

Polysaccharide Krestin Is a Novel TLR2 Agonist that Mediates Inhibition of Tumor Growth via Stimulation of CD8 T Cells and NK Cells

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Abstract

Purpose: Polysaccharide krestin (PSK) is a mushroom extract that has been long used in Asia and recently in Western countries as a treatment for cancer due to its presumed immune potentiating effects. Although there have been reports of clinical responses after patients have ingested PSK, the mechanism of action of the agent remains undefined. The current study was undertaken to investigate the mechanism of the antitumor actions of PSK.

Experimental Design: The immunostimulatory effect of PSK was first evaluated *in vitro* using splenocytes from neu transgenic mice and Toll-like receptor (TLR) 2 knockout (TLR2^{-/-}) mice. Then the immunostimulatory and antitumor effect of PSK was determined using tumor-bearing neu transgenic mice, TLR2^{-/-}, and wild-type C57BL/6 mice.

Results: We demonstrate that PSK is a selective TLR2 agonist, and the activation of dendritic cells (DC) and T cells by PSK is dependent on TLR2. Oral administration of PSK in neu transgenic mice significantly inhibits breast cancer growth. Selective depletion of specific cell populations suggests that the antitumor effect of PSK is dependent on both CD8⁺ T cell and NK cells, but not CD4⁺ T cells. PSK does not inhibit tumor growth in TLR2^{-/-} mice suggesting that the antitumor effect is mediated by TLR2.

Conclusion: These results demonstrate that PSK, a natural product commonly used for the treatment of cancer, is a specific TLR2 agonist and has potent antitumor effects via stimulation of both innate and adaptive immune pathways. *Clin Cancer Res*; 17(1); 67-76. ©2010 AACR.

Introduction

In the United States, it is reported that 42% to 69% of people with cancer use both conventional and complementary and alternative medicine (CAM) to combat their disease (1, 2). Annual expenditures associated with use of CAM are estimated to be approximately \$13.7 billion (3). Mushroom extracts are among one of the most commonly used CAM therapies. Mushrooms are a popular cancer treatment in Asian countries for presumed immune potentiating and antitumor effects (4). Recently, a phase I/II trial of a polysaccharide extract from *Grifola frondosa* (Maitake mushroom) was performed in breast cancer patients in the United States and demonstrated that the oral intake of mushroom extract resulted in increased cytokine produc-

tion [interleukin (IL)-2, tumor necrosis factor (TNF)- α , and IL-10] by immune cells to more than 50% of baseline (5). However, the specific mechanism by which medicinal mushroom extracts modulate the immune system has not been defined.

Polysaccharide krestin (PSK) is one of the most commonly used mushroom extracts (6). It was approved as a prescription drug for the treatment of cancer in Japan in 1977. By 1987, PSK accounted for more than 25% of total national expenditure for anticancer agents in Japan (7). It is a commercially available, highly purified extract of *Trametes (Coriolus) versicolor* strain CM-101. The major component is β -glucan compounds ranging in size from 94 to 100 kDa (6). Clinical trials have suggested that PSK may significantly extend survival in cancers of the stomach (8, 9), colorectum (10-12), and lung (13). A number of publications suggest that PSK may be an immune modulator, inducing gene expression of IL-8 in peripheral blood mononuclear cells (PBMC) after oral administration (14), stimulating T-cell proliferation (15), and improving the function of CD4⁺ T cells in gut-associated lymphoid tissue (16). The specific receptor/pathway via which PSK might stimulate immune cells remains unknown.

The current study was undertaken to determine whether PSK could directly stimulate immune system cells, to assess the antitumor activity of PSK in a transgenic mouse model

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Translational Relevance

It has been reported that more than half of the cancer patients have tried both conventional and complementary and alternative medicine (CAM) to combat their disease; yet, the mechanism of CAM therapies remain poorly understood. In this study, we demonstrated that polysaccharide krestin (PSK), a highly purified mushroom extract, is a potent and selective Toll-like receptor (TLR) 2 agonist. PSK activates dendritic cells (DC) and T cells in a TLR2-dependent manner. *In vivo* administration of PSK in a mouse model of breast cancer has potent antitumor effect that is dependent on CD8⁺ T cells and NK cells. PSK is a natural product with a proven safety profile. Results from this study provide important information for future clinical trials testing the antitumor potential of this novel TLR2 agonist.

of breast cancer, and to identify the mechanism of action of the extract.

Materials and Methods

Animals

A colony of neu transgenic mice harboring the nontransforming rat neu [strain name, FVB/N-TgN (MMTVneu)-202Mul] was established in our animal facilities from breeding pairs (Jackson Laboratory) and maintained as previously described (17). Toll-like receptor (TLR) 2^{-/-} and TLR4^{-/-} mice were originally obtained from Dr. Shizuo Akira. Wild-type C57BL/6 mice were obtained from Jackson Laboratory. Mice were maintained under strict inbreeding conditions. All of the procedures were performed in compliance with the University of Washington Institutional Animal Care and Use Committee guidelines.

Reagents

Reagents used included fetal bovine serum (FBS; Gemini Bioproducts), RPMI 1640, phosphate-buffered saline (PBS), penicillin-streptomycin, and L-glutamine (Invitrogen Life Technologies), fluorochrome-conjugated monoclonal antibodies (mAb) targeting CD3, CD4, CD8, CD11c, NK1.1, Foxp3, and TLR2 (eBiosciences), [³H]-thymidine (PerkinElmer), RNAqueous4PCR kit for RNA extraction (Ambion), primers and probes and gene expression master mix for real-time PCR (Applied Biosystems). PSK was purchased from Kureha Pharmaceuticals. PSK was dissolved in PBS at a stock concentration of 10 mg/mL. Aliquots of 100 μ L were stored at -80°C. The frozen aliquots were thawed immediately before use.

