Published in final edited form as:

Innate Immun. 2014 November ; 20(8): 857–866. doi:10.1177/1753425913513814.

Protein-bound polysaccharide-K induces IL-1 β via TLR2 and NLRP3 inflammasome activation

Yi Yang^{1,*}, Carol Inatsuka^{1,*}, Ekram Gad¹, Mary L Disis¹, Leanna J Standish², Nirmal Pugh³, David S Pasco³, and Hailing Lu¹

¹Tumor Vaccine Group, Center for Translational Medicine in Women's Health, University of Washington, Seattle, WA, USA

²Bastyr University, Kenmore, WA, USA

³National Center for Natural Products Research, University of Mississippi, University, MS, USA

Abstract

Inflammasome activation has been shown to regulate both innate and adaptive immune responses. It is important to investigate whether immune-enhancing natural products can also activate inflammasome. The current study examined the potential of protein-bound polysaccharide-K (PSK), a hot water extract from *Trametes versicolor*, to activate inflammasome. Using THP-1 cells, we have demonstrated that PSK induces both pro-IL-1 β and mature IL-1 β in THP-1 cells in a caspase 1- and NLRP3-dependent manner. PSK also induces IL-1 β and IL-18 in human PBMC. Cathepsin B is required for PSK-induced inflammasome activation as CA-074-Me, a cathepsin B inhibitor, significantly decreased PSK-induced IL-1 β . PSK induces NLRP3 at both mRNA and protein level. Comparison of PSK-induced IL-1 β in bone marrow-derived macrophages from wild type C57BL/6 mice, TLR2^{-/-}, P2X7R^{-/-} and NLRP3^{-/-} mice demonstrated that PSK-induced IL-1 β is dependent on both TLR2 and NLRP3. P2X7R is not required for PSK-induced inflammasome activation, but enhances PSK-induced caspase-1 activation and IL-1 β induction. Altogether, these results demonstrated that PSK induces inflammasome activation and production of IL-1 β in a TLR2- and NLRP3-dependent mechanism. These results provide novel insights into the mechanisms of the immune modulatory effects of PSK.

Keywords

TLR2; NLRP3 inflammasome; PSK

© The Author(s) 2013

Corresponding author: Hailing Lu, Tumor Vaccine Group, Center for Translational Medicine in Women's Health, University of Washington, 850 Republican St, Rm 220, Seattle, WA 98109, USA. hlu@u.washington.edu.

*These authors contributed equally to this article.

Conflict of interest

The authors do not have any potential conflicts of interest to declare.

Introduction

It is now recognized that activation of the inflammasome and induction of IL-1 β is a critical step in activating innate and adaptive immunity.¹ The inflammasome is a multi-protein complex within the cytosol that is comprised of a sensor, an adaptor and the pro-form of IL-1 β converting enzyme, pro-caspase-1. The activation of inflammasome leads to caspase-1 activation involving the autocatalytic processing of pro-caspase-1 to caspase-1, which, in turn, can cleave pro-IL-1 β and pro-IL-18 into their mature forms.^{2,3} The activation of inflammasome plays an important role in host protection by inducing immune responses and pyroptosis, which limit microbial invasion.⁴ Inflammasome activation has also been shown to mediate the adjuvant activity of alum-based adjuvants.^{5,6} Furthermore, inflammasome activation and the release of IL-1 β and IL-18 have been shown to have tumor protection effects.^{7,8}

Protein-bound polysaccharide-K (PSK) is a partially purified hot water extract of *Trametes (Coriolus) versicolor* strain CM-101, and is one of the most widely used mushroom extracts.⁹ This extract contains a mixture of β -glucan compounds ranging in size from 94 to 100 ku. The anti-tumor effect of PSK has been shown in animal models and some clinical trials in Asia.^{10–16} A meta-analysis of data from 1094 patients showed that PSK as an adjuvant to chemotherapy improved both overall survival and disease-free survival of patients with colorectal cancer.¹⁷ However, the mechanism of anti-tumor actions of PSK remained unclear. Our recent studies have demonstrated that PSK is a selective and potent TLR2 agonist. It has a potent anti-tumor effect that is dependent on both NK cells and CD8 T-cells.^{18,19} The effect of PSK on innate immune cells is represented by the induction of DC maturation and secretion of pro-inflammatory cytokines such as TNF- α , IL-12 and IL-6.^{18,20} PSK also enhances the adaptive immune response as shown by its ability to augment Ag-specific T-cell proliferation and IFN- γ secretion when administered as an adjuvant to OVA peptide vaccine.²⁰

Given the important role of inflammasome activation in regulating immune response, natural products with immune stimulatory potentials are beginning to be investigated for their ability to activate the inflammasome. For example, the study by Kankkunen et al.²¹ indicates that yeast (*Saccharomyces cerevisiae*) β -(1 $\rightarrow$ 3)-linked glucan can activate the NLRP3 pathway.²¹ Although PSK contains different β -glucans (1 $\rightarrow$ 3/1 $\rightarrow$ 4/1 $\rightarrow$ 6-linkages), we hypothesized that PSK may also activate the inflammasome. The current study was undertaken to examine the potential of PSK to activate inflammasome and the mechanism.

