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ORIGINAL ARTICLE

# Curdlan blocks the immune suppression by myeloid-derived suppressor cells and reduces tumor burden

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**Abstract** Tumor-elicited immunosuppression is one of the essential mechanisms for tumor evasion of immune surveillance. It is widely thought to be one of the main reasons for the failure of tumor immunotherapy. Myeloid-derived suppressor cells (MDSCs) comprise a heterogeneous population of cells that play an important role in tumor-induced immunosuppression. These cells expand in tumor-bearing individuals and suppress T cell responses via various mechanisms. Curdlan, the linear (1 → 3)-β-glucan from *Agrobacterium*, has been applied in the food industry and other sectors. The anti-tumor property of curdlan has been recognized for a long time although the underlying mechanism still needs to be explored. In this study, we investigated the effect of curdlan on MDSCs and found that curdlan could promote MDSCs to differentiate into a more mature state and then significantly reduce the suppressive function of MDSCs, decrease the MDSCs in vivo and down-regulate the suppression in tumor-bearing mice, thus leading to enhanced anti-tumor immune responses. We, therefore, increase the understanding of further mechanisms by which curdlan achieves anti-tumor effects.

**Keywords** Curdlan · Myeloid-derived suppressor cells · Immune suppression · Anti-tumor immunity

## Introduction

Curdlan is a linear extracellular polysaccharide (EPS) produced by a strain of *Alcaligenes faecalis* [1]. It is a neutral water-insoluble bacterial polysaccharide composed primarily of linear β-(1,3)-glycosidic linkages. Curdlan is tasteless, colorless and odorless and has gel-forming properties [1, 2]. In addition, curdlan has been suggested to be a molecule that triggers an immune response with interesting effects, including anti-infection, anti-tumor, anti-inflammation, wound repair and anticoagulation [3]. Dectin-1 is a natural killer (NK)-cell-receptor-like C-type lectin that has been reported to recognize curdlan and many other β-glucans, which are reported to be identified in dendritic cells, macrophages and monocytes/neutrophils [4, 5]. The receptor activates signaling cascades, which regulate specific genes related to the removal of foreign microorganisms. Indeed, many fungal β-glucans are known to stimulate both innate and adaptive immune responses, and some of them are used for cancer treatment. β-Glucans could activate various immune cells, including macrophages and dendritic cells, to enhance the function and cytokine production via different pathways [6–11]. β-Glucans are regarded as immunomodulators to inhibit

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tumor development mainly by improving the anti-tumor immune responses or direct cytotoxicity [12–15].

Accumulating immune suppressive cells and T cell defects are two factors responsible for the tumor-associated immune evasion. These suppressive cells inhibit the activation of immune responses and limit the effect of immunotherapy meant to improve anti-tumor immunity. Myeloid-derived suppressor cells (MDSCs), a heterogeneous population of early myeloid progenitors that are generated in the bone marrow, are considered as essential contributors to the suppressive tumor microenvironment by inhibiting efficient anti-tumor immune responses [16, 17]. MDSCs are precursors of macrophages, dendritic cells, granulocytes and myeloid cells [18–21]. The accumulation of MDSCs in lymphoid organs, blood and tumor sites has been observed in tumor-bearing hosts and is usually associated with large tumor burdens [22, 23]. MDSCs are broadly defined as $CD11b^{+}Gr\text{-}1^{+}$ cells in the mouse. MDSCs utilize various mechanisms to suppress T cell function, including a high level of arginase activity, nitric oxide (NO) as well as reactive oxygen species (ROS) production [17, 18, 24]. MDSCs can also suppress immune activation by inducing the development of regulatory T cells [25]. MDSCs are capable of suppressing T cell activation and proliferation, inducing $CD8^{+}$ T cell tolerance and inhibiting natural killer (NK) cell activity, thus leading to the suppression of anti-tumor immunity [26]. Therefore, therapies that reducing the number or the suppressive activity of MDSCs are considered to restore T cell activation and stimulate anti-tumor immune responses.

Our previous work has investigated the anti-tumor properties of yeast-derived β-glucan whole glucan particles (WGPs) and found that WGPs could directly promote the differentiation of MDSCs through the NF-κB pathway, thus effectively abrogating MDSC-associated immune suppression and improving anti-tumor responses [27]. In this study, we investigated the anti-tumor effects of another bacteria-derived β-glucan, curdlan, and we wonder whether different sources of β-glucan can also regulate MDSCs. Then, we found that curdlan could promote the maturation of MDSCs, and then, the suppressive capacity of the cells was impaired in vitro. Furthermore, administration of curdlan to tumor-bearing mice could decrease the number of MDSCs in vivo, which is probably caused by the maturation of MDSCs. And then, the reduction in suppressive effect could favor the enhancement of the CTL and Th1 cell responses and finally delay the tumor progression. Therefore, promoting the maturation, reducing the suppressive effect or the number of MDSCs by curdlan might be considered as new strategies for enhancing anti-tumor immunity.