PSK activation of splenocytes *in vitro*

The proliferation of splenocytes from neu transgenic mice after *in vitro* PSK treatment was evaluated using a [³H]-thymidine incorporation assay in 96-well plates as previously described (18). In brief, splenocytes were

cultured in 96-well plates (200,000 cells per well) in RPMI and incubated with PSK (1–200 μ g/mL) for 96 hours. [³H]-thymidine (1 μ Ci per well) was included for the last 16 hours of culture. For time-course treatment, splenocytes were stimulated with PSK (100 μ g/mL) for 24, 48, 72, or 96 hours. Cytokine levels in the culture supernatant were measured using a mouse Th1/Th2 cytokine kit according to the manufacturer's instructions (Meso Scale Discovery). To measure the percentage of each immune subset (CD4 and CD8 T cells, NK cells, and B cells), PSK or control PBS-treated splenocytes were stained with fluorochrome-conjugated anti-CD4, CD8, NK1.1, and CD19 antibodies using standard procedures as previously described (17). To measure PSK-stimulated IFN- γ production from NK cells, splenocytes from TLR2^{-/-} mice or wild-type (WT) C57BL/6 mice were treated with PSK (100 μ g/mL) for 24 hours. Intracellular staining was used to evaluate IFN- γ production by NK cells, using similar method as described in our previous publication (19). To measure the expression of TLR2 on each immune subset, splenocytes were stained with anti-TLR2-PE mAb (eBiosciences) following the manufacturer's instructions and the cells were costained with mAbs against CD3, NK1.1, CD19, and CD11c. The expression of TLR2 on mouse mammary carcinoma (MMC) tumor cells was measured using similar method.

PSK activation of dendritic cell *in vitro*

Cells were collected from the bone marrow of femurs of mice under aseptic conditions and cultured in 24-well plates in culture medium (RPMI with 10% FBS, 50 μ mol/L of β -mercaptoethanol, and penicillin/streptomycin) after lysing red blood cells. Recombinant mouse IL-4 and GM-CSF (granulocyte macrophage colony stimulating factor) was included at a final concentration of 20 ng/mL after removal of nonadherent cells. On day 7, cells were split and cultured in the presence of PSK (200 μ g/mL), lipopolysaccharide (LPS; 100 ng/mL, positive control), or PBS (negative control). After a 48-hour treatment, culture supernatant from each well was collected for analysis of IL-12p40 and IL-12p70 using ELISA kits (eBiosciences). The adherent cells were detached and 1 million cells per well were stained with anti-CD11c-APC, anti-CD80-PE, and anti-CD86-PerCP, and analyzed on a FACS Canto analyzer. FACS data analysis was performed using FlowJo software (Tree Star).

Assessment of TLR activation using HEK293 cell transfectants

Human embryonic kidney cells (HEK293) expressing TLR2, TLR3, TLR4, TLR5, TLR7, TLR8, or TLR9 (InvivoGen) were cultured in DMEM (Cambrex) containing 4.5 g/L of L-glucose (Sigma-Aldrich) and 10% FBS. To measure NF- κ B activation, the TLR-transfected HEK cells were cotransfected with a plasmid that has NF- κ B inducible reporter construct secretory alkaline phosphatase (SEAP) activity. The cells were then incubated with different doses of PSK (0.5–1,000 μ g/mL) in 1:3 dilution or with serial dilutions of the

known agonist for each TLR as a positive control [heat-killed listeria monocytogenes (HKLM) for TLR2, poly (I:C) for TLR3, LPS for TLR4, flagellin for TLR5, resiquimod for TLR7 and 8, CpG ODN2006 for TLR9]. After overnight incubation, the culture supernatant was collected and the SEAP activity in the supernatant was measured using a Quanti-blue assay (InvivoGen).

Treatment of tumor-bearing mice with oral PSK

The antitumor effect of PSK was evaluated in neu transgenic mice in both implanted tumor and spontaneous tumor setting, and in TLR2^{-/-} and WT C57BL/6 mice with implanted tumors. For implanted tumor in the neu transgenic mice, 1 million MMC cells, a syngeneic tumor cell line derived from a spontaneous tumor in these mice (18), was injected to the mice subcutaneously. PSK treatment was started 2 weeks after the implantation when the implanted tumor just became palpable (average size = 50 mm³). For spontaneous tumors, neu transgenic mice with palpable tumors (average size = 50 mm³) were randomly assigned to be treated with PSK (2 mg per mouse; equivalent to 100 mg/kg, 3 times per week for 4 weeks) or control PBS (*n* = 5 per group). For implanted tumors in C57BL/6 and TLR2^{-/-} mice, the tumor was started by subcutaneous injection of 40,000 TC-1 cervical cancer cells (C57BL/6 background; originally obtained from ATCC). PSK or control PBS treatment was started 10 days after the injection when tumors just became palpable. PSK was dissolved in PBS and given via oral gavage in a 200- μ L volume. Mice in the control group received oral gavage of PBS of the same volume. Tumors were measured every other day with vernier calipers and tumor volume was calculated as the product of length \times width \times height \times 0.5236. *In vivo* data are presented as mean \pm SEM. In some experiments, mice were depleted of specific lymphocytes (CD4⁺, CD8⁺ T cells, or NK cells) during PSK treatment using standard procedure as described in our previous publication (19).

Evaluation of IL-12 production by DC using intracellular cytokine staining

Mesenteric and tumor-draining lymph nodes (TDLN) were collected from control or PSK-treated mice. The LNs were minced over a 70- μ m cell strainer and washed in RPMI. Then, the LN cells were stimulated with phorbol myristate acetate (50 ng/mL) and ionomycin (1 μ mol/L) for 16 hours in the presence of brefeldin A (10 μ g/mL) before staining, as has been previously described (20). Cells were first stained with antibodies against surface antigen CD11c, then fixed and permeabilized with Perm/Fix solution (eBioscience). The cells were then stained with anti-IL-12-PE (clone 17.8; eBioscience). Samples were acquired on a FACS Canto analyzer and data were analyzed using FlowJo.

Measuring tumor-specific T cells using enzyme-linked immunospot (ELISPOT) assay

IFN- γ ELISPOT assay was used to determine the frequencies of tumor-specific T cells in splenocytes from PSK

and control PBS-treated mice using methods similarly as previously described (21). Splenocytes (200,000 cells per well) were stimulated in 6-well replicates using irradiated syngeneic tumor cells (MMC) or ConA (10 ng/mL) as positive control. The plates were cultured at 37°C in 5% CO₂ for 2 days. On day 3, the cells were removed and addition of secondary antibody and plate development were carried out similarly as previously described (21). Dried plates were read on an ELISPOT reader (Cell Technology).