Materials and methods

Animals

C57BL/6 mice, NLRP3 mutant mice (strain NLRP3^{tm1Hhf/j}) and P2X7R mutant mice (strain P2rx7^{tm1Gab/J}) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and maintained in our animal facilities. TLR2^{-/-} mice were originally obtained from Dr. Shizuo Akira. Dectin-1^{-/-} mice were originally obtained from Dr. Yoichiro Iwakura. Mice were maintained under specific pathogen-free conditions. All of the procedures were

performed in compliance with the University of Washington Institutional Animal Care and Use Committee guidelines.

Reagents

RPMI culture media, PBS, penicillin–streptomycin (Pen–Strep), and L-glutamine were purchased from Invitrogen Life Technologies (Grand Island, NY, USA). FBS was purchased from Gemini Bioproducts (West Sacramento, CA, USA). Acetone was purchased from Fisher Scientific (Houston, TX, USA). ATP disodium salt hydrate was purchased from Sigma-Aldrich (St. Louis, MO, USA).

THP-1 cell culture and treatment

Human monocytic cell line THP-1 and THP-1/NLRP3^{def} (deficient for NLRP3) were purchased from InvivoGen (San Diego, CA, USA) and maintained in RPMI-1640 with 10% heat-inactivated FBS and Pen–Strep. For Western blot analysis, the THP-1 cells were differentiated with 200 nM TPA for 3 h,^{22,23} then washed and re-suspended in serum-free RPMI with PSK (200 µg/ml) or LPS (100ng/ml), and incubated for 1, 3, 6 or 24 h. To compare PSK-induced IL-1β in THP-1 and THP-1/NLRP3^{def} cells, the two cell lines were treated with various concentrations of PSK (0.5–1500.0 µg/ml) overnight (18–20 h). The levels of IL-1β in the THP-1 cell culture supernatant were measured by using a cytokine reporter cell line HEK-Blue-IL-1β (InvivoGen) or by ELISA using a kit from eBiosciences (San Diego, CA, USA) following the manufacturer's instructions. To study the role of the cathepsin B inhibitor (CA-074-Me), the THP-1 cells were treated with CA-074-Me (20 µg/ml) for 1 h before the addition of PSK; then, the cells were incubated overnight and the culture supernatant was collected for measurement of IL-1β.

Acridine orange staining of lysosomes by FACS analysis

Assessment of lysosome integrity was done similarly to that described in Hornung et al.²⁴ THP-1 cells were labeled with acridine orange (1 µg/ml) for 15min at 37°C and washed three times. Then, 2×10^6 labeled THP-1 cells were treated with PBS and PSK (10, 50 or 250 µg/ml) overnight at 37°C. Cells were washed and run on FACS Canto II to evaluate lysosomal integrity, through the shift in acridine orange emission spectra. Data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Measurement of pro-IL-1β and IL-1β by Western blot

THP-1 lysate were prepared by using the RIPA lysis buffer (0.15 M NaCl, 1% NP-40, 0.1% SDS, 50 mM Tris pH 8.0) with protease inhibitors (cOmplete Mini; Roche, Mannheim, Germany). Proteins from THP-1 lysate or culture supernatant (precipitated with acetone) were separated on a 12% SDS-PAGE gel (Invitrogen) and transferred to a nitrocellulose membrane. The membrane was first probed with anti-IL-1β mAb (clone 3ZD, from NCI-FCRDC) and anti-β-actin (clone 13E5; Cell Signaling Technology, Beverly, MA, USA). After washing, the membrane was incubated with HRP-labeled secondary Abs allowing the signal for IL-1β to be detected by enhanced chemiluminescence (SuperSignal West Pico; Thermo Scientific, Fremont, CA, USA). For β-actin, the membrane was incubated with Alexafluor-labeled secondary Ab and the signal was detected using LI-COR Odyssey.

Real-time RT-PCR analysis of pro-IL-1 β and NLRP3 mRNA expression

Total RNA was extracted from THP-1 cells or PBMC treated with PSK (200 μ g/ml for 1, 3, 6 or 24 h) using RNAqueous4PCR kit from Ambion (Austin, TX, USA). cDNA was synthesized using the Superscript III reverse transcription kit (Invitrogen). Real-time Taqman PCR was run on an ABI7900HT instrument using primer and probes from Applied Biosystems (Foster City, CA, USA). The levels of IL-1 β and NLRP3 mRNA expression were normalized to β -actin using the delta Ct method as previously described.²⁵

PBMC collection and treatment

Blood samples from healthy donors were collected by Astarte Biologics (Redmond, WA, USA) with informed consent under institutional review board-approved protocol. PBMC were isolated by Ficoll gradient centrifugation, suspended in RPMI with 10% human AB sera and Pen–Strep, placed in 96-well plates (3×10^5 cells/well) and treated with serial dilutions of PSK overnight. The culture supernatant was analyzed for levels of IL-1 β or IL-18 by ELISA. To determine the mechanism of inflammasome activation, various inhibitors were added to the culture medium 1 h before adding PSK. The PBMC were treated overnight, and the levels of IL-1 β and TNF- α were analyzed by ELISA using kits from eBiosciences.

