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β-Glucan enhances antitumor immune responses by regulating differentiation and function of monocytic myeloid-derived suppressor cells

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Myeloid-derived suppressor cells (MDSCs) accumulate in tumor-bearing hosts and play a major role in tumor-induced immunosuppression, which hampers effective immunotherapeutic approaches. β-Glucans have been reported to function as potent immunomodulators to stimulate innate and adaptive immune responses, which contributes to their antitumor property. Here, we investigated the effect of particulate β-glucans on MDSCs and found that β-glucan treatment could promote the differentiation of M-MDSCs (monocytic MDSCs) into a more mature CD11c$^+$ F4/80$^+$ Ly6C^{low} population via dectin-1 pathway in vitro, which is NF-κB dependent, and the suppressive function of M-MDSCs was significantly decreased. Treatment of orally administered yeast-derived particulate β-glucan drastically downregulated MDSCs but increased the infiltrated DCs and macrophages in tumor-bearing mice, thus eliciting CTL and Th1 responses, inhibiting the suppressive activity of regulatory T cells, thereby leading to the delayed tumor progression. We show here for the first time that β-glucans induce the differentiation of MDSCs and inhibit the regulatory function of MDSCs, therefore revealing a novel mechanism for β-glucans in immunotherapy and suggesting their potential clinical benefit.

Keywords: Dectin-1 · Dendritic cells · β-Glucan · Myeloid-derived suppressor cells · Tumor immunotherapy

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Introduction

Tumor-elicited immunosuppression is one of the crucial mechanisms of tumor escape. It is probably a pivotal element contributed to the failure in cancer immunotherapy. Indeed, accumulating evidence has shown that a population of cells with suppressive activity called myeloid-derived suppressor cells (MDSCs) contributes to the negative regulation of immune responses and plays an essential role in tumor-induced immunosuppression [1–3]. MDSCs are a phenotypically heterogeneous cell population that includes myeloid progenitor cells and immature myeloid cells. In healthy individuals, MDSCs are generated in bone marrow

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quickly differentiate into mature DCs, macrophages or granulocytes. In contrast, in pathological cases, a partially block in the differentiation of MDSCs into myeloid cells results in the expansion of this population. In mice, MDSCs were characterized by the coexpression of two markers CD11b and Gr-1 (constituted by Ly6G and Ly6C) [4]. Recently, MDSCs were subdivided into two different subsets by the expression of Ly6G and Ly6C epitopes: Granulocytic MDSCs (G-MDSCs) have a $CD11b^+Ly6G^+Ly6C^{low}$ phenotype, whereas monocytic MDSCs (M-MDSCs) are $CD11b^+Ly6G^-Ly6C^{hi}$ [4, 5]. MDSCs have been shown to accumulate in tumor-bearing mice and patients with cancer, thus efficiently suppress T-cell function. They strongly inhibit the antitumor responses of $CD4^+$ T cells, $CD8^+$ T cells and NK cells, resulting in tumor-related immune suppression [6, 7]. Furthermore, it has recently been reported that MDSCs are capable of inducing the expansion of Treg cells [8–10]. MDSCs utilize a myriad of mechanisms to suppress T-cell function, including high levels of arginase activity, production of NO and ROS [11]. These main pathways are associated with different subsets of MDSCs. M-MDSCs mediate suppression by NO and arginase, whereas G-MDSC-mediated suppression is via ROS [3, 5]. All these data suggest that elimination of MDSCs may significantly improve antitumor immune responses and elicit an effective cancer immunotherapy.

β-Glucans are main components of the cell wall of various yeast, fungi, or certain bacteria, which are recognized as pathogen-associated molecular patterns (PAMPs). They are glucose polymers with a backbone of linear β-1,3-linked D-glucose molecules (β-1,3-D-glucan) and β-1,6-linked side chains of varying sizes with distribution frequency [12, 13]. The immunostimulatory properties of β-glucans have been identified for centuries [13]. They are regarded as biological response modifiers (BRMs) that enhance the innate immune system and stimulate tumor rejection [14]. Dectin-1, a non-Toll-like pattern recognition receptor for β-glucan that is mainly expressed on myeloid cells, including dendritic cells, monocytes/macrophages, neutrophils, and a subset of T cells [15, 16]. Recognition of dectin-1 by β-glucan can activate Raf-1 and Syk kinase signaling pathway, leading to the phosphorylation of MAP kinase, NFAT, and NF-κB [17–20], and finally inducing plenty of cellular responses [21, 22]. Among all these possible pathways, Syk is reported to be the main downstream signaling components after dectin-1 activation. Previous studies have demonstrated that β-glucan is capable of regulating both innate and adaptive immunity [23–25], which suggests that it can be used as an effective adjuvant in immunotherapy.

Promoting MDSCs differentiate into mature myeloid cells without suppressive capacity might be considered one of the most promising approaches in cancer immunotherapy. Previous studies have shown that, WGPs (whole β-glucan particles), the particulate β-glucans derived from yeast *Saccharomyces cerevisiae* can activate DCs and macrophages via dectin-1 signaling, eliciting Th1, and CTL priming and differentiation, thus leading to the augmented antitumor immune responses [12, 13]. Therefore, we hypothesized that WGP treatment through dectin-1 pathway may circumvent MDSC-mediated immune suppression by reducing MDSC numbers, modifying MDSC function or promoting MDSC differentiation. To solve this issue, we investigate the effect of WGP treatment on MDSCs in vitro and in murine tumor models.

Results

WGP induces the differentiation and maturation of M-MDSCs via dectin-1 pathway in vitro

To investigate the effect of WGP on MDSCs, we first examined whether the receptor for β-glucan, dectin-1 was expressed in MDSCs. Splenic M-MDSCs and G-MDSCs were isolated from tumor-bearing C57BL/6 mice. Both RT-PCR and flow cytometry analysis showed that MDSCs expressed dectin-1 (Fig. 1A).

Next, we evaluated the effect of WGP treatment on MDSCs in vitro. To this end, sorted M-MDSCs and G-MDSCs were stimulated with WGP in vitro and the proportions of certain populations were assessed. As indicated in Figure 1B, survival cells were enhanced upon WGP stimulation. However, in WGP-treated M-MDSCs group, the proportion of MDSCs was significantly reduced. Interestingly, we found that a population of $CD11c^+F4/80^+$ cells emerged in the WGP-treated M-MDSCs group, but not in the G-MDSC group. This result may imply that part of M-MDSCs was induced to differentiate into $CD11c^+F4/80^+$ cells by WGP. Furthermore, the expression of Ly6C was lower on $CD11c^+F4/80^+$ cells than $CD11c^-F4/80^-$ cells, which indicated that the newly emerged $CD11c^+F4/80^+$ cells were more mature cells. And, the expression of costimulatory molecules CD40, CD80, CD86, MHC class II (MHCII) was significantly upregulated on those $CD11c^+F4/80^+$ cells whereas the $CD11c^-F4/80^-$ cells remained low levels upon WGP stimulation. Next, analysis of arginase activity and NO release indicated that WGP stimulation could downregulate the suppressive factors produced by M-MDSCs, but IL-12 expression was upregulated after WGP treatment (Fig. 1C). These data indicate that WGPs may induce the differentiation and maturation of M-MDSCs in vitro and the immunostimulatory capability is strongly upregulated.