## Materials and methods

### Cell line, mice and tumor model

The Lewis lung carcinoma (LLC) cells were acquired 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, C57BL/6 mice were treated with 200 μl of curdlan (4 mg/ml in PBS; total 800 μg) or 200 μl PBS given every 2 days with an intragastric gavage needle for 7 days. Then, mice were implanted subcutaneously (s.c.) with LLC cells ($3 \times 10^{6}$/mouse). After tumor challenge, therapy was continuously administered for 3 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 killed.

### Isolation of MDSCs

Murine MDSCs were isolated from the spleens of LLC tumor-bearing mice using anti-CD11b conjugated to biotin followed by anti-biotin microbeads (Miltenyi Biotec), and the isolated cells were subjected to stain with fluorochrome-conjugated anti-CD11b Ab and anti-Gr-1 Ab. The purity of $CD11b^{+}Gr\text{-}1^{+}$ cells in the isolated cells was confirmed by flow cytometry.

### RNA extraction and reverse transcript PCR (RT-PCR)

Cells were discharged into TRIzol (Invitrogen), total RNA was isolated and reversed-transcribed with ReverTra Ace qPCR RT kit (TOYOBO) according to the manufacturer's instructions. The reverse transcript PCR and qRT-PCR were performed as described previously [28]. The sequences for the primers used are as follows: dectin-1, 5′-GAAAC GAGTTGGGGAAGAAT-3′ (forward), 5′-TCTTGCCTTC CTAATTGGAT-3′ (reverse); IL-12p35, 5′-TGACATGGT GAAGACGGC-3′(forward), 5′-GCCTGGAACTCTGTCT GGTA-3′ (reverse); and β-actin, 5′-TGGAATCCTGTGG CATCCATGAAAC-3′ (forward), 5′-TAAAACGCAGCT CAGTAACAGTCCG-3′ (reverse); Relative quantification of mRNA expression was calculated by the comparative threshold cycle (Ct) method.

### Flow cytometry

For surface markers, single-cell suspensions were stained with relevant fluorochrome-conjugated CD11b, Gr-1, CD11c, F4/80, CD40, CD80, CD86, MHCII mAbs (eBioscience). Anti-dectin-1 (Invivogen) and FITC-conjugated goat anti-rat IgG (KPL) were used to detect the dectin-1 expression. Cells were harvested, blocked by incubation in HB197 supernatant for Fc receptor, and 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, 50 ng/ml), ionomycin (Enzo, 1 μg/ml), monensin (Enzo, 2 μg/ml). After 5 h, cells were stained with anti-CD3 and anti-CD8 mAbs (eBioscience), fixed, permeabilized and stained with anti-IFN-γ mAb (eBioscience) according to the Intracellular Staining Kit (Invitrogen) instructions. Flow cytometry was performed using FACSCalibur flow cytometer (Becton–Dickinson).

### Detection arginase activity and NO production

Arginase activity was determined by a quantitative colorimetric assay employing a QuantiChrom Arginase Assay kit (BioAssay systems) to measure the conversion of arginine to ornithine and urea. 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).

### Enzyme-linked immunosorbent assay (ELISA)

IFN-γ concentration in the supernatants from cell cultures was detected by sandwich ELISA (R&D).

### MDSC suppression assay

For evaluation of MDSCs suppressivity, MDSCs isolated from spleens of tumor-bearing mice were cultured in the presence or absence of curdlan for 48 h, and then, curdlan was 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), FITC-conjugated anti-CD8 mAb (BD Pharmingen) and anti-FITC microbeads (Miltenyi Biotec). Responder cells were co-cultured with different ratios of curdlan-treated or untreated MDSCs in U-bottomed 96-well plates (Costar) in the presence of anti-CD3 (10 μg/ml) and anti-CD28 (Biolegend, 5 μg/ml) mAbs for 72 h and pulsed with $^3$H-thymidine (Pharmacia, 1 μCi/well) for the last 16 h of culture. The capacity of MDSCs to suppress T cells was calculated by cpm (MDSCs+T cells)-cpm MDSCs, and the average values of MDSC cpm under different treatment were <120.