Culture and treatment of mouse bone marrow-derived macrophage

Mouse bone marrow-derived macrophages (BMM) were cultured using bone marrow from 6–10-wk-old mice following published protocols.²⁶ The bone marrow from femur and tibia of the hind legs were dissected and flushed with PBS. The bone marrow cells were passed through a 0.7- μ m cell strainer, erythrocytes lysed by ACK lysis buffer, washed and re-suspended in medium containing complete RPMI 1640 (10% FBS, 50 μ M β -ME, penicillin/streptomycin) and 30% of L929 cell-conditioned medium as a source of macrophage colony stimulating factor. Every 2–3 d, cells were washed twice with DPBS and fresh media was added for a total of 7 d incubation at 37°C with 5% CO₂. FACS analysis has shown that these cells are F4/80⁺ and CD11b⁺. BMM were incubated with PSK (200 μ g/ml) or LPS (1 μ g/ml) for 6 or 24 h. When ATP (5 mM) was added, it was for the last 30 min of treatment. Then, the cells were lysed in RNA for later RNA extraction and RT-PCR analysis, or lysed in RIPA buffer for Western blot analysis. The culture supernatant was harvested for ELISA analysis of cytokine induction, or precipitated with acetone for Western blot analysis.

Statistical analysis

Statistical analysis was performed using GraphPad software (GraphPad, San Diego, CA, USA). Difference between two treatment groups was analyzed using the two-tailed Student's *t*-test or Mann–Whitney test. Correlation between two parameters was analyzed using Pearson correlation analysis. A value of *P* < 0.05 was considered statistically significant.

Results

PSK induces both pro-IL-1 β and mature IL-1 β in THP-1 cells in a caspase- and NLRP3-dependent manner

Treatment of THP-1 cells with PSK induces IL-1 β at both mRNA and protein levels (Figure 1A, B). As shown in Figure 1A, PSK (200 μ g/ml for 1, 3 or 6h) induced IL-1 β mRNA to a level that was comparable to LPS (100ng/ml). Western blot analysis showed an induction of pro-IL-1 β in THP-1 cell lysate after 3 or 6h of PSK treatment and an induction of mature IL-1 β in the cell culture supernatant after 6h of PSK treatment (Figure 1B). ELISA analysis using culture supernatant also showed induction of secreted IL-1 β after treatment with PSK or LPS for 6 or 24 h (Figure 1C). We also observed induction of active caspase-1 (p20) after 6h of PSK treatment, which returned to baseline level after 24 h of treatment (Figure 1D). The presence of caspase inhibitor, z-VAD-FMK, significantly inhibited PSK-induced IL-1 β production (Figure 1E). To examine the role of NLRP3 inflammasome, we used THP-1 cells deficient in NLRP3 and treated them side-by-side with regular THP-1 cells with PSK (0.6–1500.0 μ g/ml) for 24h. As shown in Figure 1F, PSK failed to induce IL-1 β in THP-1 cells deficient for NLRP3, demonstrating the induction of mature IL-1 β by PSK is dependent on activation of NLRP3 inflammasome.

PSK induces both pro-IL-1 β and mature IL-1 β in human PBMC

Treatment of human PBMC with PSK (42 or 250 μ g/ml for 6 or 24 h) resulted in significant induction of IL-1 β mRNA (Figure 2A). PSK (0.6–1500.0 μ g/ml, 24 h) also induced IL-1 β at the protein level, as demonstrated by a concentration-dependent induction of secreted IL-1 β in PBMC culture supernatant. The presence of z-VAD-FMK (10 μ g/ml) significantly inhibited PSK-induced IL-1 β production in PBMC (Figure 2B). In addition to IL-1 β , PSK also induced IL-18 secretion in a dose-dependent manner, which was also inhibited by the presence of z-VAD-FMK (Figure 2C).

Cathepsin B is required for PSK-induced IL-1 β production

To investigate the mechanism by which PSK induces IL-1 β , we first evaluated the role of cathepsin B, a lysosomal cysteine protease that can be released upon lysosomal disruption and bind to NLRP3 to induce inflammasome activation.²⁷ The presence of a cathepsin B inhibitor, CA-074-Me (20 μ g/ml for THP-1 cells and 10 μ g/ml for PBMC), significantly decreased PSK-induced IL-1 β , in both THP-1 cells and PBMC (Figure 3A), but had a negligible effect on PSK-induced TNF- α (Figure 3B). To investigate the potential effect of PSK on lysosome, we used acridine orange staining as reported in the literature.²⁴ As shown in Figure 3C, PSK induces lysosome disintegration in a concentration-dependent manner. Interestingly, pretreatment of cells with bafilomycin A, an inhibitor of the vacuolar H-ATPase, did not affect PSK-induced IL-1 β , indicating that PSK may induce cathepsin B release through other mechanism in addition to lysosome disintegration (data not shown). The complete cellular mechanism that triggers cathepsin B release remains to be investigated.