In order to confirm whether the differentiation and maturation of M-MDSCs were mediated by dectin-1, we used anti-dectin-1 antibody to block and found that the effect that WGP induced was partially reversed. As indicated in Figure 1D, after the dectin-1 was inhibited, the proportion of $Gr\text{-}1^+CD11b^+$ MDSCs was increased while the extent of differentiation and maturation was inhibited. The proportion of $CD11c^+F4/80^+$ cells was decreased by the blockade of dectin-1. In addition, upregulation of arginase, NO, and decreased IL-12 were observed in anti-dectin-1 group when compared with the control one. Taken together, these data suggest that WGP can promote the differentiation and maturation of M-MDSCs via dectin-1 signal, leading to a less suppressive environment.

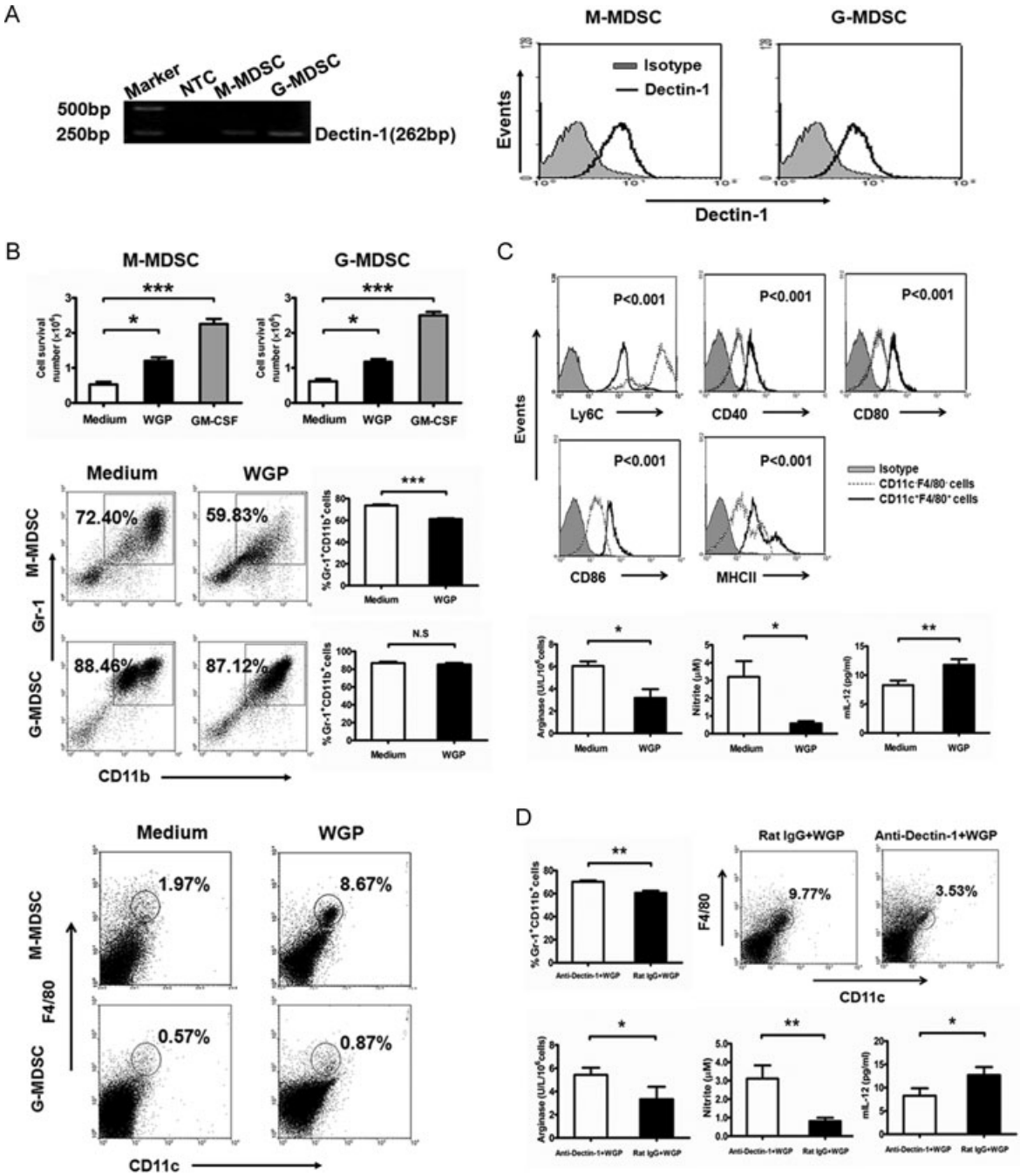

Figure 1. WGP induces maturation and differentiation of M-MDSC via dectin-1 signal. A total of 5×10^6 Lewis lung carcinoma cells were injected s.c. into C57BL/6 mice. After 4 weeks, splenocytes were collected. (A) M-MDSCs and G-MDSCs were sorted. RNA isolated from two subsets was subjected to RT-PCR to measure dectin-1 mRNA expression (left). MDSCs were stained for dectin-1 with anti-dectin-1 antibody (thick line histogram) or rat IgG2b (solid gray histogram) and then analyzed using flow cytometry (right). (B) Sorted M-MDSCs and G-MDSCs were cultured in the presence or absence of WGP (100 μg/mL) or GM-CSF (20 ng/mL) for 48 h, the survival number of cells was calculated, and the $Gr\text{-}1^+CD11b^+$ MDSCs, $CD11c^+$, $F4/80^+$ cells were analyzed by flow cytometry. (C) WGP-treated MDSCs were stained with specific Abs against Ly6C, CD40, CD80, CD86, and MHCII, cells were gated on the $CD11c^-F4/80^-$ (dot line histogram) or $CD11c^+F4/80^+$ (thick line histogram) population, respectively (top and middle). Significant differences in the mean marker expression between $CD11c^-F4/80^-$ and $CD11c^+F4/80^+$ populations were determined by Student's *t*-test. Arginase activity was measured as described in *Materials and methods*. Supernatants from WGP-stimulated MDSCs or untreated MDSCs were collected and assayed for nitrites and IL-12. (D) The purified M-MDSCs were pretreated with anti-dectin-1 mAb or rat IgG (5 μg/mL) for 1 h at 37°C and then treated with 100 μg/mL WGP, after 48 h stimulation, cells were subjected to analyze the proportions of MDSCs, $CD11c^+F4/80^+$ cells, the activity of arginase and the concentration of nitrite and IL-12. All data are shown as the mean + SD of four samples pooled from three independent experiments. Student's *t*-test was used for the statistical analysis. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, N.S. no significance.

