans from yeast WGP, Shitake and Maitake exhibited significant cytotoxic activity. β-Glucan from Reishi also showed significant cytotoxicity but at a somewhat lower level as compared to WGP, Shitake and Maitake. However, β-glucan from AHCC did not trigger any appreciable levels of cytotoxic activity. These data suggest that mushroom β-glucans from Reishi, Shitake or Maitake in combination with anti-tumor mAbs are capable of eliciting CR3-dependent cellular cytotoxicity.

Discussion

In this study, we demonstrate that yeast-derived β-glucan WGP as well as mushroom β-glucan extracts exhibit significant anti-tumor therapeutic efficacy when used in conjunction with anti-tumor mAb. As compared to mushroom β-glucans, yeast-derived β-glucan WGP demonstrates much stronger adjuvant activity with respect to long-term tumor-free survival. The disparity observed between short-term tumor regression and long-term survival suggests that particulate β-glucan WGP has much longer effects than mushroom extracts. It is possible that tumor-bearing mice continuously treated with mushroom extracts may achieve similar long-term tumor-free survival as demonstrated with WGP therapy. In addition, WGP was superior to most mushroom β-glucan extracts with respect to production of proinflammatory cytokines such as TNFα, IL-6 and IL-12. The lower levels of inflammatory cytokine production, particularly IL-12 production, by mushroom β-glucan stimulation may be responsible for the lower therapeutic efficacy in tumor therapy as compared to yeast-derived β-glucan WGP. Furthermore, we demonstrate that yeast-derived β-glucans as well as β-glucans from Shitake, Maitake and Reishi in combination with anti-tumor mAb elicit CR3-dependent cellular cytotoxicity.
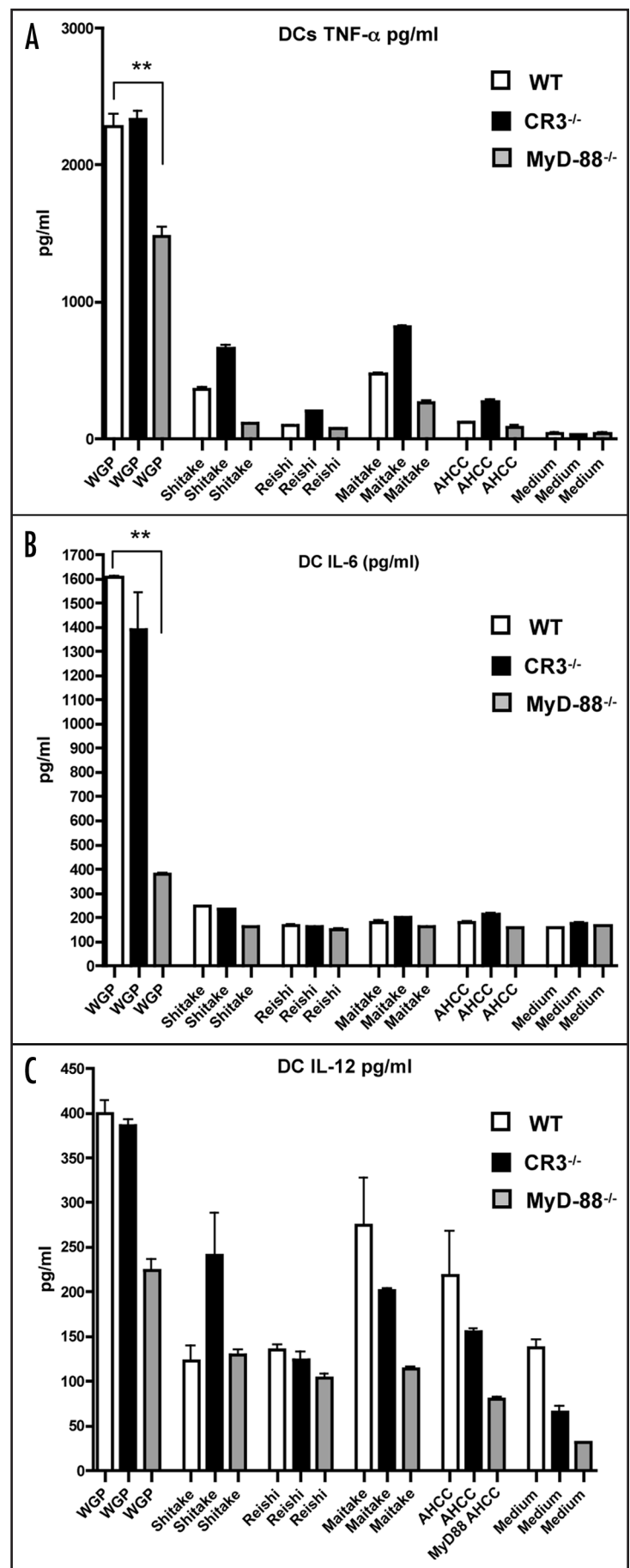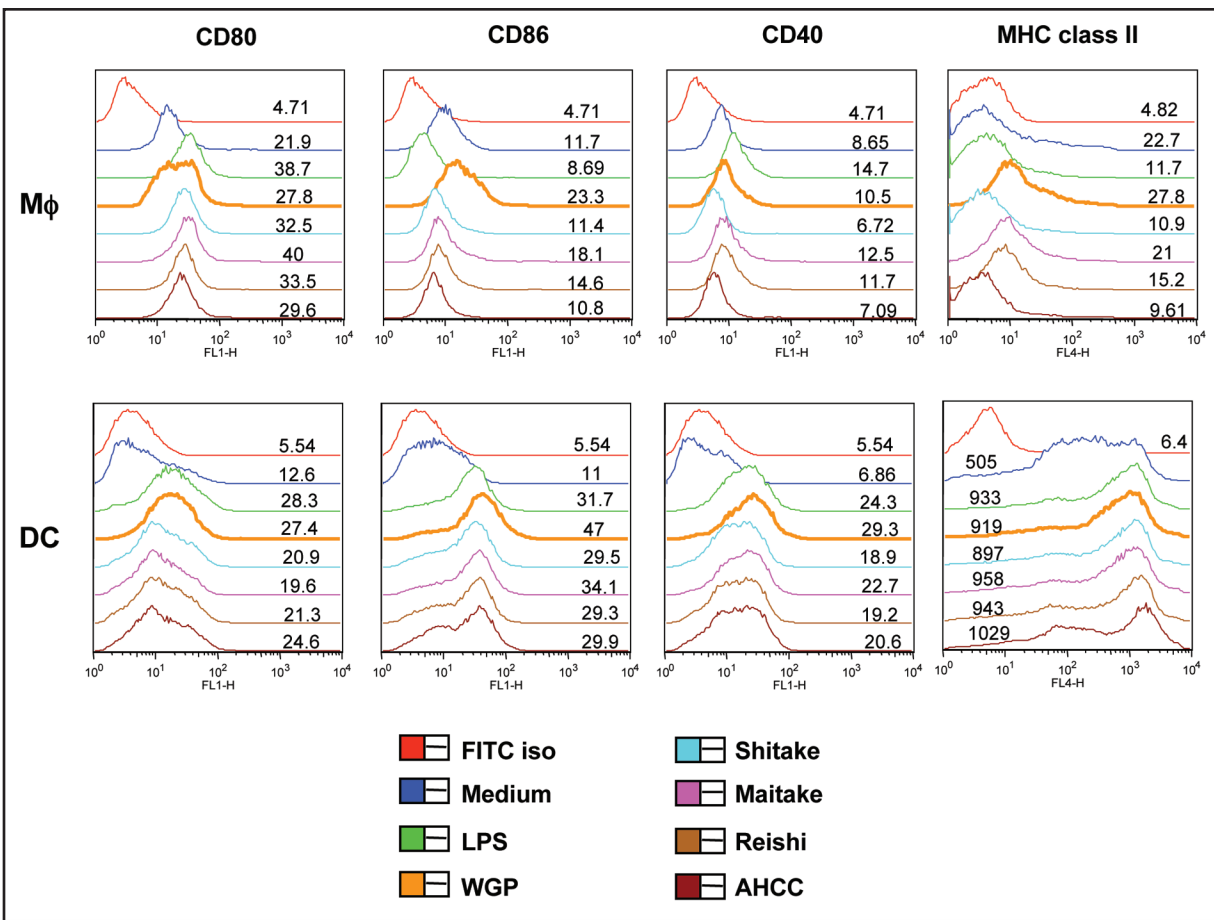
Figure 4. Surface marker expression on murine resident peritoneal macrophages. Resident peritoneal macrophages or BMDCs from C57Bl/6 WT mice were collected and stimulated with varying β-glucans (100 µg/ml). BMDCs were stimulated for 24 h while macrophages were stimulated for 48 h. The cells were harvested and stained with indicated mAbs or isotype controls. Cells cultured in the medium alone were used as controls. The histograms are one representative of three independent experiments. Mean fluorescence intensity (MFI) was shown in each line.
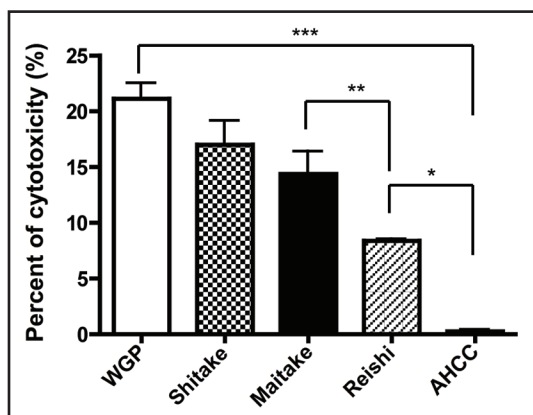