PSK up-regulates NLRP3 expression, and the induction of IL-1 β by PSK is dependent on both TLR2 and NLRP3

It has previously been shown that NLRP3 expression is a limiting factor for NLRP3 inflammasome activation,²⁸ so we investigated the potential of PSK to induce NLRP3 expression. As shown in Figure 4 (A–C), PSK induces NLRP3 mRNA expression in THP-1 cells, PBMC and mouse BMM. Using Western blot, we confirmed that NLRP3 protein was also induced by PSK (200 μ g/ml, 24 h) in BMM from wild type (WT) BL6 mice (Figure 4D, E). The induction of NLRP3 by PSK is significantly decreased in BMM from TLR2^{-/-} mice compared with WT mice. As expected, BMM from NLRP3^{-/-} mice had no detectable NLRP3 protein (Figure 4D, E). We also compared the induction of pro-IL-1 β and IL-1 β by PSK in BMM from TLR2^{-/-} and NLRP3^{-/-} mice. As shown in Figure 4F, PSK only induced mature IL-1 β (17 ku) in culture supernatant from WT BMM, but not in the culture supernatant of BMM from TLR2^{-/-} or NLRP3^{-/-} mice. Analysis of BMM cell lysate showed that the induction of pro-IL-1 β by PSK was decreased in BMM cells from TLR2^{-/-} mice, but not decreased in BMM from NLRP3^{-/-} mice. These data suggest that the failure of PSK to induce IL-1 β in TLR2^{-/-} mice occurs at the pro-IL-1 β level, while the failure of PSK to induce IL-1 β in NLRP3^{-/-} mice occurs at the processing step, where pro-IL-1 β is cleaved to mature IL-1 β . ELISA analysis of culture supernatant from the different BMM cultures confirmed that PSK-induced IL-1 β is significantly decreased in BMM from both TLR2^{-/-} and NLRP3^{-/-} mice compared with WT BL6 mice (Figure 4G). Thus, the induction of IL-1 β by PSK requires both TLR2 and NLRP3. We have previously shown that PSK does not activate TLR4,¹⁸ so the activation of NF- κ B and induction of pro-IL-1 β is not due to LPS contamination.

It has previously been reported that β -glucan-induced NLRP3 inflammasome activation is dependent on the dectin-1/Syk signaling pathway. Because PSK contains β -glucan, we questioned whether dectin-1 may be involved in PSK-induced inflammasome activation and IL-1 β production. We used splenocytes from WT, TLR2^{-/-} and dectin-1^{-/-} mice, and treated them side-by-side with different concentrations of PSK. As shown in Figure 4H, PSK-induced IL-1 β is comparable between WT and dectin-1^{-/-} mice, but significantly decreased in TLR2^{-/-} mice, demonstrating that TLR2, but not dectin-1, is involved in PSK-induced IL-1 β . PSK is a hot water extract and mainly contains soluble β -glucan. Our finding that PSK does not activate dectin-1 is consistent with a previous report that particulate, but not soluble, β -glucan activates dectin-1.²⁹

Potassium efflux contributes to PSK-induced IL-1 β , but P2X7R is not required

It has previously been shown that potassium efflux may be a common trigger for NLRP3 inflammasome activation,³⁰ so we tested whether PSK-induced IL-1 β may be inhibited by the blockade of potassium efflux. As shown in Figure 5 (A, B), the use of extracellular KCl (25 or 50 mM) significantly decreased PSK-induced IL-1 β in PBMC, without affecting PSK-induced TNF- α . It should be noted that even the high concentration of KCl (50 mM) did not completely block PSK-induced IL-1 β . To further explore the role of potassium efflux, we used P2X7R^{-/-} mice, which are deficient for the purinergic ATP-gated P2X7 receptor. P2X7R can act as an ion channel to release intracellular potassium triggering NLRP3 inflammasome activation.^{31,32} Interestingly, comparison of PSK-induced IL-1 β in BMM

from WT and P2X7R^{-/-} mice by ELISA showed no significant difference in IL-1 β released into the culture supernatant (Figure 5C, black columns). However, there was significant augmentation of PSK-induced IL-1 β by the addition of ATP, in WT but not P2X7R^{-/-} mice (Figure 5C, slashed columns). In comparison, addition of ATP did not affect PSK-induced TNF- α induction (Figure 5D). These results indicate that the activation of P2X7R via ATP is not required, but instead can enhance PSK-induced inflammasome activation and IL-1 β production. Western blot analysis confirmed that the induction of pro-IL-1 β by PSK in the BMM cell lysate is not significantly increased by the addition of ATP. Only the induction of mature IL-1 β in culture supernatant is significantly increased in BMM culture supernatant from WT mice, but not from P2X7R^{-/-} mice (Figure 5E). Consistent with the enhanced IL-1 β induction, there was significantly enhanced caspase-1 activity when ATP was added after PSK treatment (Figure 5E).