## Results

### Dectin-1 receptor is expressed on MDSCs

To evaluate whether curdlan has any effect on MDSCs, we first analyzed the expression of the dectin-1 receptor for curdlan on MDSCs. As shown in Fig. 1a, splenic MDSCs were sorted from C57BL/6 mice bearing Lewis lung carcinoma, and the purity of the cells was more than 95 %. Then, RT-PCR was used to measure the dectin-1 mRNA level in MDSCs, and the data showed that MDSCs express dectin-1 (Fig. 1b). Moreover, flow cytometry analysis further confirmed dectin-1 was expressed on MDSCs (Fig. 1c).

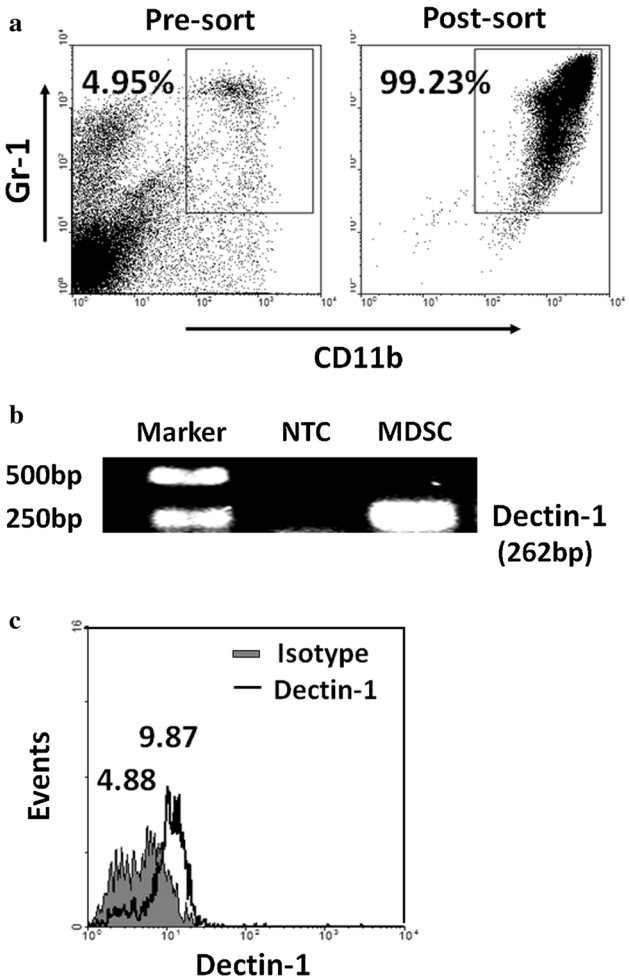



**Fig. 1** MDSCs express dectin-1 receptor. $3 \times 10^6$ LLC cells were injected s.c. into C57BL/6 mice to establish mouse Lewis lung carcinoma. After 4 weeks, splenocytes were harvested. **a** $CD11b^+Gr\text{-}1^+$MDSCs were isolated, and flow cytometry was used to analyze the purity of MDSCs. **b** RNA was extracted from MDSCs, and then, dectin-1 mRNA level was detected by RT-PCR. Marker is the DL2000 DNA marker [29], and NTC means no template control. **c** Anti-dectin-1 antibody (*thick line histogram*) or corresponding isotype control antibody (*solid gray histogram*) was used to analyze the dectin-1 expression on MDSCs by flow cytometry. Data are representative of three independent experiments

### Curdlan improves the differentiation of MDSCs

Next, we investigated the effect of curdlan on MDSCs. MDSCs isolated from splenocytes of tumor-bearing mice were stimulated with curdlan in vitro, and the levels of surface markers including CD11c, F4/80, CD40, CD80, CD86 and MHCII on MDSCs were analyzed. As shown in Fig. 2, the expression of CD11c, F4/80, CD40, CD80, CD86 and MHCII was significantly increased after curdlan treatment. The data suggest that curdlan could lead to MDSCs differentiating into more mature myeloid cells.

### Curdlan impairs the suppressive capability of MDSCs

To investigate whether curdlan could regulate the suppressive effect of MDSCs, we firstly detected the suppressive factors secreted by MDSCs, arginase and NO. As shown in Fig. 3a, b, the activity of arginase and the level of NO were strikingly decreased upon curdlan stimulation. However, the level of IL-12p35 was significantly enhanced in curdlan-treated MDSCs (Fig. 3c). Moreover, to directly determine the suppressive effect of MDSCs, we co-cultured curdlan-treated MDSCs with $CD4^+$ T cells or $CD8^+$ T cells to evaluate the ability of MDSCs in suppressing T cells. As shown in Fig. 3d, the extent of T cell proliferation in the curdlan-treated group was higher than in the control group, which suggests the suppression of MDSCs on T cells is down-regulated after curdlan stimulation. In addition, in the co-culture system, the production of IFN-γ released by T cells was also significantly higher in curdlan-treated MDSCs when compared to the control group (Fig. 3e). Together, all these data suggest that curdlan could down-regulate the suppressive capacity of MDSCs.

