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Protein-bound polysaccharide activates dendritic cells and enhances OVA-specific T cell response as vaccine adjuvant

Abbi L. Engel^{1,3}, Guan-Cheng Sun¹, Ekram Gad², Lauren R. Rastetter², Katie Strobe¹, Yi Yang², Yushe Dang², Mary L Disis², and Hailing Lu^{2,3}

¹Bastyr University, Kenmore, WA

²Tumor Vaccine Group, Center for Translational Medicine in Women's Health, University of Washington, Seattle, WA

Abstract

Protein-bound polysaccharide-K (PSK) is a hot water extract from *Trametes versicolor* mushroom. It has been used traditionally in Asian countries for its immune stimulating and anti-cancer effects. We have recently found that PSK can activate toll-like receptor 2 (TLR2). TLR2 is highly expressed on dendritic cells (DC), so the currently study was undertaken to evaluate the effect of PSK on DC activation and the potential of using PSK as a vaccine adjuvant. *In vitro* experiments using mouse bone marrow-derived DC (BMDC) demonstrated that PSK induces DC maturation as shown by dose-dependent increase in the expression of CD80, CD86, MHCII, and CD40. PSK also induces the production of multiple inflammatory cytokines by DC, including IL-12, TNF- α , and IL-6, at both mRNA and protein levels. *In vivo* experiments using PSK as an adjuvant to OVA₃₂₃₋₃₃₉ vaccine showed that PSK as adjuvant leads to enlarged draining lymph nodes with higher number of activated DC. PSK also stimulates proliferation of OVA-specific T cells, and induces T cells that produce multiple cytokines, IFN- γ , IL-2, and TNF- α . Altogether, these results demonstrate the ability of PSK to activate DC *in vitro* and *in vivo* and the potential of using PSK as a novel vaccine adjuvant.

Keywords

PSK; dendritic cells; vaccine; OVA peptide

Introduction

Dendritic cells (DC) are the main initiators of immune response, bridging innate and adaptive immunity by capturing antigens and presenting them to T cells (Banchereau et al. 2000). DCs express a repertoire of receptors essential for the innate immune response known as pattern recognition receptors (PRRs) (Joffre et al. 2009). Toll-like receptors (TLR) are a family of evolutionarily conserved PRRs that are highly expressed on DC and other innate immune cells (Palucka and Banchereau 2012). Activation of TLR leads to DC maturation and production of pro-inflammatory cytokines, which can potentiate subsequent

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³To whom correspondence should be addressed: Dr. Hailing Lu, Tumor Vaccine Group, University of Washington, Box 358050, Seattle, WA 98109. Phone: (206) 616-8448; Fax: (206) 685-3128; hlu@u.washington.edu OR Abbi L. Engel, Research Department, Bastyr University, 14500 Juanita Dr. NE, Kenmore, WA 98028. Phone: (206) 685-5428 Fax: (206) 685-3128 aengel@bastyr.edu.

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T cell responses. Therefore, TLR agonists have great potential in augmenting anti-viral and anti-tumor immune response. Although the majority of pre-clinical and clinical studies have focused on agonists of intracellular TLRs (TLR3, 7/8, and 9) (Hennessy et al. 2010, Kanzler et al. 2007), recent studies demonstrated that TLRs located on the cell surface, such as TLR2 and 4, also have immune enhancing and anti-tumor activity (Asprodites et al. 2008, Mercier et al. 2009). For example, the TLR4 agonist monophosphoryl lipid A (MPL) has been approved for use in HBV and HPV vaccines (Tritto et al. 2009). TLR2 is also highly expressed on DC and the potential of using TLR2 agonist as vaccine adjuvant has been pursued and is demonstrated in recent publications (Cheng et al. 2011, Zeng et al. 2012). The study by Cheng et al. compared the adjuvant effect of TLR2 agonist Pam2CSK4, TLR3 agonist poly (I:C), and TLR9 agonist CpG-1826 and concluded as an adjuvant to *Chlamydia* vaccine, TLR2 agonist provided the best protection (Cheng et al. 2011). Additionally, a synthetic vaccine that targets TLR2 can induce DC maturation, increase cytokine production, and promote CD8⁺ T cell or antibody response (Jackson et al. 2004).

We have recently shown that protein-bound polysaccharide-K (PSK), a hot water extract from *Trametes versicolor* mushroom, can selectively activate TLR2 but not other TLRs (Lu et al. 2011). PSK is a prescription drug in Japan. Previous clinical trials in Japan have shown that the drug is safe and can effectively prolong overall survival when used in combination with standard chemotherapy drugs (Oba et al. 2007, Ohwada et al. 2004, Yokoe et al. 1997). For example, a meta-analysis of data with center randomization from 1,094 patients enrolled in three clinical trials showed that PSK as an adjuvant to chemotherapy improved both survival and disease-free survival of patients with colorectal cancer (Sakamoto et al. 2006). Based on our recent finding that PSK can activate TLR2, we hypothesize that PSK may function as an adjuvant to vaccine. To test this hypothesis, we first characterized the effect of PSK on DC maturation and function *in vitro*, and then evaluated the effect of PSK as an adjuvant to OVA_{p323-339} vaccine *in vivo*. Results from our study provided information on the potential of using this natural product with TLR agonist activity to enhance vaccine efficacy.

Materials and Methods

Animals

All animal work was performed according to the University of Washington's Institute for Animal Care and Use Committee (IACUC) guidelines. Animals were housed under specific pathogen free conditions. DO11.10 mice [Strain name: C.Cg-Tg(DO11.10)10DIo/J], BALB/c mice, MMTV-*neu* mice [Strain name: FVB/N-Tg(MMTV*neu*)202Mul/J] were purchased from Jackson Laboratory and were inbred in our facility for multiple generations. Only female mice were used in these studies.

Antibodies and other Reagents

Fluorochrome-conjugated monoclonal antibodies against murine CD11c (clone HL3), CD80 (clone 16-10A1), CD86 (clone GL-1), CD40 (HM40-3), and MHCII (I-A^b) were from eBiosciences (San Diego, CA). Purified murine anti-CD3 (Clone 145-2C11) was purchased from University of California San Francisco Monoclonal Antibody Core. Phosphate-buffered saline (PBS), penicillin-streptomycin, and L-glutamine were obtained from Invitrogen Life Technologies (Grand Island, NY). Ovalbumin (OVA) peptide p323–339 (p323, ISQAVHAHAHAIINEAGR) was purchased from Anaspec (Fremont, CA). PSK was purchased from Kureha Pharmaceuticals (Japan). PSK was dissolved in PBS at a stock concentration of 10 mg/mL. Aliquots of 100 µl were stored at –80 °C. The frozen aliquots were thawed immediately before use.

Culture of bone marrow-derived DC (BMDC) and PSK stimulation

Bone marrow cells were collected from *neu*-transgenic (MMTV-*neu*) mice and filtered through a 0.7 μ m cell strainer. ACK solution was used to lyse red blood cells and remaining cells were added to a 6-well plate for 4 hrs. Non-adherent cells were removed and fresh culture medium (20 ng/mL IL-4 + 20 ng/mL GM-CSF) was added. On Day 3, non-adherent cells were collected and replaced with fresh medium that was added back to the culture. On Day 7, cells were treated with PSK at various concentration (5, 10, 20, 40, 80 μ g/mL) for 48 hours, or treated with a fixed concentration (40 μ g/mL) for different time (3, 12, or 24 hours). Culture supernatant was collected for cytokine analysis by ELISA or Luminex.

Flow cytometric analysis for phenotypic markers

The expression costimulatory molecules, CD80, CD86, CD40, and MHC class II were evaluated on BMDC by flow cytometry following 48 hours of treatment with PSK (5, 10, 20, 40, 80 μ g/mL) using standard procedure as described before (Lu et al. 2010). Sample acquisition was done on FACS CantoII (BD Biosciences) and the data was analyzed using FlowJo software (version 9.5.2, TreeStar, Inc.).

Real time PCR analysis of cytokine gene expression in BMDC

BMDC was treated with PBS (negative control) or PSK (40 μ g/mL) in RPMI medium for 3, 12, or 24 hours. RNA was extracted from the treated BMDC and real time RT-PCR were performed as previously described (Lu et al. 2006). The primer and probes for cytokine genes and the endogenous control, β -actin, were purchased from Applied Biosystems. Expression of the target gene was normalized to β -actin as previously described.

Measurement of cytokine production by ELISA and Luminex

The concentration of IL-12p40 and IL-12p70 in BMDC culture supernatant was measured using ELISA kits from eBiosciences (San Diego, CA). The levels of TNF- α , IL-1, IL-6, and IL-10 in BMDC culture supernatant were measured by using a Luminex kit from Millipore (Pittsburgh, PA) following the manufacturer's instruction. The levels of IL-2 and TNF- α in culture supernatant from draining lymph node cells was also measured using ELISA kits from eBiosciences.

Evaluation of *in vivo* DC activation by PSK

BALB/c mice were given a vaccine of OVA₃₂₃₋₃₃₉ peptide (p323, ISQAVHAHAHAIINEAGR; Anaspec) at the dose 0.5 μ g per mouse concurrently with an intradermal (ID) injection of either PBS, GM-CSF (5 μ g), or PSK (1000 μ g). For two days following initial injection, adjuvant was given alone (ID) once per day and animals were sacrificed on Day 4. Draining lymph nodes (dLN) were harvested when animals were sacrificed and stained for histology or FACS analysis. For histological analysis, dLNs were fixed in formalin and sections of 5 μ m thickness were cut for hematoxylin and eosin (H&E) staining. For FACS analysis, the dLNs were processed to single cell suspension and total cell numbers were recorded. Then the dLN cells were stained with the following anti-mouse antibodies: CD11c-PE-Cy7 (clone N418), CD80-PE (clone 16-10A1), CD40-APC (clone HM40-3), MHCII-FITC (clone M5/114.15.2), and CD86-PerCP (clone GL-1). Samples were acquired on FACS CantoI (BD Biosciences) and analyzed using FlowJo software (version 9.5.2, TreeStar, Inc.).

Adoptive transfer, OVA peptide immunization, and evaluation of T cell proliferation

An adoptive transfer model was used to monitor the induction of OVA peptide-specific CD4⁺ T cell responses *in vivo* using similar protocol as previously described (Dang et al. 2012). On Day 1, splenocytes from DO11.10 mice were labeled with 5 μ M

carboxyfluorescein diacetate succinimidyl ester (CFSE) (Vybrant CFDA SE Cell Tracer Kit, Molecular Probes, Invitrogen). After washing with PBS, cells (20–30 million per mouse) were transferred to recipient 6–10 week old female BALB/c mice by intravenous (IV) injection through the tail vein. After adoptive transfer, the mice were given an intradermal vaccine of OVA_{p323-339} peptide (0.5 µg peptide/mouse) and adjuvant of PBS, GM-CSF (5 µg), or PSK (1000 µg) as described above. Subsequent doses of adjuvant were given on day 2 and 3, and animals were sacrificed on Day 4 or 5. Spleens and dLN were processed to single cell suspension and total cell numbers were recorded. Before tissue processing, dLN were placed on a sanitary surface and photographed for size comparison. Some splenocytes and dLN cells were set aside for evaluation of OVA-specific T cells by ELISpot (2×10⁵ cells/well). The remaining splenocytes were stained with anti-mouse antibodies CD4-PerCP (clone RM4–5, BD Pharmingen), DO11.10 TCR-APC (Clone KJ1–26, eBioscience), and CD3-PE-Cy7 (clone 145-2C11, eBioscience). Splenocytes were analyzed FACS CantoI (BD Biosciences) and analyzed using FlowJo software (version 10.0.4, TreeStar, Inc.) by gating the CD4+DO11.10+ populations and measuring the CFSE+ cells.

IFN-γ ELISpot

IFN- responses to OVA peptides in splenocytes and dLN cells were evaluated using a standard ELISpot assay as previously described (Park et al. 2008). The ELISpot plates (MAIPS 4510, Millipore) were wetted with 70% ethanol, then coated with anti-IFN- (1 µg/mL) antibody (clone AN18, Mabtech), and incubated at 4°C overnight. The next day plates were washed and blocked with (DPBS plus 2% BSA). Splenocytes or pooled dLN cells (2×10⁵ cells/well) were added to the plates and stimulated with 10 µg/mL OVA_{p323-339} (Anaspec) or an irrelevant peptide (tetanus toxoid or HepB peptide, Genemed Synthesis) of the same concentration. Murine anti-CD3 (3.2 ng/mL, UCSF Monoclonal Antibody, Clone 145-2C11) and Concanavalin A (2.5 µg/mL, Sigma) were included as positive controls. Cells were incubated in ELISpot plates for 48h at 37°C. After incubating, cells were removed and a biotinylated anti-IFN- antibody (clone R4-6A2-Biotin, Mabtech) was added. The IFN- secreting spots were detected with streptavidin-HRP and AEC substrate (BD Biosciences). The resultant spots were counted on an ELISpot reader (Cell Technology) and reported as spots per well. Supernatant from the dLN ELISpot culture plates was collected and frozen at –20°C, then later analyzed for TNF- and IL-2 cytokines with ELISA kits (eBioscience) as mentioned previously.

Statistical analysis

Statistical analysis was performed using GraphPadv5.03 (GraphPad Software, San Diego, CA). Error bars indicate mean ± standard error of the mean (SEM) unless otherwise indicated. Data were analyzed using the two-tailed Student's *t* test. A value of *p* < 0.05 was considered statistically significant. Outliers in any data set were determined by GraphPad software Grubb's test with a cutoff at alpha=0.05, and if the value was a significant outlier, it was excluded from the data set. Additionally, if the F-test indicated a difference in variances, the Welch's correction was applied to the *t*-test. Asterisks indicate *p*-value significance of treatment compared to PBS control where *, *p* < 0.05, **, *p* < 0.01 and ***, *p* < 0.001 and the summary graphs display the mean±SEM.

Results

In vitro treatment of BMDC with PSK stimulates DC maturation and production of pro-inflammatory cytokines

Mouse BMDC exhibit the phenotype of immature DCs, displaying endocytic activity and expression of MHCII and CD11c (Dearman et al. 2009). PSK treatment (5–80 µg/mL, 48 hours) resulted in a dose-dependent increase in the expression of co-stimulatory molecules

(CD80, CD86, and CD40) and MHCII on BMDC (Figure 1). The potential of PSK to stimulate cytokine production from BMDC was evaluated at both RNA and protein levels. RNA was extracted from BMDC after 3, 12, or 24 hours of treatment with PSK (40 $\mu\text{g/mL}$). As shown in Figure 2, there was significant induction of IL-12p40, IL-12p35, TNF- α , IL-6, IL-1 β , and IL-23p19 in PSK-treated BMDC as compared to control BMDC (cultured in medium alone) at different time points. ELISA and Luminex analysis of the culture supernatant of BMDC treated with different doses of PSK (5–80 $\mu\text{g/mL}$, 48 hours) showed that PSK induced IL-12p40, IL-12p70, TNF- α , IL-6, and IL-1 β in a dose-dependent manner (Figure 3A-E). In contrast, PSK did not induce IL-10 (Figure 3F).

***In vivo* administration of PSK as adjuvant to OVA-peptide vaccine activates DC in the draining lymph nodes (dLN)**

BALB/c mice received adoptive transfer of 20–30 million splenocytes from DO11.10 mice followed by vaccination with OVA_{p323} peptide combined with different adjuvants (PBS control, 5 μg GM-CSF, and 1000 μg PSK). As shown in Figure 4A, dLNs from mice that received GM-CSF or PSK as adjuvant were enlarged compared to dLNs from mice in the PBS control group. H&E staining showed that dLNs from mice treated with GM-CSF and PSK had expanded germinal centers and paracortex regions, though dLN from the PSK group were consistently largest of the three groups (Supplemental Figure 1). Total cell counts were significantly higher in both GM-CSF ($p=0.004$) and PSK ($p<0.0001$) groups as compared to the PBS control group, and PSK group was higher than GM-CSF group ($p<0.0001$) (Figure 4B). FACS analysis showed that the percentage and number of CD11c⁺ DC were significantly increased in GM-CSF (Figure 4C $p=0.04$ and Figure 4D $p=0.008$) and PSK treated mice (Figure 4C and 4D, $p<0.0001$ between PBS and PSK groups in both figures). The expression of activation markers, CD80, CD86, MHCII, and CD40 were also up-regulated in dLNs from PSK treated animals; $p=0.003$, $p=0.0002$, $p=0.0004$, and $p<0.0001$ respectively (Figure 5A and 5B). Whether PSK-induced enlargement of dLN is a result of increased DC migration remains unknown. A recent publication reported that TLR agonists stimulate the expression of CCR7 on DC leading the increased DC trafficking (Schmid et al. 2011). We also evaluated the expression of CCR7 after PSK or GM-CSF treatment in our experiment via FACS and RT-PCR, but did not detect any significant changes (data not shown).

PSK as vaccine adjuvant stimulates proliferation of OVA-specific T cells and augments OVA-specific cytokine induction

FACS analysis of splenocytes after adoptive transfer and vaccination showed that the percentages of proliferating OVA-specific T cells were significantly higher in mice that received GM-CSF or PSK as vaccine adjuvant as compared to control PBS group (Figure 6A and B). Pooled data from three independent experiments with a total number of 17 mice per group showed that the percentage of proliferating cells was 16 ± 3 in PBS group, 37 ± 4 in GM-CSF group ($p=0.0002$ from PBS control), and 36 ± 3 in PSK group ($p<0.0001$ from PBS control) (Figure 6B).

To evaluate the function of the T cells after vaccination, we harvested splenocytes and dLN cells and evaluated their response to *ex vivo* OVA_{p323} stimulation using IFN- γ ELISpot. As shown in Figure 6C, the number of IFN- γ ⁺ spots per well was significantly higher in both splenocytes and dLN cells from mice that received GM-CSF or PSK as adjuvant compared to PBS control. The number of IFN- γ ⁺ spots per well from splenocytes was 23 ± 6 in PBS group, 63 ± 10 in GM-CSF group ($p=0.001$), 71 ± 12 in PSK group ($p=0.001$, p -values compared to PBS). There was similar increase in IFN- γ production from dLN cells where the number of spots per well were 17 ± 5 for PBS, 88 ± 15 for GM-CSF ($p=0.01$), and 151 ± 17 in PSK ($p=0.002$) treatment groups (p -values compared to PBS control) (Figure 6D). The

culture supernatant from dLN ELISpot experiments was used for analysis of other Th1 cytokines, IL-2 and TNF- α . As shown in Figure 6E and F, the cells from mice treated with GM-CSF or PSK secreted significantly higher amount of IL-2 after OVA_{p323} stimulation. IL-2 concentrations (pg/mL) averaged 180 ± 122 for PBS, while GM-CSF was 866 ± 80 ($p=0.0093$) and PSK was 2562 ± 452 ($p=0.007$). This is nearly 5-fold increase in IL-2 production in GM-CSF compared to PBS control, and 14-fold increase in the PSK treated mice. TNF- α production was much lower in all treatment groups, but had a measurable (though not significant) increase in the PSK group after OVA_{p323} stimulation. There were also detectable levels of IL-4 and IL-17 in the PSK group after *ex vivo* OVA_{p323} stimulation of dLN cells, but the levels were much lower as compared to IL-2 and the increase was not significant compared to PBS control (data not shown).

Discussion

Dendritic cells are key regulators of innate and acquired immunity. They sense the presence of invading pathogens, engulf pathogens, and present the antigens to T cells after intracellular degradation (Mellman and Steinman 2001). The goal of vaccination is to generate a strong immune response that provides long-term protection against infectious agents or cancer antigens, and the development of new adjuvant for vaccination has become a field of intensive research (Aguilar and Rodriguez 2007, McDonnell et al. 2010). TLRs have been the focus of vaccine strategy due to their high expression on DC, monocytes, and macrophages (Hornung et al. 2002, Kadowaki et al. 2001, Muzio et al. 2000). We recently discovered that PSK, a hot water extract containing β -glucan from the *Trametes versicolor* mushroom, can selectively activate TLR2 (Lu et al. 2011). In this study we have shown that PSK can activate DC *in vitro* and *in vivo* (Figures 1 and 5). Furthermore, PSK as an adjuvant to OVA_{p323} vaccination induces proliferation of antigen-specific T cells, resulting in augmented number of antigen-specific T cells, in both spleen and dLNs, and enhanced cytokine response after *ex vivo* stimulation with OVA_{p323}. To our knowledge, our study represents the first report on PSK as a DC-stimulating vaccine adjuvant.

The DCs activated *in vitro* by PSK can produce multiple proinflammatory cytokines, including IL-12, TNF- α , and IL-6 (Figures 2 and 3). The panel of cytokines that is induced by PSK is similar to what has been previously reported for other TLR agonists (Napolitani et al. 2005). This cytokine response is different from other glucan-containing molecules like yeast glucan particles (YGP). YGP did not produce significant IL-12p70 and appears to work through dectin-1 (Huang et al. 2009). Although dectin-1 has been reported to be a major receptor of β -glucan (Brown et al. 2002, Brown et al. 2003), we have found that PSK does not activate dectin-1 (Yang et al, manuscript in preparation) and does induce significant amounts of IL-12p70 (Figure 2&3). IL-12p70 and TNF- α are known as Th1-polarizing cytokines. In our study, we found that T cells from mice that received PSK as vaccine adjuvant not only have enhanced IFN- γ response, but also have enhanced IL-2 and TNF- α production, in response to *ex vivo* challenge with OVA_{p323} (Figure 6E), consistent with the ability of PSK to induce Th1-polarizing cytokines. This also indicates that PSK may induce multifunctional Th1 cells, which have been shown to define a correlate of vaccine-mediated protection against infection (Darrah et al. 2007). Polyfunctional T cells have clinical importance and have been shown to increase anti-tumor activity and lead to better clinical outcomes in melanoma patients (Yuan et al. 2008). Future experiments will use intracellular staining to confirm the Th1 cytokines are produced by multifunctional T cells. In addition to the Th1-polarizing cytokines, IL-12 and TNF- α , PSK induces the production of Th17 promoting cytokines in BMDC, IL-23 and IL-6 (Figure 2 & 3) and (Mangan et al. 2006, Perona-Wright et al. 2009). It has been shown that TLR2-activated human Langerhans cells promote Th17 polarization via IL-1, TGF- β , and IL-23 (Aliahmadi

et al. 2009). Whether *in vivo* PSK treatment could also induce Th17 cells remains to be investigated.

Priming DCs allows a flexibility in stimulating multiple cell types including influencing T cell responses (Beacock-Sharp et al. 2003). We found that animals treated with OVA_{p323-339} and PSK had an increase in CD4⁺ T cell proliferation, including an increase in antigen specific T cells (Figure 6). When the OVA_{p257-264} peptide (H2-K^b restricted epitope) was given with the same vaccination regimen, we found resulting induction of antigen specific CD8⁺ T cells (data not shown), similar to the CD4⁺ T cell response. Although our study did not evaluate antibody response, a previous study by Ragupathi et al. showed that PSK as an adjuvant to the model antigens globo H and GD3 conjugated to keyhole limpet hemocyanin (KLH) generates a robust antibody response (Ragupathi et al. 2008). This is consistent with other research showing synthetic TLR2 ligand, Pam₃Cys, can increase CD8⁺ T cell response and induce high levels of antibody production with a soluble peptide vaccine (Chua et al. 2011).

An adjuvant like PSK, with its complex proteoglycan composition, could potentially activate multiple pathways to stimulate the immune system similar to delta-inulin (AdvaxTM) (Honda-Okubo et al. 2012) and have very little side effect (Cheng and Leung 2008). However, with both GM-CSF and PSK adjuvant treatments *in vivo*, we see some mice that do not respond to treatment (note the few mice near zero in Figure 6B&C). These mice appear to have the ability to be stimulated, as T cells in ELISpot can be activated by ConA positive control (not shown). It is unclear why these non-responders occur and if it is a phenomenon due to induction of suppressive cells or T cell anergy. The minimal responders may be prime targets for combination therapy, which may provide an even wider range of immune support.

The efficacy of PSK as a DC priming agent would need further evaluation with clinically relevant antigens as well as evaluating any differences in mechanism of action based on route of administration. OVA_{p323}, a non-self antigen, induces a robust T cell response. However, most tumor antigens are self-antigens and can be less immunogenic, even in combination with a potent vaccine adjuvant. We have preliminary data supporting that PSK also increases the anti-tumor effect of a *neu*-peptide vaccine (manuscript in preparation). This study uses intradermal doses of PSK as a vaccine adjuvant with OVA_{p323}, though typically this product is given orally as a tea. PSK has already shown effectiveness at decreasing tumor growth when administered orally, either alone or in combination with anti-HER2/*neu* mAb therapy (Lu et al. 2011). Together with our study, these results indicate the potential of using natural products to enhance vaccine efficacy and avoid side effects, which have been associated with some synthetic TLR agonists when given as oral adjuvants (Engel et al. 2011).

In conclusion, we demonstrate PSK effectively stimulates DC to enhance antigen-specific T cell proliferation and Th1 cytokine induction. The *in vitro* and *in vivo* data support that PSK is a good candidate for cancer vaccine adjuvant and combination therapy. Our results demonstrating therapeutic potential and the established safety record in Asia, has allowed for clinical trials to begin to evaluate PSK in combination therapy in patients with late-stage prostate cancer (NCT trial #NCT01685489) and a proposed clinical trial in breast cancer patients. Whether antigen-specific T cell and antibody response could be augmented in cancer patients receiving PSK will be investigated in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BMDC	bone marrow-derived DC
CAM	complementary and alternative medicine
CFSE	carboxyfluorescein diacetate succinimidyl ester
CTL	cytotoxic T lymphocyte
DC	dendritic cells
FBS	fetal bovine serum
GM-CSF	granulocytes macrophages-colony stimulating factor
IFN-	interferon gamma
IL	interleukin
mAb	monoclonal antibody
OVA	ovalbumin
PBS	phosphate buffered saline
PRR	pattern recognition receptors
PSK	protein-bound polysaccharide-K (Kureha)
TLR	toll-like receptor TNF- , tumor necrosis factor alpha

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Figure 1.

PSK upregulates the expression of costimulatory molecules on BMDC. BMDC were stimulated with PSK (5–80 µg/mL) for 48 hours, and the expression levels of CD80, CD86, MHCII, and CD40 were analyzed by flow cytometry. Shown are representative histograms of the expression of co-stimulatory molecules on BMDC treated with different doses of PSK. The results shown are representative of three independent experiments.

Figure 2.

PSK stimulates the mRNA expression of IL-12 and other proinflammatory cytokines. BMDCs were cultured in RPMI with PSK (40 μ g/mL) or control PBS and RNA was extracted from BMDC at 3, 12, or 24 hours after treatment. Shown are the expression levels of IL-12p40, IL-12p35, TNF- α , IL-6, IL-1 β in PSK-treated (black bars) or control BMDC (white bars) at each time point. The expression levels of target genes were normalized to beta-actin using the Δ Ct method. Figure is one representative experiment and similar results were obtained from three independent experiments.

Figure 3.

PSK induced the production of IL-12 and other proinflammatory cytokines in a dose-dependent manner. Shown are the levels of IL-12p40, IL-12p70, TNF- α , IL-6, IL-1 β , and IL-10 in culture supernatant from BMDC treated with PBS (negative control), or different doses of PSK (5–80 µg/mL). All treatments were 48 hours. The columns represent mean $\pm$ SEM of cytokine levels from three independent experiments as determined by ELISA (IL-12p40 and IL-12p70) or Luminex (all other cytokines). Asterisks represent the following p-values: *, p<0.05; **, p<0.01; ***, p<0.001 by student t test.

Figure 4.

PSK as adjuvant to OVAp323 vaccination causes draining lymph node (dLN) enlargement and increased amount of DC in dLN. Mice received intradermal injection of OVAp323 (0.5 μ g) together with PBS, GM-CSF (5 μ g) or PSK (1000 μ g) on Day 1 and adjuvant alone was administered on Day 2 and 3. dLN were harvested on Day 4. Panel A shows representative lymph nodes after treatment, ruler indicates size in mm. Summary graphs of total number of dLN cells (B), the percentage of CD11c⁺ dLN cells (C), and the total number of CD11c⁺ DC (D) in each treatment group. The graphs represent pooled data from six independent experiments. Each data point represents a single mouse (total number is 24 or 25 in each group).

Figure 5.

PSK as adjuvant to OVAp323 vaccination causes DC activation in dLN. For FACS analysis, cells were first gated on CD11c⁺ cells and then gated for activation markers: CD80, CD86, MHCII, and CD40. (A) Representative histograms showing the staining of DC activation markers in PSK (unshaded histogram) or PBS group (shaded histogram). (B) Summary graph showing the percentages of DCs that are positive for each activation marker in control PBS () or PSK group (). Each data point represents a single mouse. The horizontal bar represents group average. P-values indicated by asterisks are t-test comparisons of PSK to PBS control, where ** is $p < 0.01$ and *** indicates $p < 0.001$.

Figure 6.

PSK as vaccine adjuvant stimulates the proliferation of OVA-specific T cells and augments OVA-specific cytokine induction. Panel A shows a representative graph of the T cell proliferation of the adoptive- transferred D011.10 T cells where solid black line indicates PSK, dotted line for GM-CSF, and gray histogram is PBS control. The percent proliferated cells is indicated above the corresponding histogram (gated from CD4⁺D011.10 TCR⁺ cell population) and the line indicates the populations included for the quantification of percent proliferated cells. In panel B, the data from mice receiving adoptive T cell treatment is summarized (three experiments, 17 mice per group). The increase in IFN- γ - ova-specific T cells is seen in splenocytes (C) and in pooled dLN cells (D) when incubated with OVA₃₂₃₋₃₃₉ antigen for approximately 48hrs in the ELISpot plate. Graphs show mice from six experiments, 27 mice in PBS and GMCSF groups and 26 in PSK (C). For dLN

ELISpot, averages of 6-well replicates from 3 individual pools of cells from two experiments are indicated in (D). Supernatant from the dLN ELISpot was collected and analyzed in IL-2 (E) and TNF- (F) ELISA. Cytokine concentrations (pg/mL) indicated for cells incubated *ex vivo* with zero antigen (0 Ag), OVA_{p323}, or ConA where GM-CSF and PSK groups have increased IL-2 compared to PBS control. Asterisks indicates p-value significance of treatment compared to PBS control where *p=0.01 to 0.05, **p=0.001 to 0.01 and ***p<0.001.