Evaluation of NK lytic activity using flow cytometry

Splenocytes from PSK or control PBS-treated mice were isolated and tested for NK-cell lytic activity against NK-sensitive yeast artificial chromosome (YAC)-1 tumor target cells by a standard flow cytometry assay. YAC-1 cells were labeled with 3,3'-dioctadecyloxacarbocyanine [DiOC₁₈(3); Sigma] and then cultured for 4 hours with splenocytes from PSK or PBS-treated mice in triplicate wells at 3 different effector to target (E:T) ratios (50:1, 25:1, and 12.5:1). At the end of 4 hours of incubation, cells were harvested and stained with propidium iodide (PI) and washed. Dead target cells were detected as PI⁺DiOC₃⁺ cells by flow cytometry. NK-cell activity, defined as percent cytotoxicity of each sample, was calculated at each E:T ratio using the following formula: (% PI⁺DiOC⁺ cells)_{splenocytes+YAC-1} - (% PI⁺DiOC⁺ cells)_{YAC-1 alone}.

Lytic units (LU) values were extrapolated from dose-response curves of percent-specific lysis versus log E:T ratio for each blood sample assayed using a software program provided by Dr. Theresa L. Whiteside, University of Pittsburgh School of Medicine (22, 23). Lytic units of NK-cell activity, defined as the number of cells required to cause 20% target cell lysis relative to 10⁷ effector cells, were determined by the equation: 10⁷/LU₂₀.

Real-time reverse transcription PCR

Total RNA from PSK or control PBS-treated tumors was isolated using RNA4Aqueous kit (Ambion). The integrity of RNA was tested using an Agilent BioAnalyzer. Reverse transcription and real-time PCR analysis was done similarly as previously described (17). Primer and probes for CD4 and CD8 were purchased from Applied Biosystems. Data analysis was performed using SDS 2.21 (Applied Biosystems). The mRNA expression level of the target gene was normalized to β -actin using the ΔC_T method. Level of expression = 2^{- ΔC_T} , where $\Delta C_T = C_{\text{Target gene}} - C_{\text{Tactin}}$. C_T is the cycle threshold at which the fluorescence signal crosses an arbitrary value.

Statistical analysis

Statistical analysis was performed using GraphPad (GraphPad Software). Data were analyzed using the 2-tailed Student's *t* test or ANOVA. A value of *P* < 0.05 was considered statistically significant.

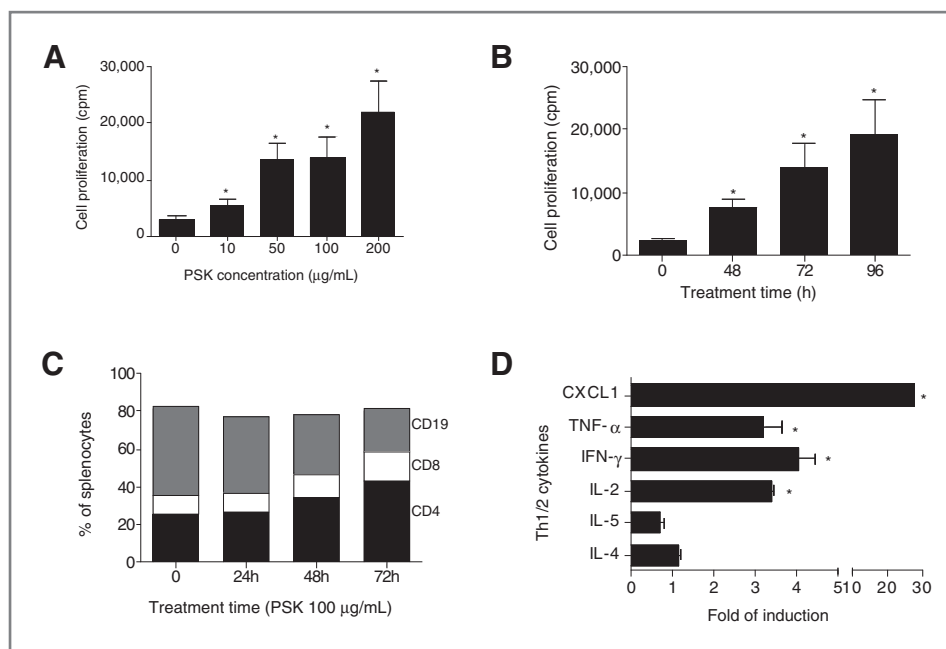


Figure 1. PSK stimulates splenocyte proliferation and secretion of Th1 cytokines. A, dose of PSK on the horizontal axis and cell proliferation in cpm on the vertical axis. *, values significantly different from control, $P < 0.05$. The bars represent the mean \pm SEM of triplicate wells in each treatment group. Similar results were observed from 2 independent experiments. B, time course of PSK-stimulated splenocyte proliferation. The bars represent the mean \pm SEM of triplicate wells in each treatment group. *, significantly different from control, $P < 0.05$. Similar results were observed from more than 2 independent experiments. C, percentages of CD4⁺ (black bar), CD8⁺ (white bar), and CD19⁺ cells (gray bar) among total splenocytes at various treatment times (x-axis). Values shown are representative of 3 replicates. D, the columns represent the fold of induction in PSK-treated samples over untreated control for each cytokine. Shown is the mean \pm SEM of duplicate wells in each treatment group. The experiment was repeated 3 times with similar results. *, $P < 0.05$.

Results

PSK treatment stimulates T-cell proliferation and Th1 cytokine secretion and induces DC maturation and IL-12 secretion