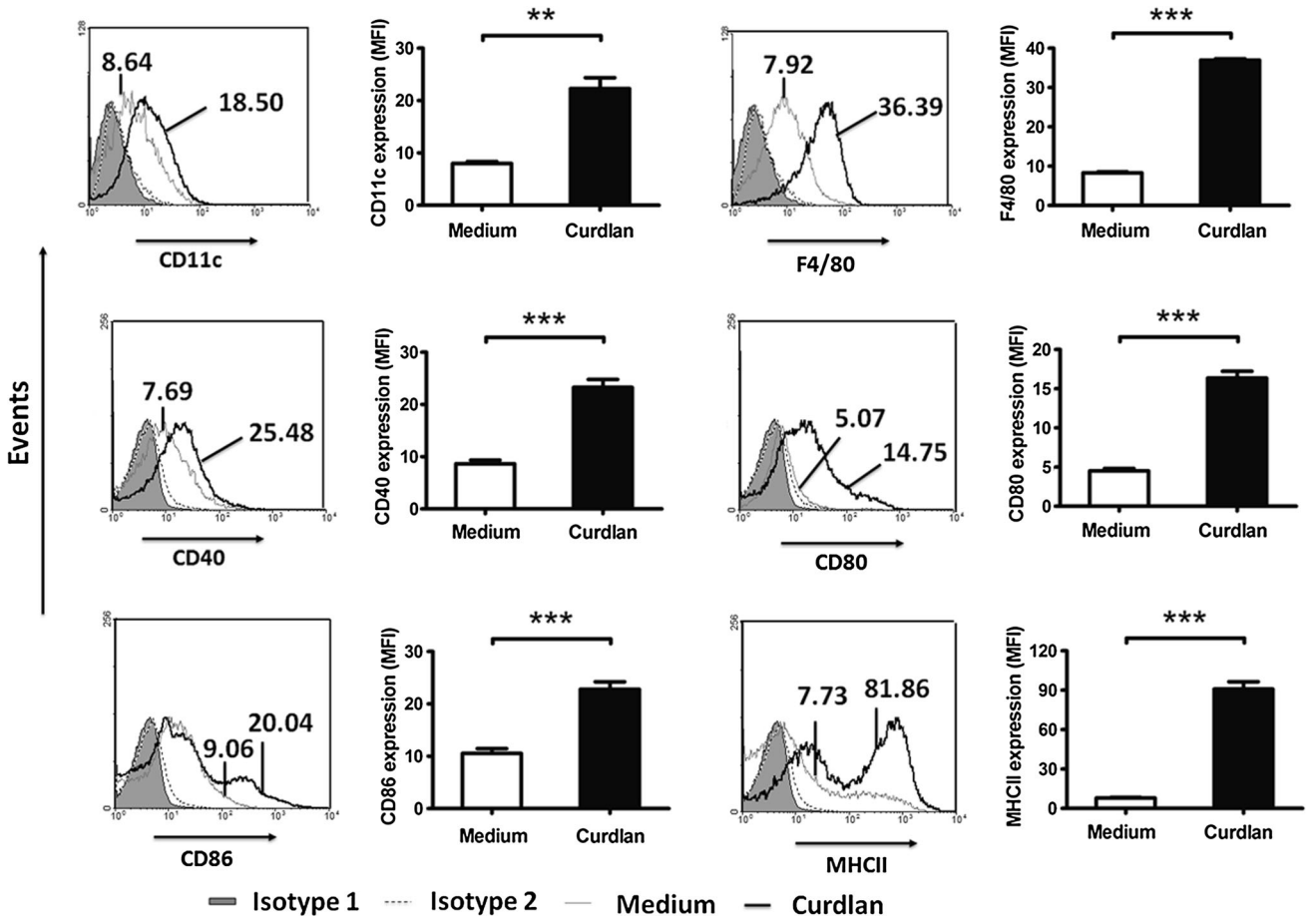



**Fig. 2** Curdlan promotes the differentiation of MDSCs. $3 \times 10^6$ LLC cells were injected s.c. into C57BL/6 mice to establish mouse Lewis lung carcinoma. MDSCs sorted from spleens of tumor-bearing mice were stimulated with or without curdlan (100 μg/ml) for 48 h, and then, cells were collected to detect the expression of CD11c, F4/80, CD40, CD80, CD86 and MHCII. The isotype 1 (*gray solid histogram*) is the isotype of medium control cells, and the isotype 2 (*dotted line histogram*) is the isotype of curdlan-treated MDSCs. Data are shown as the mean ± SD of three independent experiments. Student's *t* test was used for the statistical analysis. $***p < 0.001$, $**p < 0.01$, $*p < 0.05$