Figure 5. In vitro neutrophil-mediated CR3-dependent cellular cytotoxicity. In vitro cytotoxicity experiments suggested that β-glucans from yeast, Shitake, Maitake, Reishi in conjunction with anti-tumor mAb elicit cellular cytotoxicity of SKOV-3 tumors. No cytotoxicity was observed with AHCC. *p < 0.05, **p < 0.01, ***p < 0.001.

β-Glucans derived from different sources have some variations in their structure. For example, bacterial β-glucan consists of linear glucose polymers with β(1,3) linkage. Oat and barley β-glucans are primarily linear, with large regions of β(1,4) linkages. Mushroom β-glucans have short β(1,6) linked branches coming off a β(1,3) backbone. Yeast β-glucans have β(1,6) branches that are further elaborated with additional β(1,3) regions. These seemingly minor structural differences can have great implications for the activity of the β-glucan. Indeed, in our study, we show that yeast-derived β-glucan WGP and all mushroom β-glucans trigger macrophages to produce varying levels of IL-6 and TNFα. Even among the mushroom β-glucans, the levels of cytokine release are different. These differences may reflect the structural and conformational variations as well as bioavailability of β-glucans from these extracts. This may be also related to the different extraction methods used for different food supplements. Interestingly, only yeast-derived β-glucan WGP stimulates macrophages to secrete IL-12. IL-12 is a critical regulator of immune responses against pathogens and tumors, as it is the most potent promoter of Th1 responses.[29,30] This finding suggests that yeast-derived β-glucan WGP may have direct impact on adaptive immune responses. To further support this notion, we found that yeast-derived β-glucan WGP also stimulated DCs to secrete large amount of IL-12. This is different from β-glucan of

Candida albicans, which stimulates production of IL-10 but not IL-12 in DCs.[31] Among mushroom extracts, Maitake is the only mushroom β-glucan, which stimulates DCs for low level of IL-12 production. However, DCs and macrophages displayed different levels of cytokine secretion in response to β-glucan stimulation. As we demonstrated, macrophages produce large amounts of TNFα and IL-6 but low levels of IL-12 when stimulated by WGP, while DCs produce lower levels of TNFα and IL-6 but significantly higher amount of IL-12. The varying cytokine levels elicited by macrophages and DCs upon β-glucan treatment may also correlate with their in vivo anti-tumor therapeutic efficacies. This notion is reinforced by the finding that yeast-derived β-glucan WGP showed much stronger anti-tumor effects in terms of tumor regression as well as tumor-free survival.