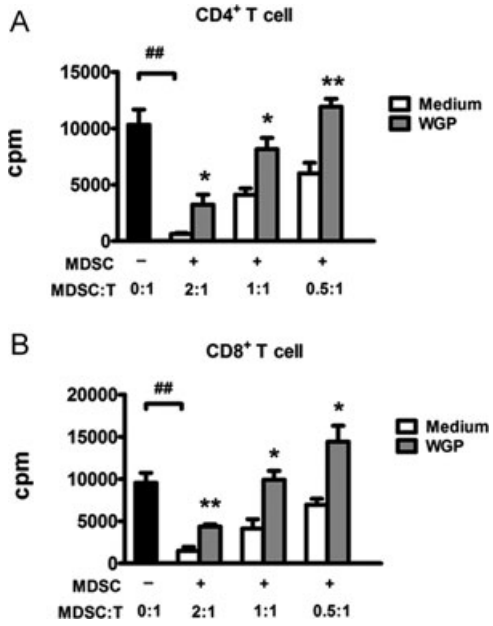

Figure 2. The suppressive capacity of WGP-treated M-MDSCs is down-regulated. (A, B) Splenic M-MDSCs purified from tumor-bearing C57BL/6 mice were used for in vitro assays. M-MDSCs were cultured in the presence or absence of WGP at 100 μg/mL for 48 h. WGPs were removed and the cells were harvested, and then cocultured with responder cells, (A) $CD4^+$ T cells or (B) $CD8^+$ T cells (MDSC: T cell ratio of 2:1, 1:1, 0.5:1) in the presence of anti-CD3 mAb and anti-CD28 mAb for 72 h. Suppression of T-cell proliferation was measured by ^{3}H-thymidine incorporation. Data are presented as mean + SD of five samples pooled from three independent experiments. Student's *t*-test was used for the statistical analysis. $^{**}p < 0.01$, $^{*}p < 0.05$, WGP versus the corresponding medium. $^{\#\#}p < 0.01$.

WGP treatment alters the suppressive capacity of M-MDSCs in vitro

In an effort to determine if WGP treatment has any effect on the suppressive function of M-MDSCs in vitro, we cocultured $CD4^+$ T cells or $CD8^+$ T cells with M-MDSCs treated with or without WGP. As shown in Figure 2, suppressive activity of M-MDSCs on $CD4^+$ T-cell (Fig. 2A) and $CD8^+$ T-cell (Fig. 2B) proliferation was significantly inhibited by WGP treatment. Thus, M-MDSCs seemed to lose their normal immunosuppressive effect and acquire stimulatory function after WGP stimulation.

WGP reduces MDSC proportions while promoting DC and macrophage infiltration in tumor-bearing mice

Having observed that WGP could differentiate M-MDSCs in vitro, we next investigated the in vivo effect. C57BL/6 mice were implanted with 3×10^6 Lewis lung carcinoma (LLC) cells. After palpable tumors were formed, mice were treated with PBS or WGP for 2 weeks. As depicted in Figure 3A, tumor growth in WGP-treated group was found to be drastically slower as compared with that in the PBS group at day 16, day 18, day 20 after tumor implantation. These data suggest that WGP can obviously delay tumor progression.

We further examined different populations in these mice. We found that WGP treatment dramatically reduced the frequencies of Gr-1^+CD$11b^+$ MDSCs both in spleens and tumors (Fig. 3B). The proportions of $CD11c^+$ DCs in draining lymph nodes and tumors (Fig. 3C), $F4/80^+$ macrophages in draining lymph nodes and tumors were significantly increased (Fig. 3D). Analysis of MHCII and Ly6C expression on $CD11c^+$ and $F4/80^+$ cells in tumors showed that the majority of enhanced $CD11c^+$ and $F4/80^+$ cells were $MHCII^+$ $Ly6C^{low}$ phenotype, which suggested their potential positive immunostimulatory function. According to what we have observed in vitro, we speculated that part of MDSCs were probably differentiated into DCs and macrophages in vivo. Next, we investigated the production of certain factors related to MDSCs suppressive activity, the levels of ROS, arginase activity, and iNOS expression were determined in tumor sites. As shown in Figure 3E, no difference of ROS levels was observed between mice treated with or without WGP. However, the activity of arginase and iNOS was decreased within the tumor milieu after WGP treatment. In addition, the expression of IL-12 was increased in WGP-treated group. All these in vivo effect was also observed in CT26 colon carcinoma (Supporting Information Fig. 1). Therefore, the data indicate that WGP could suppress the accumulation of MDSCs and promote the development of DCs and macrophages in tumor-bearing mice, which modulates the immunosuppressive tumor microenvironment.

WGP treatment enhances $CD4^+$ and $CD8^+$ T-cell responses and impairs Treg-cell suppressive capacity

Having demonstrated that WGP is able to inhibit MDSCs and increase the frequencies of DCs and macrophages, we further determined to investigate whether the enhanced CTL and Th1 priming will be induced. To address it, we analyzed the $CD8^+$IFN-γ^+ CTLs and $CD4^+$IFN-γ^+ Th1 cells in spleens, draining lymph nodes and tumors by flow cytometry. As shown in Figure 4A and B, WGP treatment contributed to the augmented CTLs and Th1-cell responses.

Next, based on recent findings showing that MDSCs have the potential to induce Treg cells [8, 10], we investigated whether the reduction of MDSCs has any impact on Treg cells in vivo. As indicated in Figure 4C, in tumor sites, WGP treatment could substantially reduced the number of $CD4^+CD25^+Foxp3^+$ Treg cells. However, we found that there was no significant difference in splenic Treg cells between two groups. Interestingly, the suppressive capacity of splenic Treg cells was obviously impaired upon WGP treatment in vivo (Fig. 4D). This suggests that, WGP could modulate the suppressive activity of peripheral Treg cells despite the stable percentages. The similar results were observed in CT26 colon carcinoma (Supporting Information Fig. 2). Taken together, these data suggest that WGP-induced differentiation of MDSCs not only promotes CTL and Th1 responses but also alters the suppressive activity of Treg cells, thus promoting antitumor immunity, leading to a more efficient defense mechanism against tumor development.

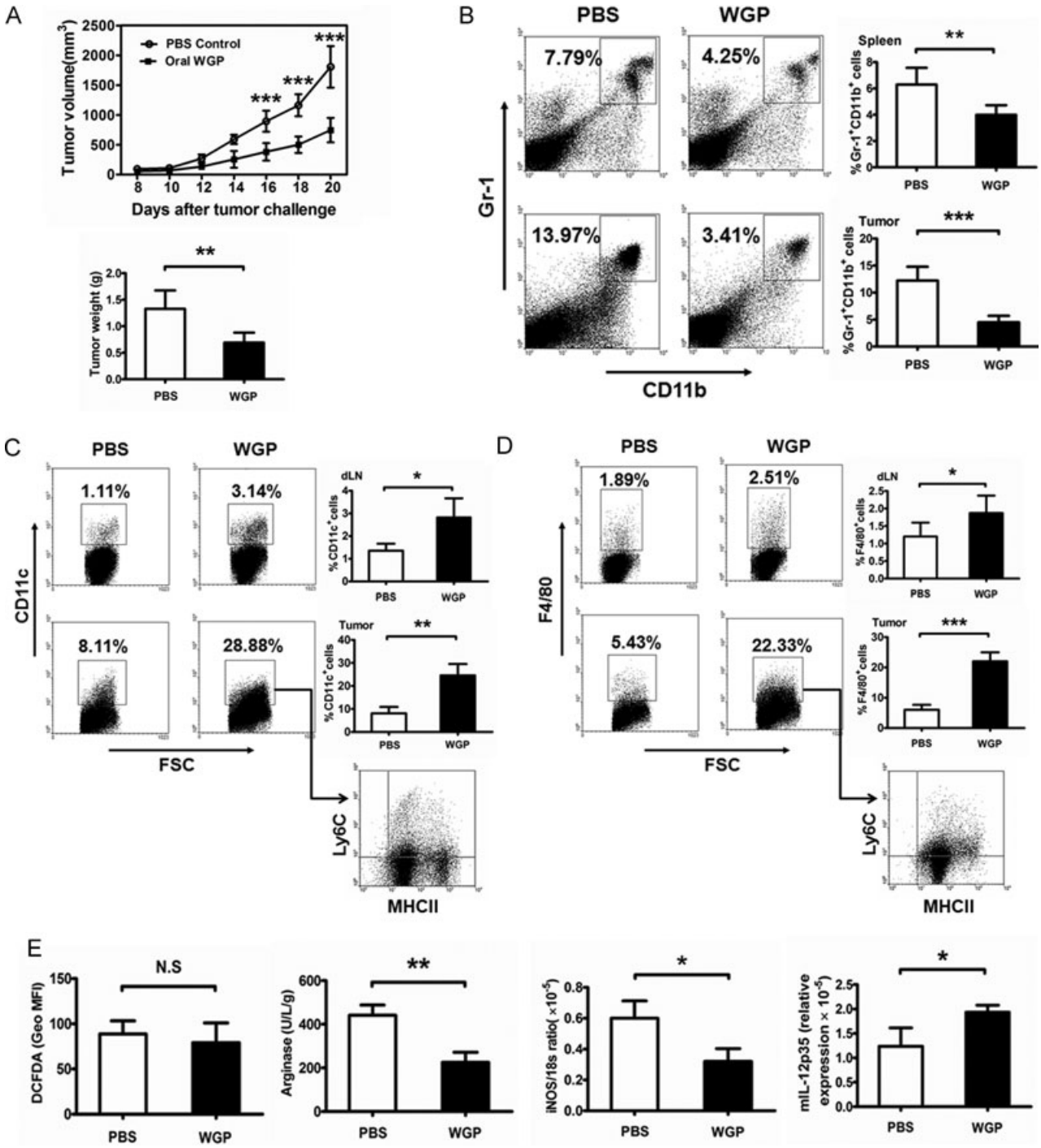

Figure 3. WGP treatment in vivo significantly decreases the MDSCs while upregulates DCs and macrophages, and delays tumor progression. Groups of mice ($n = 8$) bearing established Lewis lung carcinoma were treated either with oral WGP or PBS as a control. (A) Tumors were measured with a caliper at indicated time and weighed. $^{***}p < 0.001$, PBS versus WGP at different time points. (B) The proportions of Gr-1$^+$CD11b$^+$ MDSCs were evaluated in spleens and tumor tissues, (C) CD11c$^+$ DCs, (D) F4/80$^+$ macrophages in draining lymph nodes and tumors were analyzed using flow cytometry. MHCII and Ly6C levels were measured on CD11c$^+$ and F4/80$^+$ cells infiltrated in tumors. (E) Cells collected from the tumor sites of tumor-bearing mice treated with or without WGP were stimulated with PMA, labeled with 2.5 μM oxidation-sensitive dye 2,7-dichlorofluorescin diacetate, and stained with anti-Gr-1 and anti-CD11b mAbs. oxidation-sensitive dye 2,7-dichlorofluorescin diacetate fluorescence was measured in Gr-1$^+$CD11b$^+$ MDSCs by flow cytometry. Arginase activity of cells from tumor tissues was measured. RNA from tumor specimens treated with or without WGP was extracted and qRT-PCR was performed for iNOS and IL-12p35. Results are expressed as mean + SD of $n = 8$ representative of three independent experiments performed. Student's t-test was used for the statistical analysis. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, N.S. no significance, Geo MFI: geometric mean fluorescent intensity.

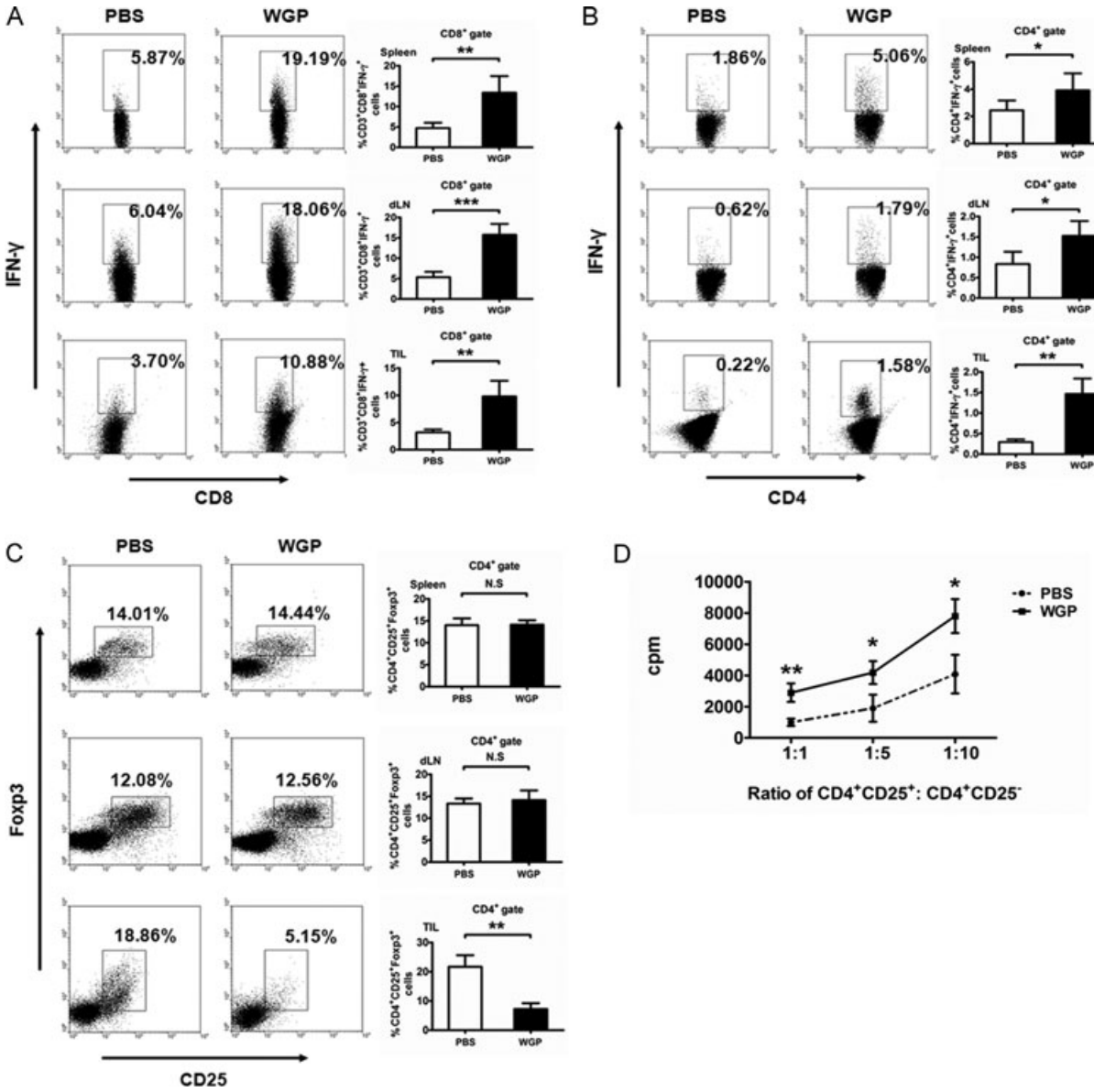

Figure 4. Enhanced T-cell responses and decreased Treg-cell suppressive function upon WGP treatment. Groups of mice-bearing established Lewis lung carcinoma were treated either with oral WGP or PBS as a control. (A–C) Single cell suspensions from spleens, draining lymph nodes, and tumor tissues ($n = 8$) were stained with corresponding antibodies to evaluate the proportions of (A) $CD8^+IFN\text{-}\gamma^+$ CTLs, (B) $CD4^+IFN\text{-}\gamma^+$ Th1 cells, and (C) $CD4^+CD25^+Foxp3^+$ Treg cells. Cells were gated on $CD8^+$ or $CD4^+$ T cells. Data are presented as mean + SD of $n = 8$ representative of three experiments performed. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, N.S. no significance. (D) $CD4^+CD25^+$ Treg cells isolated from WGP-treated or untreated tumor-bearing mice ($n = 6$) were cocultured with $CD4^+CD25^-$ effector T cells in the presence of anti-CD3 mAb and anti-CD28 mAb for 72 h. Wells were pulsed with 1 μCi/well ^{3}H-thymidine and analyzed as described in *Materials and methods*. Data are presented as mean + SD of $n = 6$ representative of three experiments performed. Student's *t*-test was used for the statistical analysis. $^{**}p < 0.01$, $^{*}p < 0.05$, PBS versus WGP at different ratios.

WGP induces differentiation of M-MDSCs via NF-κB-dependent pathway

To investigate possible molecular mechanisms in WGP-mediated M-MDSC differentiation, we examined the expression of phosphorylated Syk (P-Syk) and NF-κB. Western blot analysis of P-Syk and P-p65 in WGP-treated MDSCs showed that levels of P-Syk and P-p65 were elevated when compared with that in untreated MDSCs (Fig. 5A). Although WGP had no effect on total p65 (T-p65) protein levels in MDSCs, significant activation of P-p65 was observed upon WGP treatment. To further confirm if WGP acts through NF-κB pathway to differentiate MDSCs, WGP-treated M-MDSCs were cultured in the presence or absence of PDTC, an inhibitor of NF-κB activation, then the cells were collected and analyzed. Compared with the data shown in Figure 1B, the differentiation of M-MDSCs was strongly inhibited after the blockade of NF-κB (Fig. 5B). Here, we observed that the proportion of MDSCs was again reversed to the high level and almost only

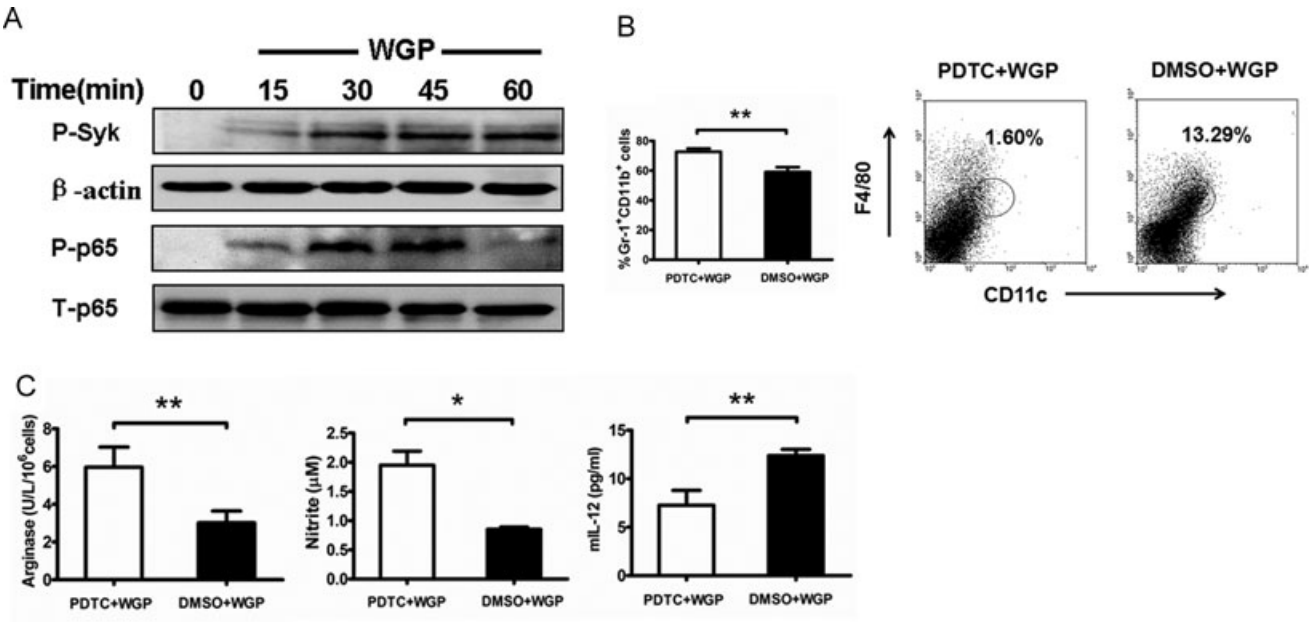

Figure 5. WGP-induced differentiation of M-MDSCs is mediated by Syk and NF-κB p65. (A) Sorted splenic MDSCs from tumor-bearing mice were treated with or without WGP (100 μg/mL) for indicated times. Cells were lysed, and western blot analysis developed with anti-phospho-Syk antibody and anti-phospho-NF-κB p65 antibody. β-Actin served as a loading control. (B) M-MDSCs were pretreated for 1 h with or without the NF-κB inhibitors PDTC (100 μM) prior to the addition of WGP. After 48 h incubation, the Gr-1$^+$CD11b$^+$ MDSCs, CD11c$^+$F4/80$^+$ cells were analyzed by flow cytometry. (C) Levels of arginase, nitrite, and IL-12 were measured in the cells or supernatants. Results are represented as mean ± SD of four samples pooled from three independent experiments. Student's *t*-test was used for the statistical analysis. $^{**}p < 0.01$, $^{*}p < 0.05$.

CD11c$^-$F4/80$^-$ cells existed in the block group. We further analyzed arginase activity and NO production, and found that groups blocked with PDTC had significant increased expression of arginase and NO. The level of IL-12 was also decreased after the inhibition of NF-κB p65 (Fig. 5C). All these data suggest that WGP is capable of inducing the differentiation of M-MDSCs, which is dependent on the activation of NF-κB p65.

Discussion

As effective immune-stimulating polymers of glucose, β-glucan has been used in cancer therapy with varying and unpredictable success. Previous studies showed that orally administered WGPs could be captured by gastrointestinal macrophages, and then interacted with immunocytes in BM, lymph node, and spleen [26]. β-Glucans play an essential role in innate immune responses, their mechanism in cancer therapy is activating macrophages and priming of neutrophil complement receptor 3 to kill iC3b-opsonized tumor cells [27]. Moreover, β-glucans are recently identified to induce adaptive immune responses. It is reported that the particulate β-glucans WGPs can activate and maturate DCs both in vitro and in vivo, leading to enhanced Ag-specific CD4 and CD8 T-cell responses [13]. Besides, the yeast zymosan β-glucan is demonstrated to promote the differentiation of Treg that is mediated by regulatory antigen-presenting cells (APCs) [28, 29]. In addition, bacterial β-glucan curdlan can activate DCs and augment Th1, CTL, and Th17 cell responses via dectin-1-dependent pathway [18, 22]. All these emerging data suggest that β-glucans has a potential modulatory capability to elicit innate and adaptive immune responses. However, most of the studies are primarily focused on the effect of β-glucans on immune cells that promote the positive immune responses, including DCs, macrophages, and neutrophils. Thus far, few data have been published to study the effect on those regulatory cells, such as MDSC, a population with potent suppressive activity that is of great importance in the development of cancers. In tumor-bearing hosts, various endogenous factors contribute to the accumulation of MDSCs, thus leading to tumor-associated immune suppression [30–32]. In our study, we first proposed that the essential receptor for β-glucans, dectin-1 was expressed on both M-MDSCs and G-MDSCs. This suggests that β-glucans are capable of interacting with MDSCs directly through dectin-1 pathway. Excitingly, we observed that the yeast-derived particulate β-glucan WGP could differentiate M-MDSCs into more mature myeloid cells, a population of CD11c$^+$F4/80$^+$Ly6C^{low} cells, leading to the decreased suppressive cells and increased effector cells. However, the similar differentiation was not observed in G-MDSCs, but this does not mean WGP could not regulate G-MDSCs. G-MDSCs were granulocytic phenotype, it is probably that WGP could promote the differentiation of G-MDSCs into mature granulocytes. Moreover, our recent data have shown that WGP could regulate the function of G-MDSCs in vitro (data not shown). Hence, investigation on the effect of WGP on G-MDSCs is to be continued in our future work. Next, we also observed that the levels of costimulatory molecules on CD11c$^+$F4/80$^+$ cells were substantially higher than the CD11c$^-$F4/80$^-$ cells population after WGP stimulation. Thus,

the high levels of costimulatory molecules (CD40, CD80, CD86, MHCII) and the low level of Ly6C indicate the relative mature state of $CD11c^+F4/80^+$ cells, which renders a potential capacity to elicit the adaptive immune responses. In addition, downregulation of arginase and NO imply the decreased suppressive capacity. Moreover, the expression of IL-12 was increased upon WGP stimulation, which acts as an essential polarizing element to promote naive $CD8^+$ T cells differentiate into effectors. Previously, several receptors for β-glucan have been identified, including CR3, dectin-1, TLR2, and TLR4 [16]. Thus, dectin-1 signaling is probably not the unique pathway to mediate the differentiation of M-MDSCs. This is consistent with what we observed. As shown in Figure 1D, inhibition of dectin-1 could not completely reverse the differentiation of M-MDSCs. Nevertheless, the role of dectin-1 played in the differentiation is relatively dominant.

We further observed that the suppressive activity of M-MDSCs was dramatically decreased after WGP stimulation. It appears that the decreased M-MDSC suppressive activity by WGP treatment is associated with the induction of maturation and differentiation of M-MDSCs. Strikingly, in some conditional groups, the proliferation of $CD4^+$ T cells or $CD8^+$ T cells was higher than the non-MDSC group. Probably, this is due to the generation of mature myeloid cells by M-MDSC differentiation. β-Glucan in the culture medium further triggered the dectin-1 signaling activation on mature myeloid cells, which promoted the maturation of the cells, thus enhancing the activation and expansion of T cells. Taken together, all these data indicate that WGP-induced MDSC differentiation can alter the suppressive environment and promote the effective adaptive immune responses.

Previous studies have shown that WGP administration could delay the tumor development [12, 13]. Here, we reaffirmed the antitumor properties of WGP shown by the abrogation of tumor progression in two murine carcinoma models. WGP administration substantially reduced the proportions of MDSCs in spleens and tumor sites, whereas the percentages of DCs and macrophages were significantly increased, especially in tumors. The decreased MDSCs accumulation in vivo probably due to the fact that WGP promoted MDSC differentiation and maturation into DCs and macrophages as we observed in vitro. However, this was not the sole possibility. Similarly, the production of suppressive factors including arginase, iNOS was significantly decreased while IL-12p35 expression was upregulated in tumor microenvironment, which is consistent with our in vitro data. Next, we investigated whether this differentiation had any effect on antitumor immune responses. We found that WGP treatment could elicit enhanced $CD4^+$ and $CD8^+$ T-cell responses and modulate suppressive tumor microenvironment by decreasing Treg cells in tumor sites and downregulating suppressive activity of Treg cells in spleens. The decreased MDSCs in tumor-bearing mice reduce their immunosuppressive effect on T cells, which allows the effector T cells (CTL and Th1 cells) persist longer and keep tumor development under control. Moreover, the enhancement of DCs and macrophages is beneficial to prime $CD4^+$ and $CD8^+$ T-cell responses, thus eliciting effective antitumor responses. It is reported that MDSCs have potential to induce Treg-cell generation, and MDSCs and Treg cells might be linked in the immunoregulatory network [4, 8]. In our study, MDSC reduction did not affect the proportions of peripheral Treg cells but impair the suppressive activity of Treg cells. However, Treg cells infiltration in tumor microenvironment was significantly decreased. The different events between peripheral organs and tumor sites are probably due to the special and complicated tumor microenvironment. Therefore, it appears that WGPs modulate the antitumor responses from two sides, one is downregulation of suppressive elements, the other is upregulation of positive immune responses, thus leading to an efficient approach to treat cancers.

In dectin-1 receptor pathway, Syk is a critical signaling molecule mediating the downstream cellular responses, including cytokine production and induction of the respiratory burst [16]. Caspase recruitment domain 9 (CARD9), which assembles with BCL10 and MALT1, has been regarded as a vital downstream adaptor linking Syk-coupled receptors to the canonical NF-κB pathway [33]. To determine a possible molecular mechanism by which WGP induces MDSC differentiation, we examined two major downstream signaling molecules Syk and NF-κB. In our study, we confirmed that Syk was also activated in MDSC upon dectin-1 triggering. Strikingly, WGP-treated MDSCs have markedly increased levels of phospho-NF-κB p65. This finding leads us to hypothesize that the differentiation of MDSCs is mediated by the NF-κB pathway. This notion is supposed by the data showing that the differentiation was inhibited after the block of NF-κB with PDTC. Thus, it is proposed that the immunomodulatory effect of WGP is mediated by the NF-κB signaling in M-MDSCs, leading to the differentiation of M-MDSCs into mature myeloid cells, which provides improved strategies to support immune-mediated antitumor therapy.

Efficient cancer immunotherapy requires elicitation of potent antitumor T-cell responses and downregulation of immunosuppressive elements such as MDSCs and Treg cells. As the suppressive function of MDSC is closely related to their immature state, the induction of differentiation will be an effective approach in reversing the suppressive environment to normal immune responses. Previous successful approaches have been undertaken to promote MDSC maturation with all-trans-retinoic acid [34], docetaxel [35], and CPG [32]. In our study, we demonstrate that particulate β-glucans directly modulate MDSCs and promote the differentiation of cells through NF-κB pathway, thus effectively abrogating MDSC-associated immune suppression and improving antitumor responses. We thus reveal a novel mechanism by which WGP modulates antitumor responses and particulate β-glucans are considered to be of great clinical value in cancer immunotherapy.

Materials and methods

Particulate yeast-derived β-glucan WGP

WGP (Biothera, Eagan, MN) was purified from the cell wall of *S. cerevisiae* through a series of alkaline and acid extractions to

yield hollow yeast cell wall ghosts composed mainly of β-1,3/1,6-glucan. The components of WGPs have been described previously [12]. WGPs were kindly gifted by Dr. Jun Yan from the University of Louisville School of Medicine.

Cell line, mice, and tumor models

The LLC cells were obtained from American Type Culture Collection. Specific pathogen-free male C57BL/6 mice were purchased from Yangzhou University. Mice were used as 6–8 weeks of age. All experiments were approved by the Institutional Committee on the Use of Animals for Research and Teaching.

For tumor models, mice were implanted s.c. with LLC (3×10^6/mouse). After palpable tumors were formed, two groups of C57BL/6 mice were treated with 200 μL of yeast-derived β-glucan WGP (4 mg/mL in PBS; total 800 μg) or 200 μL PBS given every day using an intragastric gavage needle. Therapy was continuously administered for 2 weeks. Tumor growth was monitored with bidirectional tumor measurements using calipers every 2 days and tumor volume was calculated using the formula $V = 0.5ab^2$ with "a" as the larger diameter and "b" as the smaller diameter. The weight of tumors was measured when the mice were sacrificed.

Preparation of single cell suspension from tumors

Tumors were weighed and the similar volumes were acquired from different groups and minced into small (1–2 mm^3) pieces and immersed in 10 mL of digestion mixture including 5% NCS in RPMI 1640 (GIBCO, Carlsbad, CA), 0.5 mg/mL collagenase A (Sigma-Aldrich, St. Louis, MO), 0.2 mg/mL hyaluronidase (Sigma-Aldrich, St. Louis, MO), and 0.02 mg/mL DNase I (Sigma-Aldrich, St. Louis, MO) per 0.25 g of tumor tissues. This mixture was incubated at 37°C for 1.5 h on a rotating platform. Then the cell suspensions were filtered through 70 μm cell strainers (BD Falcon, San Jose, CA) and washed twice with ice-cold PBS. Remaining red blood cells were lysed in ammonium chloride solution.

Isolation of MDSCs

Murine MDSCs were isolated from the spleens of LLC tumor-bearing mice using a mouse MDSC isolation kit (Miltenyi Biotec, Auburn, CA) following the manufacturer's protocol.