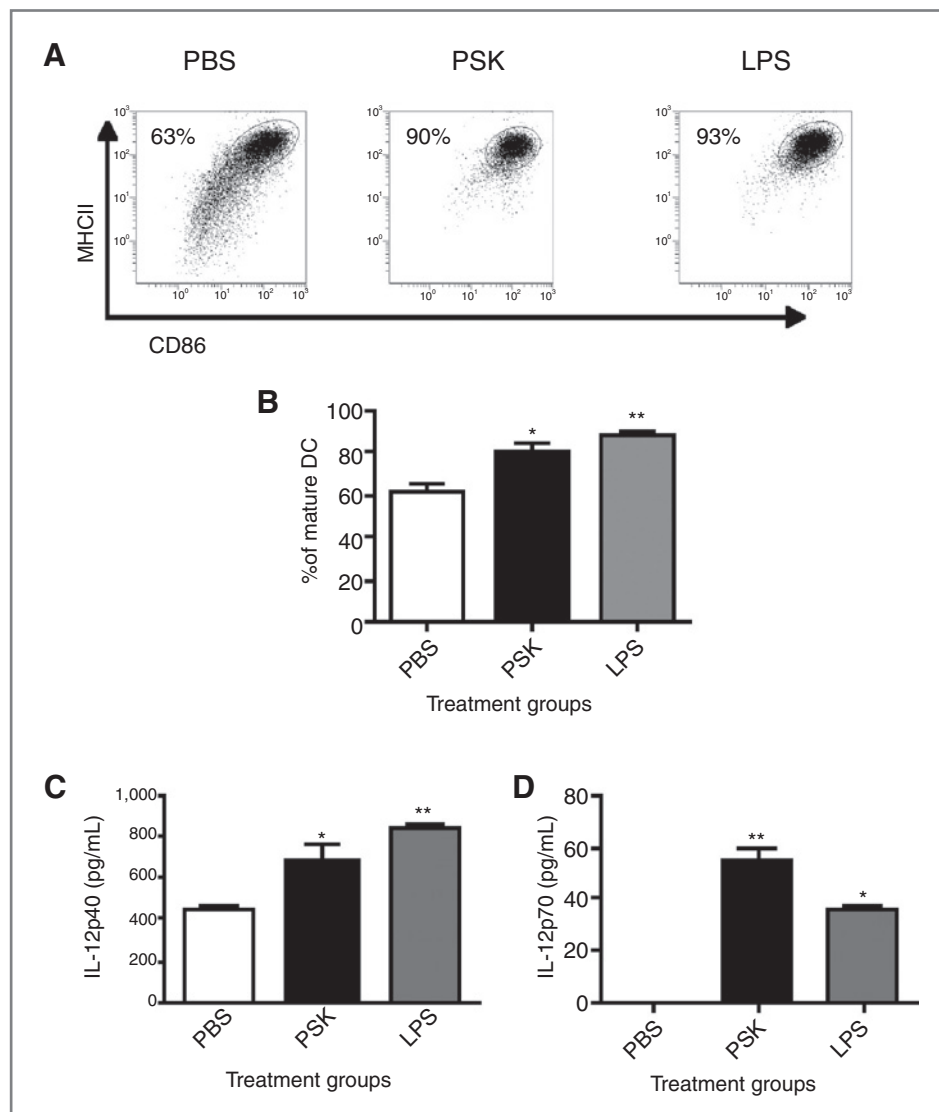
PSK treatment (10–200 $\mu\text{g/mL}$, 48–96 hours) significantly stimulated the proliferation of splenocytes in a dose- (Fig. 1A) and time-dependent manner (Fig. 1B). PSK treatment increased the percentages of CD4⁺ ($25.5 \pm 1.5\%$ in control vs. $43.2 \pm 3.9\%$ after 72 hours PSK at 100 $\mu\text{g/mL}$; $P = 0.01$) and CD8⁺ T cells ($9.9 \pm 0.5\%$ vs. $15.5 \pm 0.5\%$; $P = 0.0015$) among total splenocytes and reduced the percentage of B cells ($46.8 \pm 0.6\%$ vs. $22.4 \pm 1.9\%$; $P = 0.0003$; Fig. 1C). B-cell number also decreased, consistent with previous publication that PSK inhibits B-cell growth (24). PSK treatment (10–200 $\mu\text{g/mL}$, 48–96 hours) also induced secretion of Th1 cytokines in a dose- and time-dependent manner. After 96 hours of PSK treatment (100 $\mu\text{g/mL}$), the level of IFN- γ was increased by 4.03 ± 0.41 -fold ($P = 0.001$ from control). TNF- α was induced by 3.21 ± 0.44 -fold ($P = 0.0043$ from control). IL-2 was induced by 3.40 ± 0.06 -fold ($P = 0.0002$ from control). The levels of IL-4 and IL-5 were no different from controls ($P = 0.22$ and 0.11 , respectively; Fig. 1D). None of the innate immune subsets we tested [monocytes (CD14⁺,

macrophage (F4/80⁺), and DC (CD11c⁺)] were increased among total splenocytes after *in vitro* PSK treatment ($P > 0.05$ as compared with control). Although the number of CD11c⁺ cells was not increased after PSK treatment, we questioned whether the phenotype and functional capacity of DC was impacted by PSK. Using bone marrow-derived dendritic cells (BMDC), we found that PSK treatment (200 $\mu\text{g/mL}$, 48 hours) resulted in increased percentage of mature DC that are CD86⁺MHCII^{high} ($62.3 \pm 3.4\%$ in PBS vs. $80.1 \pm 5.0\%$ in PSK group; $P = 0.04$; Fig. 2A and B). PSK-treated DC also secreted significantly greater levels of IL-12p40 (441 ± 24 pg/mL in PBS group vs. 689 ± 78 pg/mL in PSK group; $P = 0.03$; Fig. 2C), and IL-12p70 (0.4 ± 0.1 pg/mL in PBS vs. 55.3 ± 3.6 pg/mL in PSK group; $P < 0.001$) than controls (Fig. 2 D).

PSK is a selective TLR2 agonist and the type I inflammatory response induced by PSK is dependent on TLR2 activation

It has been shown that fungal pathogens can activate TLRs (25), so we questioned whether PSK, which is a fungal product, may induce type I immunity via TLR activation. PSK specifically activates TLR2 in a dose-dependent manner and demonstrated no activity against TLR3, TLR4, TLR5, TLR7, TLR8, or TLR9 (Fig. 3A and B

Figure 2. *In vitro* PSK treatment activates DC maturation and secretion of IL-12. A, representative flow graphs of CD11c⁺ DC from PBS-, PSK-, and LPS-treated BMDC stained with anti-CD86 and anti-MHCII. The number in each graph indicates the percentage of mature DC (CD86⁺MHCII^{high}) in each sample. B, the columns represents mean \pm SEM of the percentage of mature DC in BMDC treated with PBS (white column), PSK (black column), and LPS (gray column); *, $P < 0.05$; **, $P < 0.01$. Similar results were observed in 3 independent experiments. C and D, the columns represents mean \pm SEM of the levels of IL-12p40 and IL-12p70 in culture supernatant of BMDC treated with PBS (white column), PSK (black column), and LPS (gray column); *, $P < 0.05$; **, $P < 0.01$. Similar results were observed in 3 independent experiments.



and Supplementary Fig. 1). Evaluation of TLR2 expression in different subsets of splenocytes from neu transgenic mice showed that TLR2 is expressed at nondetectable or very low levels in CD3⁺ T cells and NK1.1⁺ NK cells. The expression was detectable in CD19⁺ B cells and highly expressed in CD11c⁺ DC (Fig. 3C). TLR2 was not expressed in tumor cells from neu transgenic mice (data not shown). Preincubation with an anti-TLR2 mAb but not an anti-TLR4 mAb inhibited PSK-induced IL-12p40 production by BMDC ($P < 0.0001$; Fig. 3D). BMDC from TLR2^{-/-} mice, when stimulated with PSK, secreted significantly less IL-12p40 compared with BMDC from WT or TLR4^{-/-} mice (254 ± 8 pg/mL vs. 787 ± 21 pg/mL and 790 ± 10 pg/mL; $P < 0.0001$; Fig. 3E). Similarly, splenocytes from TLR2^{-/-} mice did not secrete TNF- α upon PSK stimulation (Supplementary Fig. 2). *In vitro* PSK stimu-

lates NK cells from WT mice but not from TLR2^{-/-} mice to secrete IFN- γ (Fig. 3F).

Oral PSK administration significantly inhibits the growth of both implanted and spontaneous breast tumors in neu transgenic mice

PSK was administered via oral gavage to neu transgenic mice bearing implanted or spontaneous tumors. As shown in Figure 4, PSK treatment significantly inhibits the growth of both the tumors. In the implant model, the tumor size after 3 weeks of treatment was 574 ± 26 mm³ in the PSK group and $1,174 \pm 41$ mm³ in the PBS group ($P < 0.0001$). In the spontaneous tumor setting, the tumor size after 3 weeks of treatment was 95 ± 39 mm³ in PSK group and 825 ± 154 mm³ in PBS group ($P = 0.0006$). We have previously reported that spontaneous tumors are different

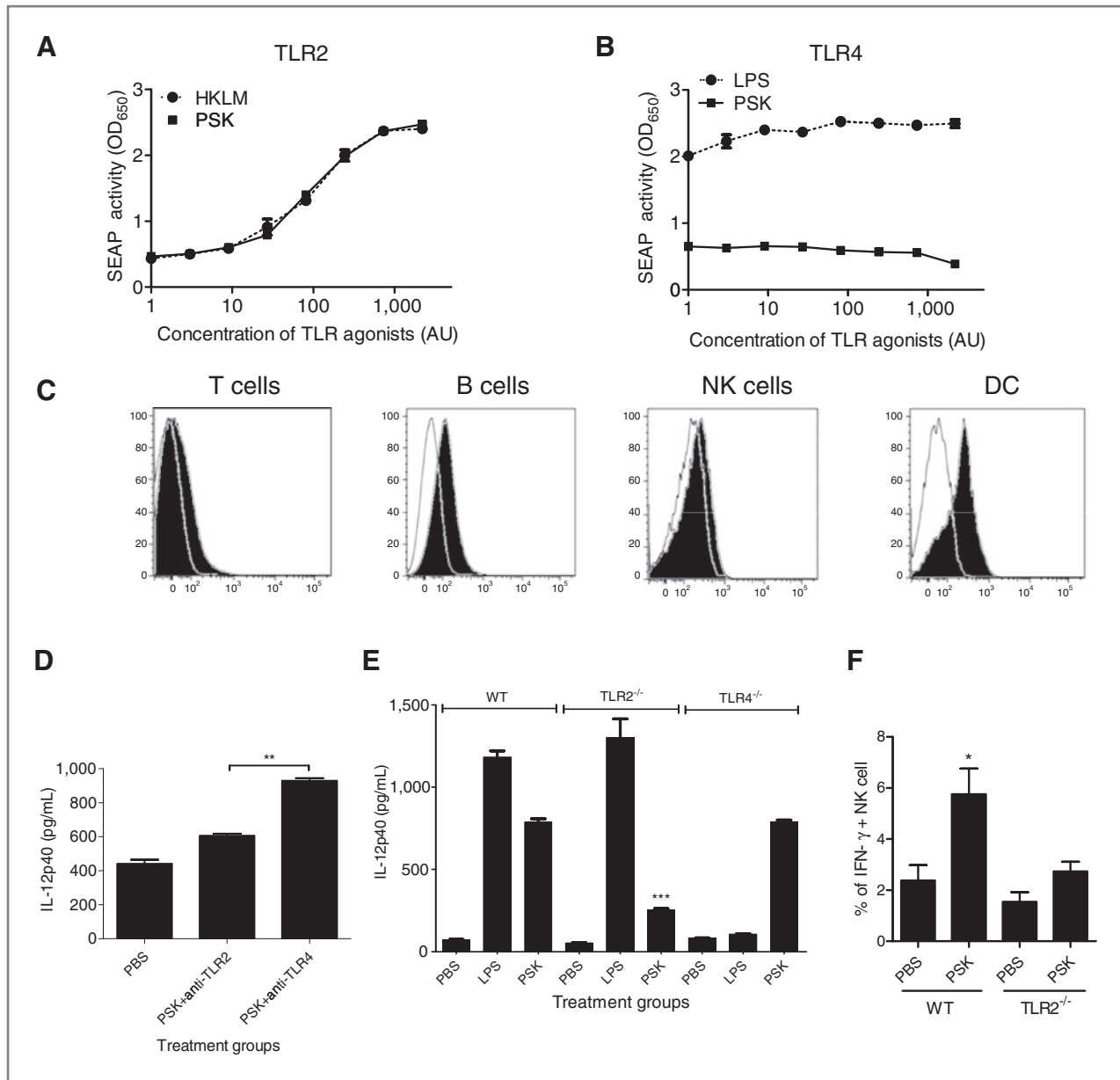


Figure 3. PSK selectively activates TLR2 and the stimulatory effect of PSK on DC is dependent on TLR2 activation. A and B shown are SEAP activity (y-axis) in the culture supernatant of TLR2- and TLR4-transfected HEK-293 cells when stimulated with serial dilutions of PSK (0.5–1,500 μg/mL, solid line with ■) or the positive control TLR agonists (HKLM for TLR2, and LPS for TLR4, shown as dotted line with ●). Each data point represents the mean ± SEM of triplicate culture wells at each concentration. Arbitrary units (AU): PSK 1 unit = 0.5 μg/mL; HKLM 1 unit = 4,500 cells; LPS 1 unit = 0.05 ng/mL. Similar results were obtained from more than 3 experiments. C, histograms showing the expression of TLR2 in T cells, B cells, NK cells, and DC from neu transgenic mice. The filled histogram represents cells stained with anti-TLR2-PE; the unfilled histogram represents cells stained with an isotype control antibody. Experiments were repeated twice with similar results. D, IL-12p40 levels (mean ± SEM) in BMDC isolated from neu transgenic mice pretreated with anti-TLR2 or anti-TLR4 monoclonal antibody (10 μg/mL) for 1 hour prior to PSK treatment (100 μg/mL for 48 hours). **, *P* < 0.01. E, the columns represent average IL-12p40 levels (mean ± SEM) in PBS-, PSK-, or LPS-treated DC from WT, TLR2^{-/-}, or TLR4^{-/-} mice. ***, *P* < 0.001 compared with PSK treatment group in WT. Experiments were repeated twice with similar results. F, percentages of IFN-γ⁺ NK cells in splenocytes from WT and TLR2^{-/-} mice treated with PSK (100 μg/mL, 24 hours) or control PBS. *, *P* < 0.05 from PBS control. Results are representative of 3 independent measurements.

from implanted tumors in these neu transgenic mice because spontaneous tumors grow slowly, have more infiltrating immune cells, are more immunogenic, and usually responded better to immunotherapy (17, 19). However, as

the mice do not develop spontaneous tumors until approximately 5 months old, the implant model is still frequently used, especially when large number of mice is required.

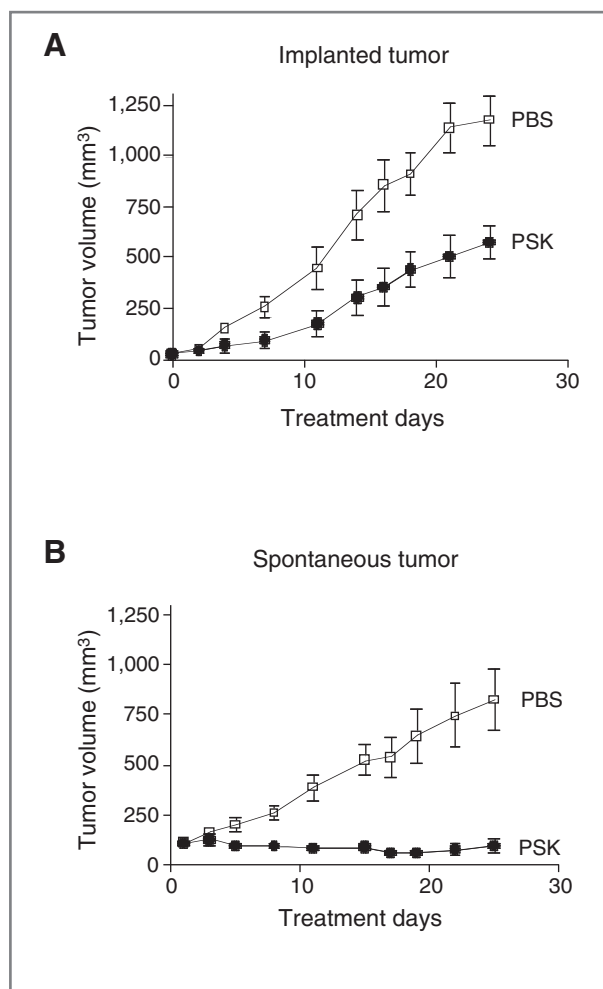


Figure 4. Oral PSK inhibits the growth of implanted and spontaneous breast tumors in neu transgenic mice. Shown are tumor growth curves in mice bearing implanted tumor (A), or spontaneous tumors (B), treated with PSK (●) or control PBS (□). Each data point represents the average tumor size (mean \pm SEM) in each group ($n = 14$ per group for implanted tumor and $n = 13$ per group for spontaneous tumor). Results shown are representative of 3 independent experiments.

Oral PSK administration augments both T-cell and NK-cell activity and the antitumor effect of PSK is dependent on CD8⁺ T cells and NK cells and is mediated by TLR2

Oral PSK induced IL-12 secretion from DC in both local mesenteric LN (mLN; $1.3 \pm 0.3\%$ vs. $3.5 \pm 0.9\%$ IL-12⁺ DC; $P = 0.03$) and DC in TDLNs ($0.4 \pm 0.2\%$ vs. $2.3 \pm 0.6\%$ IL-12⁺ DC; $P = 0.04$). Systemically, PSK not only increased the percentages of CD4⁺ ($28.1 \pm 2.5\%$ vs. $36.6 \pm 2.7\%$; $P = 0.04$) and CD8⁺ T cells ($7.5 \pm 0.6\%$ vs. $9.6 \pm 0.4\%$; $P = 0.02$) among total splenocytes *in vivo*, but augmented the number of tumor-specific T cells (37 ± 21 vs. 160 ± 32 tumor-specific T cells per million splenocytes; $P = 0.03$; Fig. 5A). In addition to T cells, PSK-treated mice also demonstrate augmented NK-cell activity as evidenced by increased lysing of YAC-1 tumor cells (1.6 ± 0.5

LU vs. 9.8 ± 2.0 LU; $P = 0.01$; Fig. 5B). Evaluation of tumor-infiltrating lymphocytes (TIL) showed a significantly increased ratio of CD8/CD4 T cells at both the mRNA and protein level in PSK-treated mice ($P = 0.014$ by RT-PCR and $P = 0.04$ by FACS as compared with control; Fig. 5C and D). The percentage of CD4⁺Foxp3⁺ regulatory T cells among total TIL decreased from $2.73 \pm 0.60\%$ to $1.02 \pm 0.16\%$ in PSK-treated mice ($P = 0.01$; Fig. 5E). To determine the role of different immune subsets in the antitumor effect of PSK, we selectively depleted CD4⁺, CD8⁺ T cells or NK cells during PSK treatment. As shown in Figure 6A, selective depletion of CD8⁺ T cells and NK cells, but not CD4⁺ T cells, significantly inhibited the antitumor effect of PSK in mice with implanted breast tumors. Both NK cells and CD8 T cells are required, as depletion of both sets during PSK treatment resulted in larger tumor than depleting either set alone (data not shown). To investigate whether the antitumor effect of PSK is mediated by TLR2 activation, we implanted the same amount of TC-1 tumor cells into TLR2 knockout mice and WT C57BL/6 mice. Then we treated the tumor-bearing TLR2^{-/-} mice or WT mice with oral PSK or control PBS. As shown in Figure 6B, PSK significantly inhibited tumor growth in WT mice ($P < 0.0001$ between PBS and PSK group), but not in TLR2^{-/-} mice ($P = 0.5$ between PBS and PSK group), indicating that TLR2 is critical in mediating the antitumor effect of PSK.

Discussion

In the current study, we have demonstrated that PSK is a potent and selective TLR2 agonist. Moreover, PSK treatment induces type I T cells potentially via its effect on the DC phenotype. Finally, the antitumor effects observed with PSK treatment are dependent on both T-cell and NK-cell activity.

Data presented here are the first evidence that PSK selectively activates TLR2. TLRs are a family of evolutionarily conserved pathogen recognition receptors that play a pivotal role in host defense by regulating both innate and adaptive immune responses (26, 27). TLR agonists have been tested intensively in tumor immunotherapy in recent years, and almost all of the studies exploring the antitumor effect of TLR agonists have focused on the ligands for TLR3, TLR7, TLR8, and TLR9 (28–30). TLR2 is a transmembrane protein receptor and can form a heterodimer with TLR1 or TLR6. The expression of TLR2 on T cells is shown to be upregulated following T-cell activation and can act as a costimulatory receptor (31). Recently, it has been found that this costimulation via TLR2 is more responsible for proliferation and survival of CD8⁺ T cells than for that of CD4⁺ T cells (32). It has also been reported that TLR2 engagement on CD8⁺ T cells enables generation of functional memory cells in response to a suboptimal TCR (T-cell receptor) signal, such as that seen against self-tumor antigens (33). Moreover, engagement of TLR1/2 on ova-specific OT-1 CTLs increased cell proliferation and the expression of various effector molecules on T cells (34). These previous reports concerning TLR2 activation are

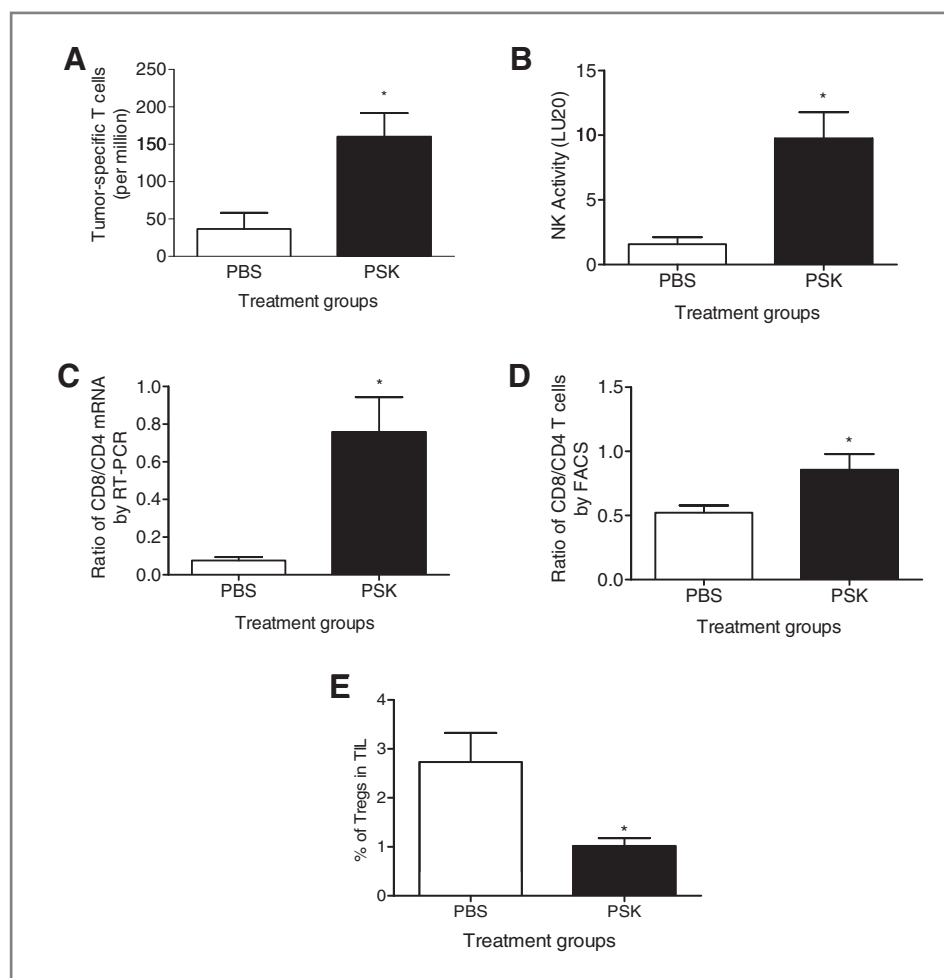


Figure 5. Oral PSK augments T cells and NK cells in spleen of PSK-treated mice and increases CD8/CD4 ratio in TIL. A, numbers of IFN- γ secreting tumor-specific T cells (mean \pm SEM) per million splenocytes in PBS- (white column) or PSK (black column)-treated mice ($n = 3$ per group). B, NK activity (LU₂₀) in splenocytes isolated from PBS- (white column) or PSK (black column)-treated mice ($n = 4$ –5 per group). C, the ratio of CD8/CD4 mRNA expression in tumors from PBS- (white column) or PSK (black column)-treated mice ($n = 4$ –5 per group). D, the ratio of CD8⁺/CD4⁺ T cells in TIL from PBS- (white column) or PSK (black column)-treated mice ($n = 4$ per group). E, the percentage of regulatory T cells (Treg; CD4⁺Foxp3⁺) among total TIL in PBS- (white column) or PSK (black column)-treated mice ($n = 5$ –6 per group). *, $P < 0.05$. All results were repeated in 2 independent experiments.

consistent with our finding that the ratio of CD8/CD4 in TIL increased in PSK-treated mice (Fig. 5) and CD8⁺ T cells, together with NK cells, mediate the antitumor effect of PSK. In addition to the effect on CD8⁺ T cells, recent literature suggests that TLR2 can abolish the suppressive capacity of regulatory T cells or make effector T cells resistant to the suppression of regulatory T cells (35, 36). We observed a decrease in regulatory T cells in tumor after PSK treatment that could also have contributed to the antitumor effect of PSK.

The finding that TLR2 is minimally expressed on T cells but highly expressed on DC (Fig. 3C) suggests that the effect of PSK on T cells could be indirect via activation of DC, secretion of IL-12, and the polarizing of a tumor-specific Th1 response. The ability of TLR agonists to augment antitumor immunity via stimulation of DC has been well described (37–39). For example, it has been shown that TLR8-primed DC can generate high-avidity antitumor T cells via IL-12 production (37). Ligation of TLR9 by CpG converts tolerogenic DC into antigen-presenting cells capable of stimulating antitumor immunity via activating Th1/

Th17 and NK-cell response (38). Our results suggest the potential of using PSK, a natural product with a demonstrated favorable safety profile, to augment antitumor immunity via stimulating DC. A recent study showed that a combination of 3 TLR ligands (TLR2/6, TLR3, and TLR9) greatly enhanced IL-15 production from DC and increased the generation of high-avidity T cells after vaccination (40). Whether combining PSK with other TLR agonists will have enhanced antitumor effect remains to be investigated.

NK cells are also activated by PSK treatment and play an important role in the antitumor activity of PSK. It is noted that TLR2 expression on NK cells is very low, although significantly higher than the expression on T cells. Thus, the question remains whether the activation of NK cells by PSK is via direct activation of TLR2 on NK cells or indirect via activation of antigen-presenting cells. It is reported that NK-cell priming requires the presence of CD11c^{high} DC (41). However, direct TLR2 signaling on NK cells has also been reported (42). The crosstalk between NK cells and DC has also been well described (43, 44). The finding that NK cells are activated by PSK indicates the potential of using this

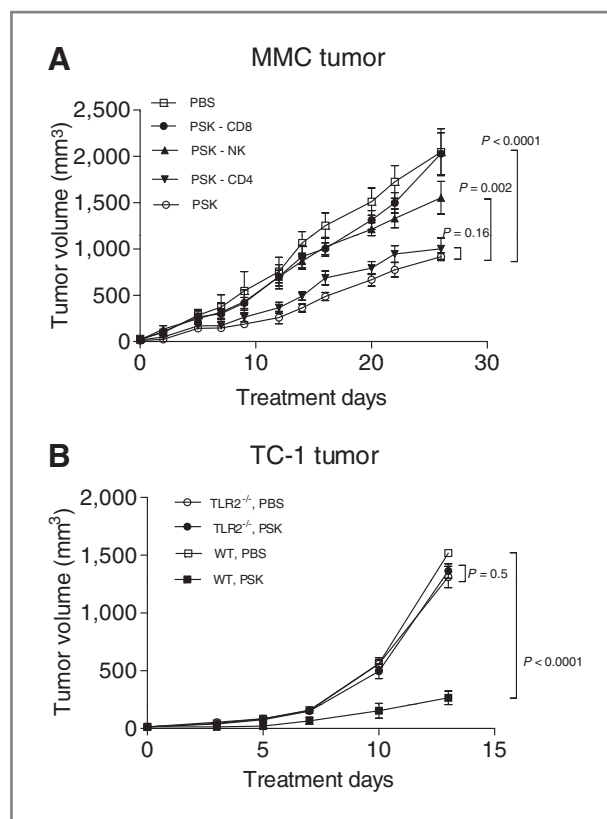


Figure 6. The antitumor effect of PSK is dependent on CD8⁺ T cells and NK cells and is mediated by TLR2. A, selective depletion of CD8⁺ T cells or NK cells but not CD4⁺ T cells abolished the antitumor effect of PSK. Shown are the growth curves of implanted MMC tumors in neu transgenic mice having received PBS (□), PSK (○), PSK with CD4 depletion (▼), PSK with CD8 depletion (●), or PSK with NK depletion (▲). Data represent 5 mice in each treatment group. Similar results were observed in 2 independent experiments. B, PSK inhibits the growth of implanted TC-1 tumors in WT C57BL/6 mice but not in TLR2^{-/-} mice. Shown are the growth curves of implanted TC-1 tumors in WT mice receiving PBS (□), WT mice receiving PSK (■), TLR2^{-/-} mice receiving PBS (○), and TLR2^{-/-} mice receiving PSK (●). *n* = 5 in each group.

natural product to enhance the therapeutic effect of human epidermal growth factor receptor 2 (HER2)-targeted mAb therapy by trastuzumab, which is currently a standard of care for patients with HER2 overexpressing breast cancer.

The major mechanism of the antitumor effect of trastuzumab is antibody-dependent cell-mediated cytotoxicity (ADCC) in which the tumor cells are coated with the mAb and lysed by NK cells via binding of the Fc receptor. NK cell-stimulatory cytokines, such as IL-12 and IL-21, have been shown to enhance ADCC (45). The potential of using TLR agonists to enhance ADCC has also been suggested in publications (46–48). Our results suggest the potential of using PSK to augment the therapeutic effect of trastuzumab in breast cancer patients. Preliminary analysis using human PBMC showed that PSK has similar stimulatory effect on human immune cells and augments the ability of NK cell to lyse tumor targets (Lu et al., unpublished data).

It is noted that PSK-induced IL-12 production from BMDC was not completely abrogated in TLR2^{-/-} mice. Thus, it is possible that receptors other than TLR2 may also be stimulated by PSK and have contributed to IL-12 production in the knockout mice. It has been reported that C-type lectins, such as dectin-1 and dectin-2, are involved in recognition of some fungal pathogens (49, 50). Whether PSK could also activate these lectin receptors remains to be investigated in future studies.

In summary, results from the current study demonstrate that PSK is a selective TLR2 agonist. The effect of PSK on DC and T cells is dependent on TLR2 activation. Oral PSK inhibits breast cancer growth in neu transgenic mice and the antitumor effect is dependent on both CD8⁺ T cells and NK cells. Results from this study elucidate the mechanism of action of this mushroom-based natural product and may lead to more effective methods of therapeutically exploiting the antitumor effects of PSK.

Disclosure of Potential Conflicts of Interests

No potential conflicts of interests were disclosed.

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