It is still intriguing which receptors are used by β-glucans to mediate its biological activity. There are at least four β-glucan receptors that have been identified: CR3,[32] lactosylceramide,[33] scavenger receptors[34] and dectin-1.[26,35] In our study, we demonstrated that the cytokine release by yeast-derived β-glucan WGP and mushroom β-glucans is independent of CR3, but largely depends on MyD-88 signaling pathway. In macrophages, TNFα and IL-6 production mediated by β-glucans is significantly decreased in MyD-88-deficient macrophages. However, the secretion of TNFα and IL-6 was not completely abrogated, suggesting that some components in the β-glucan extracts also engage other signaling pathway such as dectin-1 pathway to trigger cytokine release.[36] Indeed, *Sparassi crispa* glucan (SCG), a soluble β(1,6)-branched β(1,3)-glucan purified from the edible mushroom, induces production of TNFα and IL-12 in BMDCs. This effect was completely abrogated in dectin-1-deficient mice but not in MyD-88-deficient mice.[37] Our previous study demonstrated that soluble yeast-derived β-glucan could significantly inhibit WGP-mediated cytokine release in macrophages, indicating potential interplay between TLR signaling pathway and β-glucan-mediated signaling pathway.[21] In addition, β-glucans such as β-glucan-rich yeast particle zymosan can also stimulate alternative pathway of complement activation and release C3a and C5a thereby leading to enhanced cytokine release.[38] It is worth noting that IL-12 production by WGP in macrophages was significantly elevated in CR3-deficient macrophage. Similar observations were seen in Reishi and AHCC-induced IL-6 production in macrophages as well as mushroom extract-induced TNFα production in DCs. A recent study demonstrated that CD11b-deficiency led to enhanced IL-6 production in APCs, which subsequently promoted Th17 cell differentiation.[39] The increased cytokine production in CR3-deficient mice may reflect the breakdown of tolerance induction in APCs after complement activation production iC3b-CR3 engagement.[40,41]

Our previous study has discovered that yeast-derived β-glucan WGP was captured by macrophages and then processed to release an active moiety that primes neutrophil CR3 to elicit cytotoxicity of iC3b-opsoinzed tumor cells.[18] In this study, we found that β-glucans from WGP, Shitake, Mitake and Reishi indeed elicited CR3-dependent cellular cytotoxicity. However, AHCC did not have any appreciable level of cytotoxicity, suggesting that AHCC-mediated tumor regression and tumor-free survival are not dependent on neutrophil-mediated cellular cytotoxicity. Indeed, a previous report suggests that treatment with AHCC enhances both Ag-specific CD4 and CD8 T cell activation and increases the frequency of tumor Ag-specific IFNγ-producing CD8 T cells.[42] AHCC also stimulates NK cell and γδ T cells as well as macrophages and DCs, suggesting the role of AHCC in the regulating innate immune responses. The superior adjuvant effects observed in yeast-derived β-glucan WGP may be attributed to a combination of effects that not only engage the conventional innate and adaptive immune responses but also prime neutrophils to elicit CR3-dependent cellular cytotoxicity. This may be also true for certain mushroom β-glucans such as Shitake and Maitake.

In summary, our study describes how β-glucans from different sources affect the cytokine release by murine macrophages and DCs and their effect on tumor immunotherapy in conjunction with anti-tumor mAb. These results provide a rationale for the use of those naturally-derived polysaccharides in various clinical settings, particularly as an adjuvant for tumor immunotherapy.

Materials and Methods

Therapeutic food supplements. WGP (Whole Glucan Particles; Wellmune WGP® Biothera, Inc., Eagan, MN) were purified from baker's yeast.[4] Active hexose correlated compound (AHCC) was purchased from Amino Up Chemicals, Sapporo, Japan. PeakImmune Shitake was from Daiwa Health Development Inc., Los Angeles, CA. MycoMedicinals Maitake and Reishi mushroom extracts were purchased from New Chapter, Inc., Brattleboro, VT.