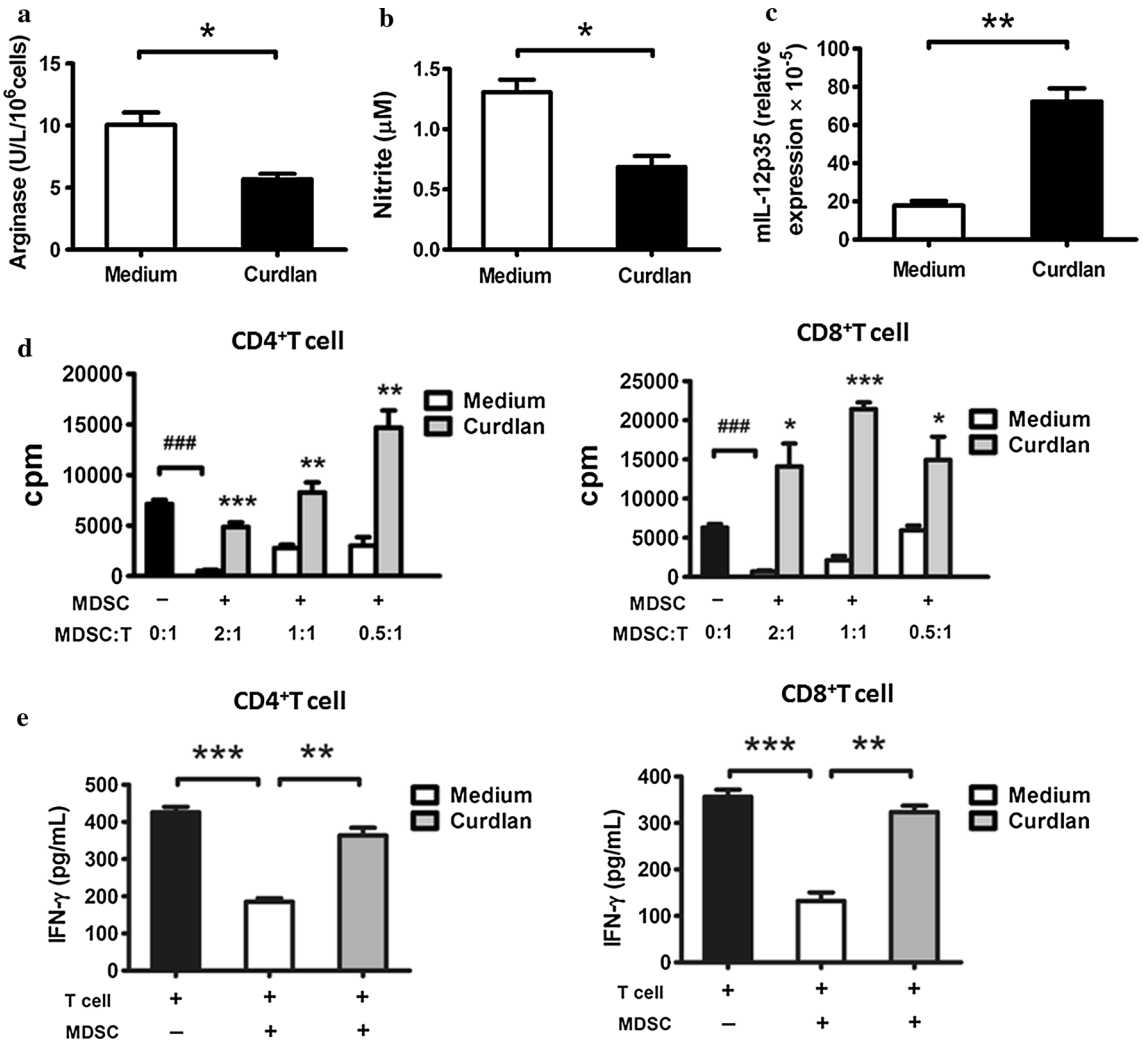