Discussion

Results from this study demonstrate, for the first time, that PSK can activate NLRP3 inflammasome to produce mature IL-1 β . Further, we showed that PSK-induced IL-1 β production is dependent on both TLR2 and NLRP3, and requires cathepsin B. These new data, together with our previous finding that PSK activates TLR2, demonstrate that this mushroom extract can modulate immune response at multiple levels, via stimulating both extracellular PRR (TLR2) and intracellular pathogen sensors (NLRP3 inflammasome).

It is generally believed that production of IL-1 β and IL-18 requires two signals. Signal one is from TLR-driven NF- κ B activation and synthesis of pro-IL-1 β and pro-IL18. Then, a second signal, such as ATP release or potassium efflux, is required for activation of the inflammasome. Our results suggest that PSK treatment is sufficient to produce both signals. Treatment of THP-1, PBMC or BMM with PSK not only induces pro-IL-1 β , but also activates caspase-1, which cleaves pro-IL-1 β to mature IL-1 β . Indeed, results from P2X7R^{-/-} mice suggest that P2X7R activation is not required for PSK-induced IL-1 β . This observation is consistent with a recent report from the Nunez group that TLR agonists can stimulate NLRP3-dependent IL-1 β production independently of P2X7R.³³ Of note, the addition of ATP that triggers K⁺ efflux significantly enhanced the IL-1 β induction by PSK. Western blot data suggest a significant induction of caspase-1 and enhanced cleavage of pro-IL-1 β to IL-1 β in the presence of ATP (Figure 5). It should be noted that PSK is frequently used in cancer patients as an adjuvant to chemotherapy, which can induce ATP release.³⁴ Therefore, the *in vivo* induction of IL-1 β is probably a result of combined PSK and ATP stimulation.

PSK not only induces IL-1 β , but also induces IL-18, also known as the IFN- γ inducing factor. Studies in the infectious disease setting have shown that inflammasome activation and induction of IL-1 β and IL-18 contribute to the activation of T-cells and NK cells.^{35,36} We hypothesize that inflammasome activation and IL-18 induction may also play a role in PSK-induced NK cell activation. Indeed, our previous publication has shown that the effect of PSK on human NK cell activation is mostly indirect and requires IL-12 secreted by accessory cells such as DC and monocytes. Based on the current finding that PSK induces

IL-18, we think IL-18 probably works synergistically with IL-12 in PSK-induced NK cell activation.

Inflammasome activation has also been shown to play an important role in the adjuvant activity of aluminum, the most commonly used vaccine adjuvant.^{5,6} A recent publication has shown that PSK stimulates the proliferation of Ag-specific T-cells when used as an adjuvant to ova peptide vaccine.²⁰ Unpublished data showed that the generation of ova-specific Ab was also enhanced by PSK. The role of inflammasome activation in this adjuvant effect of PSK is currently under investigation.

Our finding that PSK activates the NLRP3 inflammasome not only improves our understanding of the immune modulatory activity of PSK, but also has important implication as for human clinical studies on PSK. Specifically, we hypothesize that a single nucleotide polymorphism (SNP) in the *NLRP3* gene, which has recently been reported,^{37,38} may affect the clinical response to PSK. For example, the gain-of-function alteration in Q705K in NLRP3 has been shown to result in excessive IL-1 β and IL-18 production.³⁸ This extra amount of pro-inflammatory cytokines may lead to a better anti-tumor effect. However, this might be detrimental owing to an uncontrolled inflammation. Currently, there are two planned clinical trials in the USA evaluating the immune stimulatory potential of PSK, one in prostate cancer patients using PSK in combination with docetaxel and the other one in breast cancer patients in combination with trastuzumab and a HER2-targeted peptide vaccine. Results from this study would suggest the inclusion of NLRP3 SNP analysis in the clinical immune monitoring.

In summary, this study shows, for the first time, that PSK, a widely used mushroom extract, can activate the NLRP3 inflammasome and induce IL-1 β in a TLR2- and NLRP3-dependent manner. This information suggest that the pro-inflammatory and Th1 polarizing effects of PSK are not only due to the activation of TLR2/MyD88 and NF- κ B driven cytokine synthesis, but also due to caspase-1 activation and cleavage of pro-IL-1 β and pro-IL-18 to their mature form. This significantly improves our understanding of the mechanism of the immune stimulatory effects of PSK.

Acknowledgments

We thank Lauren Rastetter and Marlese Koehnlein for assistance with the animal work. We also thank Dr. Tobias Hohl for providing tissue from dectin-1^{-/-} mice.

Funding: This work was supported by NIH grants R01CA138547 (Hailing Lu), R01AT004314 (Mary L. Disis), U19AT006028 (Leanna J. Standish), and a gift to the Tumor Vaccine Group. Drs. Nirmal Pugh and David Pasco were partially supported by the USDA Agricultural Research Service Specific Cooperative Agreement No. 58-6408-1-603 (NCNPR/University of Mississippi).