Measurement of glucan concentration from different food supplements. The glucan concentration of different extracts was measured by Mushroom and Yeast Glucan Assay Kit (Megazyme International Ireland Ltd, Bray Co, Wicklow, Ireland). For β-glucan measurement, 100 mg of each mushroom or yeast dry glucans was suspended in 1.5 ml of concentrated HCl and incubated at 45°C for 30 min, then put in boiling water bath for two h. The pH was neutralized with 2 M KOH and samples were spun at 1500 xg. One-hundred μl of the supernatants was then transferred to a new tube and 0.1 ml of a mixture of exo-1,3-β-glucanase (20 U/mL) plus β-glucosidase (4 U/ml) in 200 mM sodium acetate buffer (pH 5.0) was added to each tube, vortexed and incubated at 40°C for 60 min. Then 3 mL of glucose oxidase/peroxidase mixture (GOPOD) was added. For α-glucan measurement, 100 mg of each mushroom or yeast dry glucans was suspended in 2 ml of 2M KOH and incubated at 4°C for 20 min. The sample pH was then neutralized with 8 mL of 1.2 M sodium acetate buffer (pH 3.8). The samples were added 0.2 mL of amyloglucosidase (1630 U/ml) plus invertase (500 U/mL), vortexed and incubated at 40°C for 30 min and then centrifuged for 10 min at 1500 xg. In 100 μl of supernatant, 3 mL of GOPOD was added. The samples were gently mixed and incubated at 40°C for 20 min. Absorbance was measured at 510 nm against the GOPOD reagent blank and unknowns were compared to the glucose standard to calculate percent of glucan. Glucan contents of each extract were summarized in Table 1.

Mice and tumor models. The murine tumor therapy protocols were performed in compliance with all relevant laws and institutional guidelines, and were approved by the Institutional Animal Care and Use Committee of the University of Louisville. Normal C57Bl/6 mice were purchased from NCI-Frederick (Frederick, MD). RMA-S-MUC1, a C57Bl/6 lymphoma transfected with human MUC1 and its use in tumor therapy protocols in combination with anti-MUC1 mAb and β(1,3)-glucan were previously described.[17,19]

Table 1 Glucan contents of different extracts

Material assayed	Total glucan %
Megazyme yeast glucan control	58.07015
WGP	54.787
Shitake	60.09
Maitake	53.62
Reishi	51.505
AHCC	26.635

This murine model was used to determine the therapeutic efficacy of AHCC, Reishi mushroom extract, Maitake mushroom extract, Shitake mushroom extract as compared to yeast-derived β-glucan extract WGP. The following dosages were used; WGP—22.2 mg/kg daily, AHCC—42.55 mg/kg daily, Reishi—20 mg/kg daily, Maitake—1 mg/kg daily, Shitake—1 mg/kg daily. The dosage used in this study was calculated based on human uptake doses suggested by commercial vendors. The average weight of mice was 18 grams.

Groups of male C57/Bl6 mice, six-weeks old, were injected with 100 μl of PBS containing 2×10^6 RMA-S-MUC1 cells subcutaneously in the right flank. Therapy with various groups of extracts below was initiated once mice were observed to have developed small tumors (2 mm in diameter). Mice were divided into seven groups; Group 1 (AHCC extract plus mAb), Group 2 (Reishi mushroom extract plus mAb), Group 3 (Maitake mushroom extract plus mAb), Group 4 (Shitake mushroom extract plus mAb), Group 5 (yeast-derived β-glucan extract WGP plus mAb), Group 6 (mAb alone) and Group 7 (saline control group). Extracts were given daily by oral lavage, 100 μl of each extract. The anti-MUC1 mAb BCP8 (200 μg/per mouse) was given i.v. twice a week. Therapy was carried out for a total of four weeks. Tumor measurements were taken every three days for four weeks. Mice were sacrificed when tumor diameters reached 12 mm. Mice were maintained to observe long-term survival for 100 days total time after tumor injection.

