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Role for toll-like receptor 4 in TNF-alpha secretion by murine macrophages in response to polysaccharide Krestin, a *Trametes versicolor* mushroom extract

Lisa A. Price [a,*], Cynthia A. Wenner [a], Daniel T. Sloper [b,c], Joel W. Slaton [b,c], Jeffrey P. Novack [d]

[a] Bastyr University, Department of Basic Sciences, School of Natural Health Sciences, Kenmore, WA 98028, United States
[b] Department of Urologic Surgery, University of Minnesota, Minneapolis MN 55455, United States
[c] University of Minnesota Minneapolis, MN Veterans Administration Medical Center Minneapolis, MN 55417, United States
[d] Bellevue Community College, Science Division, Bellevue, WA 98007, United States

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ABSTRACT

Woody fungi and yeast preparations show promise in cancer treatment by activating anti-tumor immune responses. Macrophages (J774A.1) were treated with PSK, Reishi extract, scleroglucan or vehicle control. Pre-incubation with TLR4 blocking antibody inhibited TNF-alpha secretion by both J774A.1 cells and primary splenocytes but had inconclusive effect on scleroglucan-induced secretion of TNF-alpha. PSK induced TNF-alpha and IL-6 secretion by wild type but not by TLR4-deficient peritoneal macrophages. We conclude that constituents from PSK act as ligands for TLR4 receptors leading to induction of TNF-alpha and IL-6 inflammatory cytokines. Receptor-mediated differences may be due to structural differences in beta glucans or non-glucan constituents.

1. Introduction

Tumor cells create a physiological microenvironment which is immunosuppressive. Immune functions that would normally recognize and trigger destruction of abnormal cells are ineffective in cancer settings. As part of the tumor-induced evasion of immunosurveillance, immune cell signaling and activation through cytokines is dampened. Woody fungi and yeast preparations show promise in adjunctive cancer treatment by activating an immunosuppressed system via stimulation of innate and adaptive immunity through highly conserved pathogen recognition receptors (PRR) that bind to various ligands in these preparations. These receptors can initiate both innate and adaptive immunological responses that are tailored to specific pathogens such as fungi, bacteria and yeast [1,2]. Common PRR ligands include lipopolysaccharide (LPS), N-acetyl glucosamine and beta glucans, which are constituents of bacterial or fungal cell walls. Binding to PRRs such as toll-like receptor-4 (TLR4), dectin-1 and complement receptor 3 (CR3) by polysaccharides can activate immune responses by enhancing the secretion of TNF-alpha, IL-6 and other inflammatory cytokines. Pathogen recognition receptors also serve to bind ligands that prime immune responses [3,4]. These immune modulating responses are of particular interest in cancer prevention and treatment research.

Yeast beta-(1–3)-D-glucans have been shown to activate anti-tumor responses via CR3 (Cd11b/CD18) and dectin-1 receptors [2,5–7]. While there has been considerable research on how isolated yeast beta-(1–3)-D-glucan activates cytokine production through dectin-1 and CR3, there are few reports on the mechanisms of how traditionally used medicinal mushroom preparations, that contain polysaccharides, interact with PRR. The few studies addressing such effects suggest that polysaccharides from *Ganoderma lucidum* (Reishi) and *Phellinus linteus* can act through TLR pathways to induce inflammatory responses in mouse cells [3,8,9]. However, there are no published reports on the effects of *Trametes versicolor* mushroom extracts on PRR pathways. Despite

* Corresponding author. Tel.: +1 425 602 3144; fax: +1 425 602 3079.
E-mail address: lprice@bastyr.edu (L.A. Price).

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considerable similarity between yeast and mushroom beta glucans, these yeast polysaccharides differ from the mushroom counterparts in size, length, branching locations, polysaccharide ratios, and protein backbones [10–14]. In addition, mushroom preparations, which contain several secondary metabolites, result in a more complex chemical mixture than yeast.

This study involved investigating the effect of three distinct mushroom preparations on inflammatory cytokine secretion by murine cells. The three preparations included polysaccharide Krestin (PSK), which is a hot water extract from *T. versicolor*; a Reishi extract from *G. lucidum*; and scleroglucan, which is a highly purified preparation from *Sclerotium rolfsii* most like beta glucan from yeast containing beta glucan with 1,3 and 1,6 linkages. PSK and Reishi, have similar beta glucan composition with primarily 1,4 and 1,6 linkages, yet differ in secondary metabolite profiles [15].

This line of research will help to direct future research to investigate pattern recognition receptor binding, and immune modulating activities of novel cancer therapeutic agents that may promote or enhance anti-tumor immunity.