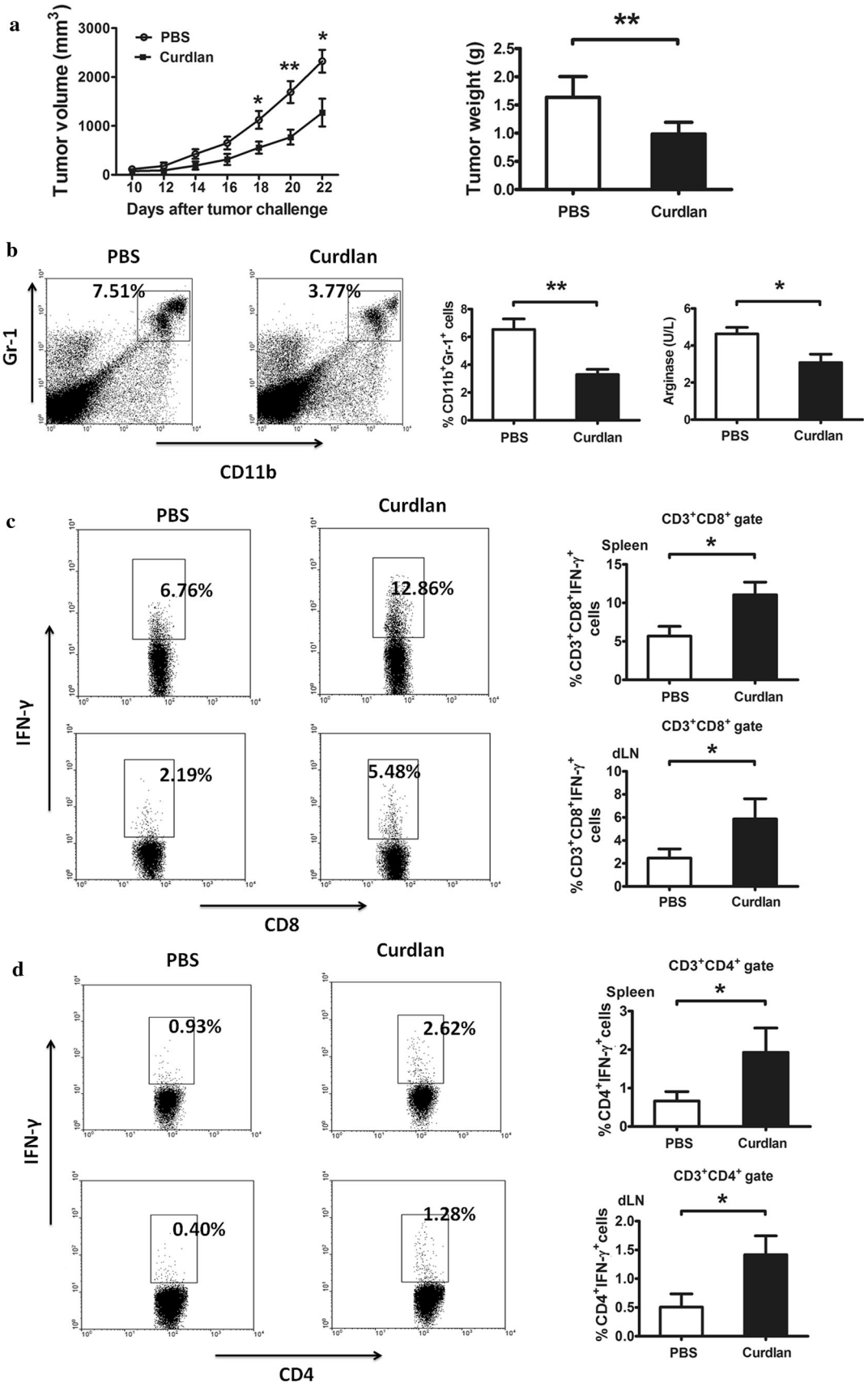