References

1. Eisenbarth SC, Flavell RA. Innate instruction of adaptive immunity revisited: The inflammasome. *EMBO Mol Med.* 2009; 1:92–98. [PubMed: 20049709]
2. Franchi L, Eigenbrod T, Munoz-Planillo R, Nunez G. The inflammasome: A caspase-1-activation platform that regulates immune responses and disease pathogenesis. *Nat Immunol.* 2009; 10:241–247. [PubMed: 19221555]

3. Martinon F, Tschopp J. Inflammatory caspases: Linking an intracellular innate immune system to autoinflammatory diseases. *Cell*. 2004; 117:561–574. [PubMed: 15163405]
4. Franchi L, Munoz-Planillo R, Nunez G. Sensing and reacting to microbes through the inflammasomes. *Nat Immunol*. 2012; 13:325–332. [PubMed: 22430785]
5. Eisenbarth SC, Colegio OR, O'Connor W, Sutterwala FS, Flavell RA. Crucial role for the nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature*. 2008; 453:1122–1126. [PubMed: 18496530]
6. Li H, Willingham SB, Ting JP, Re F. Cutting edge: Inflammasome activation by alum and alum's adjuvant effect are mediated by NLRP3. *J Immunol*. 2008; 181:17–21. [PubMed: 18566365]
7. Zaki MH, Vogel P, Body-Malapel M, Lamkanfi M, Kanneganti TD. Il-18 production downstream of the NLRP3 inflammasome confers protection against colorectal tumor formation. *J Immunol*. 2010; 185:4912–4920. [PubMed: 20855874]
8. Ghiringhelli F, Apetoh L, Tesniere A, Aymeric L, Ma Y, Ortiz C, et al. Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. *Nat Med*. 2009; 15:1170–1178. [PubMed: 19767732]
9. Fisher M, Yang LX. Anticancer effects and mechanisms of polysaccharide-k (PSK): Implications of cancer immunotherapy. *Anticancer Res*. 2002; 22:1737–1754. [PubMed: 12168863]
10. Ebina T, Murata K. Antitumor effect of psk at a distant site: Tumor-specific immunity and combination with other chemotherapeutic agents. *Jpn J Cancer Res*. 1992; 83:775–782. [PubMed: 1517151]
11. Matsunaga K, Aota M, Nyunoya Y, Hakozaki M, Ishikawa Y, Ohhara M, et al. Antitumor effect of biological response modifier, PSK, on c57bl/6 mice with syngeneic melanoma b16 and its mode of action. *Oncology*. 1994; 51:303–308. [PubMed: 8208511]
12. Ueno Y, Kohgo Y, Sakamaki S, Itoh Y, Takahashi M, Hirayama Y, Niitsu Y. Immunochemotherapy in b-16-melanoma-cell-transplanted mice with combinations of interleukin-2, cyclophosphamide, and PSK. *Oncology*. 1994; 51:296–302. [PubMed: 8196915]
13. Kidd PM. The use of mushroom glucans and proteoglycans in cancer treatment. *Altern Med Rev*. 2000; 5:4–27. [PubMed: 10696116]
14. Ohwada S, Ikeya T, Yokomori T, Kusaba T, Roppongi T, Takahashi T, et al. Adjuvant immunochemotherapy with oral tegafur/uracil plus psk in patients with stage II or III colorectal cancer: A randomised controlled study. *Br J Cancer*. 2004; 90:1003–1010. [PubMed: 14997197]
15. Yokoe T, Iino Y, Takei H, Horiguchi J, Koibuchi Y, Maemura M, et al. HLA antigen as predictive index for the outcome of breast cancer patients with adjuvant immunochemotherapy with PSK. *Anticancer Res*. 1997; 17:2815–2818. [PubMed: 9252721]
16. Oba K, Teramukai S, Kobayashi M, Matsui T, Koda Y, Sakamoto J. Efficacy of adjuvant immunochemotherapy with polysaccharide k for patients with curative resections of gastric cancer. *Cancer Immunol Immunother*. 2007; 56:905–911. [PubMed: 17106715]
17. Sakamoto J, Morita S, Oba K, Matsui T, Kobayashi M, Nakazato H, Ohashi Y. Efficacy of adjuvant immunochemotherapy with polysaccharide k for patients with curatively resected colorectal cancer: A meta-analysis of centrally randomized controlled clinical trials. *Cancer Immunol Immunother*. 2006; 55:404–411. [PubMed: 16133112]
18. Lu H, Yang Y, Gad E, Wenner CA, Chang A, Larson ER, et al. Polysaccharide krestin is a novel TLR2 agonist that mediates inhibition of tumor growth via stimulation of CD8 T cells and NK cells. *Clin Cancer Res*. 2011; 17:67–76. [PubMed: 21068144]
19. Lu H, Yang Y, Gad E, Inatsuka C, Wenner CA, Disis ML, Standish LJ. TLR2 agonist PSK activates human NK cells and enhances the antitumor effect of HER2-targeted monoclonal antibody therapy. *Clin Cancer Res*. 2011; 17:6742–6753. [PubMed: 21918170]
20. Engel AL, Sun GC, Gad E, Rastetter LR, Strobe K, Yang Y, et al. Protein-bound polysaccharide activates dendritic cells and enhances ova-specific t cell response as vaccine adjuvant. *Immunobiology*. 2013; 218:1468–1476. [PubMed: 23735481]
21. Kankkunen P, Teirila L, Rintahaka J, Alenius H, Wolff H, Matikainen S. (1,3)-beta-glucans activate both dectin-1 and nlrp3 inflammasome in human macrophages. *J Immunol*. 2010; 184:6335–6342. [PubMed: 20421639]