There is much scientific evidence dating back to the 1970s regarding inflammatory cytokine induction by mushroom preparations. To date, only one study found that a different mushroom, Reishi, binds to TLR4. Our results show that the mushroom preparations interact with distinct PRRs to induce TNF-alpha and IL-6 secretion in J774A.1 cell cultures. The objective of the study is to determine the involvement of mushroom ligands with PRRs.

2. Material and methods

2.1. Materials

Polysaccharide Krestin is a proteopolysaccharide produced from the mushroom *T. versicolor*. Compositional analysis of PSK was performed at the Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia. PSK was found to be composed of 92.9% carbohydrate, mainly mannose and glucose. Linkage analysis showed mainly 22% of 4-Glc, 19% of 3-Glc, 13% of terminal glucose and 20% of doubly linked residue indicating branching.

PSK whole dried powder from Kureha, Inc., (Tokyo, Japan) was solubilized in 20% anhydrous alcohol and 80% endotoxin free water to create 10 mg/mL final concentration. Batched aliquots were stored at −80 °C until use. LPS and polymyxin B sulfate was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Reishi extract was provided by JHS, Natural Products (Eugene, Oregon, USA). Whole beta glucan particle was generously donated by Dr. Gary Ostroff, University of Massachusetts. Macrophage J774A.1 murine cells were originally from ATCC (Manassas, Virginia, USA). RPMI 1640 was supplemented with 2 mM glutamine, 10% FBS, and 2 mM sodium pyruvate. Ficoll was obtained from GE Healthcare, USA, and PBS from Mediatech, Inc. (Herndon, Virginia, USA). Murine monoclonal blocking antibodies anti-CR3 (clone M1/70), anti-TLR4 (clone MTS510), anti-TLR2 (clone 6C2), were obtained from eBioscience, USA, and anti-dectin-1(clone 2A11) from Cell Sciences, USA (dectin-1; clone 2A11). TNF-alpha and IL-6 ELISA Duoset kits were obtained from R&D Systems (Minneapolis, Minnesota, USA). ELISA plates were analyzed using a Molecular Devices Spectramax Plus microplate reader.

2.2. Animals

TLR4+/+ and TLR4−/− C57BL/6 male mice were obtained from Jackson Laboratories (Bar Harbor, Massachusetts). The animals were maintained and cells were harvested at the University of Minnesota Veterans Administration Hospital. All mice were maintained in a laminar airflow cabinet under pathogen-free conditions and sacrificed at 8–12 weeks. Animal facilities were approved by the American Association for Accreditation of Laboratory Animal Care in accordance with the current regulations and standards of the U.S. Department of Agriculture, U.S. Department of Health and Human Services, and NIH.

2.3. Murine peritoneal macrophage and splenocyte preparation

Following intraperitoneal injection of 1.5 mL of the thioglycolate, peritoneal macrophages were obtained from sacrificed mice at 24 h via intraperitoneal irrigation with 3 mL of media. The abdomens were excised and spleens harvested. The macrophages and spleens were washed, then shipped in separate 10 mL sterile vials filled with media from University of Minnesota to Bastyr University for experiments. Upon receipt, macrophages were suspended in PBS and then centrifuged at 1400 rpm, washed again in PBS, and again suspended in media.

Murine spleens were macerated in steel mesh splenoctye suspensions, then collected and overlaid onto Ficoll and centrifuged at 25 °C and 2000 rpm for 25 min. Buffy coats were harvested and washed twice in PBS, and splenocytes resuspended in media.

2.4. Establishment of a standard response curve for PSK

J774A.1 cells were placed in 24 well plates at 5×10^5 cells/mL in 0.5 mL of media. Cells were incubated for 20 to 24 h at 37 °C, and 5% CO_2 and visually observed at 20–24 h for viability, morphological changes, and contamination at which time media was replaced and treatment conditions added. PSK was added to the media and cells at concentrations of 0, 62.5, 125, 250, 500, and 1000 μg/mL. Cells were incubated at 37 °C and 5% CO_2 for 24 h, then observed for morphology and viability. Supernatants were collected and frozen at −80 °C until time of assay for TNF-alpha production by ELISA. Concentrations of test substances that resulted in sub-maximal TNF-alpha secretion (between 80 and 90% of the maximal concentration) were selected in order to maximally inhibit TNF-alpha secretion in the receptor-specific blocking antibody studies.

2.5. Endotoxin analysis

Endotoxin (LPS) is a well established ligand of TLR4 and therefore a possible contaminant in the extracts under study which if present could contribute to TLR4 dependent cytokine secretion, confounding results. Results of independent endotoxin testing done on PSK and Reishi by Associates of Cape Cod, Inc. yielded the following results: less than 0.002 ng/mL of endotoxin was detected at 62.5 μg/mL PSK, and less than