Measurement of csytokine secretion in murine peritoneal macrophages and bone-marrow-derived dendritic cells (BMDCs). Peritoneal macrophages and BMDCs were obtained from C57Bl/6 wildtype mice (WT), MyD88-deficient mice (MyD88^{-/-}), or CR3-deficient mice (CR3^{-/-}). For BMDCs, bone marrow cells were isolated by flushing femurs and tibiae with DMEM medium supplemented with 10% heat-inactivated FBS. The cells were then passed through a 70-μm cell strainer. The cells were centrifuged and re-suspended at 10^6 cells/ml in DMEM medium in the presence of Flt3-ligand (200 ng/ml) as described.[22,23] The preparation of peritoneal macrophages was described in our previous study.[21] LPS-free extracts of each Maitake, Shitake, Reishi, AHCC and WGP were made by placing 1 gram each in a conical vial and adding 200 mM (5 mL 0.2 M) NaOH and incubating 20 min at room temperature. Vials were centrifuged at 2000 rpm x 10 min and then washed with molecular grade/Endotoxin-free water four times. 100 μg/mL of each extract was added with macrophages and DCs overnight. Supernatants were collected and immediately used for ELISA cytokine assay. Cytokine levels of TNFα, IL-6 and IL-12 were measured by ELISA Max Deluxe kits (Bio-Legend, San Diego, CA). Standard curves were used for calculation of cytokine concentrations.

Surface marker expression on macrophages and DCs. Murine resident macrophages or BMDCs were stimulated with WGP, Shitake, Maitake, Reishi, AHCC (100 μg/ml) or LPS (10 μg/ml). After 24 h culture for BMDCs or 48 h culture for macrophages, cells were harvested and stained with fluorochrome-labeled mAbs F4/80, CD11c, CD80, CD86, CD40 and MHC class II (eBioscience, San Diego, CA). Cells were collected with a FACSCalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Preparation of active moiety of β-glucans from macrophages. Murine resident peritoneal macrophages were maintained in a bioreactor flask (Integra Biosciences; Chur, Switzerland) in macrophage growth serum-free medium (Invitrogen, Grand Island, NY). Yeast-derived β-glucan WGP or mushroom extracts of Reishi, Maitake, Shitake and AHCC was added into culture. Following two weeks of cell culture, the cell-free fluid from the lower chamber of the bioreactor was collected and these fractions were measured for hexose concentration by the phenol-sulfuric acid method.[24]

In vitro neutrophil-mediated CR3-dependent cellular cytotoxicity. In vitro cytotoxicity of SKOV-3 cells by β-glucan-primed human neutrophils was analyzed using a real-time (RT-CES) measurement of the impedance of electrical current by viable target cells adhered to a conductor on the bottom of wells in a 16 well plate, according to manufacturer's instruction (Acea Biosciences, San Diego, CA). SKOV-3 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). SKOV-3 cells constitutively express high levels of human Her-2/neu on the surface. Our previous study demonstrated that anti-Her-2/nue mAb binds to Her-2/neu on SKOV-3 cells leading to complement activation.[25] For cytotoxicity assay, 5×10^3 SKOV-3 cells were placed into the wells of the Acea 16-well plates for 24 h. Following this, fresh human serum and sufficient anti-Her-2/neu mAb were added to the adherent SKOV-3 cells. The cells were incubated for 30 min at 37°C to permit complement activation and deposition of human iC3b. Human neutrophils were added to achieve E:T cell ratios of 20:1 with or without hexose from macrophage-digested WGP, Reishi, Shitake, Maitake, or AHCC (10 μg/ml). Cells were incubated at 37°C in a humidified 5% CO_2 incubator for 12 h. Cytotoxicity was calculated by measuring the relative decrease in current impedance among wells containing iC3b-opsonized SKOV-3 cells and hexose-primed neutrophils and wells containing iC3b-opsonized SKOV-3 cells and non-hexose-primed neutrophils.[19]

Graphing and statistical analysis of data. Data from mouse therapy protocols were entered into Prism 4.0 (Graph Pad Software, San Diego, CA) to generate graphs of tumor regression or survival and to determine the significance of differences between data sets. One-way analysis of variance (ANOVA) with Fisher's least significant difference (LSD) was employed to compare differences between two tumor regression curves, whereas the log rank test was used to determine the significance of differences between two survival curves. Cytokine data were also entered into Prism 4.0 to generate bar graphs. One-way ANOVA was used to compare the significance of differences between two groups.

Conflict of interest

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