Flow cytometry

For surface markers, single cell suspensions were stained with relevant fluorochrome-conjugated CD11b, Gr-1, Ly6C, CD11c, F4/80, CD40, CD80, CD86, MHCII mAbs (eBioscience, San Diego, CA). Anti-dectin-1 (Invivogen, San Diego, CA) and FITC-conjugated goat anti-rat IgG (KPL, Gaithersburg, MD) were used to detect the dectin-1 expression. Cells were harvested, Fc receptors were blocked by incubation in HB197 supernatant, stained with above antibodies in PBS for 30 min at 4°C. For intracellular cytokine staining, single cell suspensions were stimulated with PMA (Sigma-Aldrich, St. Louis, MO, 50 ng/mL), ionomycin (Enzo, Farmingdale, NY, 1 μg/mL), monensin (Enzo, Farmingdale, *NY*, 2 μg/mL). After 5 h, cells were stained with anti-CD3 and anti-CD8 mAbs (eBioscience, San Diego, CA), fixed, permeabilized, and stained with anti-IFN-γ mAb (eBioscience) according to the Intracellular Staining Kit (Invitrogen, Carlsbad, *CA*) instructions. For Treg cells staining, anti-CD4, anti-CD25, and anti-Foxp3 mAbs (eBioscience) were performed following Foxp3 Staining Buffer Set (eBioscience) protocols. Flow cytometry was performed using FACSCalibur Flow Cytometer (Becton Dickinson, Sparks, MD).

Reverse transcript PCR and quantitative real-time PCR

Cells were discharged into TRIzol (Invitrogen, Carlsbad, CA), total RNA was isolated and reversed-transcribed with ReverTra Ace qPCR RT kit (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. The reverse transcript PCR and quantitative real-time PCR (qRT-PCR) were performed as described previously [36]. The sequences for the primers used are: dectin-1, 5′-GAAAC GAGTTGGGGAAGAAT-3′ (forward), 5′-TCTTGCCTTCCTAATTGG AT-3′ (reverse); IL-12p35, 5′-TGACATGGTGAAGACGGC-3′ (forward), 5′-GCCTGGAACTCTGTCTGGTA-3′ (reverse); β-actin, 5′-TG GAATCCTGTGGCATCCATGAAAC-3′ (forward), 5′-TAAAACGCAG CTCAGTAACAGTCCG-3′ (reverse); iNOS, 5′-GAGCCCTCAGCA GCATCCAT-3′ (forward), 5′-GGTGAGGGCTTGGCTGAGTG-3′ (reverse); 18s, 5′-TCCGGAGAGGGAGCCTGA GA-3′ (forward), 5′-GCACCAGACTTGCCCTCCAA-3′ (reverse). Relative quantification of mRNA expression was calculated by the comparative threshold cycle (Ct) method.

Detection of ROS levels, arginase activity, and NO production

The oxidation-sensitive dye 2,7-dichlorofluorescin diacetate (Invitrogen, Carlsbad, *CA*) was used to measure ROS production by MDSCs. Cells were simultaneously cultured with 2.5 μM oxidation-sensitive dye 2,7-dichlorofluorescin diacetate with 30 ng/mL PMA in PBS for 30 min. Cells were then labeled with anti-CD11b and anti-Gr-1 mAbs and evaluated by flow cytometry.

Arginase activity, measuring the conversion of arginine to ornithine and urea, was determined by a quantitative colorimetric assay employing a QuantiChrom Arginase Assay kit (BioAssay systems, Hayward, CA). The arginase activity was calculated according to the manufacturer's instructions.

The amount of NO was assessed by determining the concentration of nitrite accumulated in culture supernatants using the colorimetric Griess reaction (Promega, Madison, WI).

Enzyme-linked immunosorbent assay

IL-12 content in the supernatants from cell cultures were detected by sandwich ELISA (R&D, Minneapolis, MN).

MDSC suppression assay

For evaluation of MDSCs suppressivity, MDSCs isolated from spleens of tumor-bearing mice were cultured in the presence or absence of WGPs for 48 h, then WGPs were removed. For responder cells, $CD4^+$ T cells and $CD8^+$ T cells were sorted from wild-type C57BL/6 mice spleens using CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), FITC-conjugated anti-CD8 mAb (BD Pharmingen, San Diego, CA), and anti-FITC microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Responder cells were cocultured with different ratios of WGP-treated or untreated MDSCs in U-bottomed 96-well plates (Costar, Corning, NY) in the presence of anti-CD3 (10 μg/mL) and anti-CD28 (Biolegend, San Diego, *CA* 5 μg/mL) mAbs for 72 h and pulsed with ^{3}H-thymidine (Pharmacia, Stockholm, Sweden, 1 μCi/well) for the last 16 h of culture.

In vitro proliferation assays

Single cell suspensions were prepared from spleens and red blood cells were lysed. $CD4^+CD25^+$ Treg cells from WGP-treated or untreated tumor-bearing C57BL/6 mice, $CD4^+CD25^-$ T cells from wild-type C57BL/6 mice were isolated with a $CD4^+$ T-cell negative selection kit (Invitrogen, Carlsbad, CA), FITC-conjugated anti-CD25 antibody (BD Pharmingen, San Diego, CA) and anti-FITC microbeads. The purity of isolated $CD4^+CD25^+$ Treg cells and $CD4^+CD25^-$ T cells was more than 90% (data not shown). Suppression assays were performed in U-bottomed 96-well plates in a final of 200 μL per well. $CD4^+CD25^-$ T cells were plated at 5×10^4 as responder cells with $CD4^+CD25^+$ suppressor cells at the following ratios of suppressor cells: responder cells 1:1, 1:5, 1:10. Cells were incubated in the presence of anti-CD3 (10 μg/mL) and anti-CD28 (5 μg/mL) mAbs for 72 h and pulsed with ^{3}H-thymidine (1 μCi/well) for the last 16 h of culture.

Western blot analysis

Proteins extracted from cells were prepared as described previously [36]. Proteins were separated by SDS-PAGE, transferred onto immobilon polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA), and probed with rabbit phospho-Syk antibody (CST, Danvers, MA), rabbit phospho-NF-κB p65 antibody (CST, Danvers, MA), rabbit NF-κB p65 antibody (CST, Danvers, MA) and mouse β-actin antibody (Abcam, Cambridge, UK) followed by chemiluminescent detection (Champion Chemical, Whittier, CA).

Graphing and statistical analysis of data

Data from all experiments were entered into GraphPad Prism 5.0 (GraphPad, San Diego, CA) to generate bar graphs or graphs of tumor regression. The statistical significance of differences between groups was determined by the Student's *t*-test. All analyses were performed using SPSS11.5 software. Differences were considered significant at a p level less than 0.05.

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Abbreviations: **LLC**: Lewis lung carcinoma · **MDSC**: myeloid-derived suppressor cell · **WGP**: whole β-glucan particle

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