22. Jun HK, Lee SH, Lee HR, Choi BK. Integrin alpha5beta1 activates the nlrp3 inflammasome by direct interaction with a bacterial surface protein. *Immunity*. 2012; 36:755–768. [PubMed: 22608495]
23. Zhou R, Tardivel A, Thorens B, Choi I, Tschopp J. Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nat Immunol*. 2010; 11:136–140. [PubMed: 20023662]
24. Hornung V, Bauernfeind F, Halle A, Samstad EO, Kono H, Rock KL, et al. Silica crystals and aluminum salts activate the nalp3 inflammasome through phagosomal destabilization. *Nat Immunol*. 2008; 9:847–856. [PubMed: 18604214]
25. Lu H, Knutson KL, Gad E, Disis ML. The tumor antigen repertoire identified in tumor-bearing neu transgenic mice predicts human tumor antigens. *Cancer Res*. 2006; 66:9754–9761. [PubMed: 17018635]
26. Weischenfeldt J, Porse B. Bone marrow-derived macrophages (BMM): Isolation and applications. *CSH Protoc*. 2008 pdb.prot5080.
27. Bruchard M, Mignot G, Derangere V, Chalmin F, Chevriaux A, Vegran F, et al. Chemotherapy-triggered cathepsin b release in myeloid-derived suppressor cells activates the nlrp3 inflammasome and promotes tumor growth. *Nat Med*. 2013; 19:57–64. [PubMed: 23202296]
28. Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, et al. Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J Immunol*. 2009; 183:787–791. [PubMed: 19570822]
29. Qi C, Cai Y, Gunn L, Ding C, Li B, Kloecker G, et al. Differential pathways regulating innate and adaptive antitumor immune responses by particulate and soluble yeast-derived betaglacans. *Blood*. 2011; 117:6825–6836. [PubMed: 21531981]
30. Petrilli V, Papin S, Dostert C, Mayor A, Martinon F, Tschopp J. Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration. *Cell Death Differ*. 2007; 14:1583–1589. [PubMed: 17599094]
31. Ferrari D, Pizzirani C, Adinolfi E, Lemoli RM, Curti A, Idzko M, et al. The p2x7 receptor: A key player in il-1 processing and release. *J Immunol*. 2006; 176:3877–3883. [PubMed: 16547218]
32. Petrilli V, Dostert C, Muruve DA, Tschopp J. The inflammasome: A danger sensing complex triggering innate immunity. *Curr Opin Immunol*. 2007; 19:615–622. [PubMed: 17977705]
33. He Y, Franchi L, Nunez G. Tlr agonists stimulate NLRP3-dependent IL-1beta production independently of the purinergic P2X7 receptor in dendritic cells and in vivo. *J Immunol*. 2013; 190:334–339. [PubMed: 23225887]
34. Aymeric L, Apetoh L, Ghiringhelli F, Tesniere A, Martins I, Kroemer G, et al. Tumor cell death and atp release prime dendritic cells and efficient anticancer immunity. *Cancer Res*. 2010; 70:855–858. [PubMed: 20086177]
35. Soudja SM, Ruiz AL, Marie JC, Lauvau G. Inflammatory monocytes activate memory CD8(+) T and innate NK lymphocytes independent of cognate antigen during microbial pathogen invasion. *Immunity*. 2012; 37:549–562. [PubMed: 22940097]
36. Mitchell AJ, Yau B, McQuillan JA, Ball HJ, Too LK, Abtin A, et al. Inflammasome-dependent IFN-gamma drives pathogenesis in *Streptococcus pneumoniae* meningitis. *J Immunol*. 2012; 189:4970–4980. [PubMed: 23071286]
37. Pontillo A, Brandao L, Guimaraes R, Segat L, Araujo J, Crovella S. Two SNPs in NLRP3 gene are involved in the predisposition to type-1 diabetes and celiac disease in a pediatric population from northeast Brazil. *Autoimmunity*. 2010; 43:583–589. [PubMed: 20370570]
38. Verma D, Sarndahl E, Andersson H, Eriksson P, Fredrikson M, Jonsson JI, et al. The q705k polymorphism in NLRP3 is a gain-of-function alteration leading to excessive interleukin-1beta and IL-18 production. *PLoS One*. 2012; 7:e34977. [PubMed: 22529966]

Figure 1.