**Fig. 3** Curdlan impairs the suppressive capacity of MDSCs. MDSCs sorted from spleens of tumor-bearing mice were stimulated with or without curdlan (100 μg/ml) for 48 h. **a**, **b** Arginase activity and nitrites were analyzed as described in "Methods and materials" section. **c** IL-12p35 mRNA level was measured in MDSCs treated with or without curdlan. **d** MDSCs treated with or without curdlan were harvested to co-culture with $CD4^+$ T cells (*left*) or $CD8^+$ T cells (*right*) (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. **e** The level of IFN-γ level in the supernatants (MDSC: T cell ratio of 1:1) was evaluated using ELISA. Data are shown as the mean ± SD of three independent experiments. Student's *t* test was used for the statistical analysis. $^{\#\#\#}p < 0.001$ (T cells group versus medium group), $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ (curdlan group versus medium group)

## Curdlan delays tumor progression by reducing MDSCs and enhancing the CTL and Th1 responses in vivo

Having demonstrated that curdlan could down-regulate the suppressive effect of MDSCs in vitro, we next investigated the effect of curdlan in tumor-bearing mice. C57BL/6 mice were treated with PBS or curdlan for 1 week, and then, mice were challenged with LLC tumor cells. After tumor challenge, therapy was continuously administered for 3 weeks. As shown in Fig. 4a, tumor growth in the curdlan-treated group was significantly slower than in the PBS group, especially at day 18, day 20, day 22 after tumor challenge. In addition, the weight of tumors also decreased after curdlan treatment. These data demonstrate that curdlan can obviously slow the tumor progression. Next, we detected the proportion of $CD11b^+Gr\text{-}1^+$MDSCs in spleen and found that the percentage of MDSCs significantly decreased after curdlan treatment. Moreover, the arginase level in the serum of mice was also down-regulated (Fig. 4b), which suggests curdlan could reduce the MDSC-induced suppression in tumor-bearing mice. Then, we detected the $CD3^+CD8^+IFN\text{-}\gamma^+$ CTLs and $CD3^+CD4^+$ $IFN\text{-}\gamma^+$ Th1 cells in spleens and draining lymph nodes. Consistent with the in vitro data, we found that proportions of CTL and Th1 cell responses were significantly enhanced


a
Tumor volume (mm3)
PBS
Curdlan
Days after tumor challenge
Tumor weight (g)
b
PBS
Curdlan
7.51%
3.77%
Gr-1
CD11b
% CD11b+Gr-1+ cells
Arginase (U/L)
c
6.76%
12.86%
2.19%
5.48%
IFN-γ
CD8
CD3+CD8+ gate
Spleen
dLN
% CD3+CD8+IFN-γ+ cells
d
0.93%
2.62%
0.40%
1.28%
CD4
CD3+CD4+ gate
% CD4+IFN-γ+cells

◀**Fig. 4** Curdlan reduces MDSCs and delays the tumor development. C57BL/6 mice were treated with 200 μl curdlan (800 μg) or PBS every 2 days with an intragastric gavage needle for 7 days ($n = 6$). Then, mice were implanted subcutaneously (s.c.) with LLC cells ($3 \times 10^6$/mouse). After tumor challenge, therapy was continuously administered for 3 weeks. **a** Tumors were measured with a caliper at indicated time and weighed after sacrificing. **b** The proportion of $CD11b^+Gr\text{-}1^+$ MDSCs in spleen and the arginase level in the serum of tumor-bearing mice treated with or without curdlan were analyzed. Single-cell suspensions from spleens and draining lymph nodes (dLN) were collected to analyze the proportions of $CD3^+CD8^+IFN\text{-}\gamma^+$ CTLs (**c**) and $CD3^+CD4^+IFN\text{-}\gamma^+$ Th1 cells (**d**). Cells were gated on $CD3^+CD8^+$ or $CD3^+CD4^+$ T cells. Data are presented as mean ± SD. $^{**}p < 0.01$, $^{*}p < 0.05$

in both spleens and draining lymph nodes in tumor-bearing mice treated with curdlan (Fig. 4c, d). Taken together, curdlan could reduce the MDSC-induced suppression and augment the CD4 and CD8 T cell responses and finally delay the tumor progression.

## Discussion

Owing to the recent striking successes of cancer immunotherapy in human melanoma, human chronic lymphocytic leukemia, and some other tumor types [30–32], most researchers have recognized that immune suppression plays an essential role in promoting tumor development and contributes to the failure of cancer immunotherapy. Indeed, it has become increasingly clear that eliminating suppressive factors from the individual might be an effective cancer immunotherapy. As MDSCs are one of the prominent immunosuppressive elements in cancer, various therapeutic strategies that target these cells are now being explored, including promoting the differentiation of MDSCs, inhibition of MDSC function, inhibition of MDSC expansion as well as elimination of MDSCs. In this study, we have found that curdlan could promote the maturation of MDSCs and then reduce the suppressive effect and the number of MDSCs, and thereafter enhancing the anti-tumor T cell responses in tumor-bearing mice.

β-Glucans consisting of a β-(1,3)-linked main chain are known to be an immune stimulator [33, 34]. Administration of β-glucans derived from plenty of organisms, such as yeasts, mushrooms and plants, is shown to exhibit a variety of beneficial effects and is usually used as food supplements. However, from a medical point of view, the anti-tumor capability may be the most prominent effect of β-glucans. Indeed, β-glucan has been used as the main component of some anti-tumor drugs and already applied in cancer treatment, such as Lentinan [35], Krestin and Sizofiran [36]. In our study, we investigated the novel mechanism for the anti-tumor capacity of curdlan, the β-glucan derived from bacteria. Many previous studies have reported the application of curdlan in food [37]. Recently, accumulating researches have focused on the immunological effect of curdlan. In 1978, Sasaki et al. have already described the anti-tumor effect of curdlan. Actually, many previous studies have demonstrated different mechanisms for the anti-tumor property of various β-glucans or curdlan. Ding et al. [38] and Leibundgut-Landmann et al. [39] both demonstrated that glucan could activate the dendritic cells, promote the maturation of DCs and enhance cytokine production, which further elicits CTL responses. Wu et al. [40] also described the regulation of curdlan on DCs in tumor sites, enabling the DCs to produce IL-12p70 and favor the generation of Th1 cells and $CD103^+CD8^+$ mucosal T cells. Min et al. [41] found that curdlan has a synergistic effect with GM-CSF to promote the maturation of dendritic cells. In addition, Chiba et al. [42] demonstrated that dectin-1 expressed by DCs and macrophages could recognize *N*-glycan structures on tumor cells and signals to activate IRF5 pathway, thereby activating NK cells to kill tumor cells. Most of these studies have investigated the regulation of glucan on DCs or macrophages via dectin-1, and then, the activated DCs stimulate the innate or adaptive immune responses. However, in our study, we revealed the mechanism of anti-tumor capacity via the regulation of curdlan on immunosuppressive cells MDSCs, and we found that curdlan could impair the function of MDSCs. Although the specific mechanisms are various, the effects are consistent, and they all demonstrated that curdlan could enhance the anti-tumor immune responses by enhancing innate immunity or adaptive immunity, no matter via enhancing the activation of DCs or reducing the suppressive effect induced by MDSCs. During the regulation of β-glucans on immune cells, dectin-1 activation is an essential signaling pathway. Curdlan can activate NF-kB, mitogen-activated protein kinases and NFAT transcription factors via Src-/Syk-dependent pathway to activate dendritic cells, neutrophils and macrophages after upon activation of dectin-1 [43]. Xu et al. [44] demonstrated that dectin-1 could elicit $Ca^{2+}$ signaling and that PLCγ2 is a critical player in the dectin-1 signal transduction pathway. In addition, the triggering of dectin-1 by β-glucans has been reported to lead to Syk-dependent formation of the CARD9-Bcl-10-MALT1 scaffold that resulted in IL1B transcription and the activation of a MALT1–caspase-8–ASC complex that mediated the processing of pro-IL-1β [45]. Besides, curdlan is also reported to modulate the course of autoimmune diseases. Curdlan induces dendritic cell maturation and secretion of the proinflammatory cytokines including TNF-α, IL-6 and IL-23, which are important in the induction of Th17 cell differentiation, thus modulating the development of

experimental autoimmune encephalomyelitis [46–48]. Taken together, curdlan is a biological response modifier, which can modulate both innate and adaptive immune responses. In addition, it is reported that the structure and the source of β-glucans are associated with the bioactivity of β-glucans. Different structures and sources may determine different functions. Masuda et al. [49] have previously investigated the soluble β-glucan extracted from maitake named MD-Fraction, which has β-1, 6-main chain with β-1, 3-branches, could relieve the myelosuppression and enhance the generation of progenitor cells in bone marrow, and the increased GM-CSF, M-CSF could stimulate hematopoiesis in vivo and promote the maturation and differentiation of progenitor cells in bone marrow. However, our previous work has investigated the anti-tumor property of WGPs, which 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 [50]. We found that WGPs could directly promote the differentiation of splenic MDSCs through NF-κB pathway, thus effectively abrogating MDSC-associated immune suppression and improving anti-tumor responses [27]. Thereafter, we aim to investigate whether curdlan, the different sources of β-glucan with different structure, has the similar effect on the peripheral MDSCs. In this study, we found that the bacteria-derived β-glucan with β-1,3-linked D-glucose molecules also can modulate the suppressive capacity and the number of MDSCs and then delay the tumor progression.

In our study, we first found that curdlan could promote the maturation of MDSCs, and then probably leading to the impairment of the suppressive ability of MDSCs as well as the reduction in the undifferentiated MDSCs, then down-regulation of the suppressive effect in vivo, and therefore enhancing the anti-tumor immunity, and finally leading to the delay of tumor development. We reveal a novel mechanism of anti-tumor property of curdlan and suggest its clinical potential application. However, with the increasing demand for curdlan, chemical derivations of curdlan, such as its sulfated and carboxymethylated forms, rendering curdlan derivatives to be water-soluble, would increase the versatility of curdlan and thus expand the commercial market for curdlan.

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**Compliance with ethical standards**

**Conflict of interest** The authors have no financial conflicts of interest.

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