PSK induces both pro-IL-1 β and mature IL-1 β in THP-1 cells. (A) The level of IL-1 β mRNA (normalized to β -actin) in THP-1 cells treated with control PBS (white column), PSK (200 μ g/ml, black column) or LPS (100 ng/ml, gray column) for 1, 3 or 6 h. The level of IL-1 β was normalized to β -actin using the Ct method. (B) Western blot showing the level of IL-1 β (17 ku) in culture supernatant, and pro-IL-1 β (35 ku) and β -actin in the lysate of THP-1 cells treated with PSK or LPS for 1, 3 or 6 h. (C) The level of IL-1 β in THP-1 culture supernatant as measured by ELISA. (D) Western blot showing caspase-1 (20 ku) in THP-1 culture supernatant and β -actin in THP-1 cell lysate after treatment with control PBS, PSK or LPS for 6 or 24 h. (E) The levels of IL-1 β in culture supernatant from THP-1 cells treated with serial dilutions of PSK or PSK in the presence of z-VAD-FMK (10 μ g/ml) for 24h. (F) Comparison of IL-1 β levels in PSK-treated THP-1 cells (●) and THP-1 cells deficient for NLRP3 (▲).

Figure 2.

PSK induces both IL-1 β and IL-18 in human PBMC in a caspase-dependent manner. (A) Induction of IL-1 β mRNA in human PBMC by PSK (42 or 250 μ g/ml) or LPS (100 ng/ml) after 6 h (gray column) or 24 h (black column) treatment. (B) Dose-response of PSK-induced IL-1 β in PBMC culture supernatant in the absence or presence of caspase inhibitor z-VAD-FMK (10 μ g/ml). (C) Dose-response of PSK-induced IL-18 in PBMC culture supernatant in the absence or presence of caspase inhibitor z-VAD-FMK (10 μ g/ml).

Figure 3.

Cathepsin B is required for PSK-induced IL-1 β production. (A) Dose-dependent induction of IL-1 β by PSK (●) in THP-1 cells and human PBMC. The presence of CA-074-Me significantly decreased PSK-induced IL-1 β (○) in both THP-1 and PBMC. (B) PSK-induced TNF- α in THP-1 and PBMC without (■) or with CA-074-Me (□). (C) Acridine orange staining showing the percentages of disintegrated lysosome in control and THP-1 cells treated with different concentrations of PSK.

Figure 4.

NLRP3 is induced by PSK, and both TLR2 and NLRP3 are required for PSK-induced IL-1 β . (A) Induction of NLRP3 mRNA by PSK (200 $\mu\text{g/ml}$, black column) and LPS (100ng/ml, gray column) in THP-1 cells. (B) Induction of NLRP3 mRNA by PSK and LPS in PBMC from two different donors. (C) Induction of NLRP3 mRNA by PSK and LPS in BMM from C57BL/6 mice. mRNA level was normalized to human or mouse β -actin. (D) Western blot showing the level of NLRP3 in control or PSK-treated BMM from WT (BL6), TLR2^{-/-}, or NLRP3^{-/-} mice. (E) Summary graph showing the quantification of NLRP3 expression

levels in Western blot. Each data point represents result from an independent experiment. The expression of NLRP3 was normalized to β -actin after quantification with ImageJ software. Empty circle: relative expression level of NLRP3 in control PBS group; filled circle: expression level in PSK group. (F) Western blot showing the levels of pro-IL1 β (35 ku) and IL-1 β (17 ku) in BMM culture supernatant and cell lysate from WT (BL6), TLR2^{-/-} and NLRP3^{-/-} mice. β -Actin in BMM lysate is included as control. (G) IL-1 β in culture supernatant from control or PSK-treated BMM from WT (BL6), TLR2^{-/-} or NLRP3^{-/-} mice. Results shown represent the summary data of three independent experiments. (H) PSK-induced IL-1 β in splenocytes from WT, TLR2^{-/-} and dectin-1^{-/-} mice. Splenocytes were treated with different concentrations of PSK (0.005–1500.0 ug/ml) for 96 h. The culture supernatant was collected and the level of IL-1 β was measured by ELISA. * $P < 0.05$ by two-tailed Student's t -test.

Figure 5.

Potassium efflux contributes to PSK-induced IL-1 β , but P2X7R is not required. (A) Extracellular KCl (25 or 50 mM) inhibits PSK-induced IL-1 β in PBMC. Shown are dose–response curves of PSK-induced IL-1 β in PBMC without extracellular KCl (●), with 25 mM KCl (○) or 50mM KCl (□). (B) Extracellular KCl does not decrease PSK-induced TNF- α in PBMC. (C) The level of IL-1 β in control or PSK-treated BMM from WT (BL6) or P2X7R $^{-/-}$ mice without ATP (solid columns) or with the addition of 5mM ATP after PSK treatment (slashed columns). (D) The level of TNF- α in culture supernatant of BMM.

Results shown are the summary of three independent experiments. $**P<0.01$; $***P<0.001$ by two-tailed Student's *t*-test. ns: non-significant. (E) Western blot analyses show the levels of pro-IL-1 β (35 ku), caspase-1 (p10), and control β -actin in BMM from BL6 mice or P2X7R $^{-/-}$ mice treated with PSK with or without ATP (5 mM). The levels of pro-IL-1 β (35 ku) and IL-1 β (17ku), pro-caspase-1 (45 ku) and caspase-1 (p10) in culture supernatant are also shown.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript