**RESULTS**

1. PSK inhibits the growth of spontaneous breast tumors in neu transgenic mice. Shown are average tumor size (measurments, n=5 in each group) in PSK (A) and control PBS (B) group. PSK (100mg/kg, oral gavage, 3 times/week) was given for 4 weeks. The average tumor size was

2. PSK stimulates splenocytes proliferation and cytokine secretion. (A) PSK (1.200ug/ml) stimulates the proliferation of splenocytes from neu-tg mice dose-dependently. (B) PSK stimulates the secretion of multiple inflammatory cytokines, including IL12, CXCL1 (IL-8), and TNF.

3. PSK activates bone marrow-derived dendritic cells. (A) PSK stimulates CD11c+ DC maturation, resulting in higher percentage of CD86+MHCIIhigh DC. (B) PSK-treated DC secretes higher amount of IL12p40. (C) PSK-treated DC secretes higher amount of IL12p70. *, p<0.05

4. PSK activates TLR2 but not other TLRs. (A) PSK (0.5-1500ug/ml, overnight incubation) activates TLR2 but not other TLRs. The known agonist for the other TLRs (positive control) worked as expected (data not shown).

5. The immunostimulatory effect of PSK is mediated through TLR2. (A) Splenocytes from TLR2-/- mice and WT mice were treated with PSK (25-400ug/ml, 72 hour). PSK stimulated the secretion of TNF in splenocytes from WT mice but not from TLR2-/- mice. *, p<0.05, ** p<0.01.

**CONCLUSIONS**

- PSK inhibits the growth of spontaneous breast tumors in neu-tg mice.
- PSK stimulates splenocytes from neu-tg mice to proliferate and secrete multiple proinflammatory cytokines.
- PSK activates DC maturation and IL-12 secretion.
- PSK activated TLR2 but not other TLRs.
- The effect of PSK on activating T cells and DC is dependent on TLR2 activation.

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**INTRODUCTION**

- Polysaccharide Krestin (PSK), a high MW polysaccharide (95-100kDa), is a hot water extract of Coriolus Versicolor (Bunzi), a medicinal mushroom.
- PSK has been widely used in Asia for its anti-cancer and immunostimulatory effect. We studied the mechanisms of the anti-tumor effect of PSK using neu transgenic mice, a model of human HER2+ breast cancer.
- PSK inhibits tumor growth and activates DC and T cells in this mouse model. The current study aim to investigate the role of Toll-like receptors (TLRs) in the immunostimulatory effect of PSK.
- TLRs are a family of pathogen recognition receptors that plays an important role in linking innate to adaptive immunity. TLR ligation is critical in mediating anti-viral and anti-tumor immunity.

**MATERIALS AND METHODS**

- **Mouse model**: neu-transgenic mice [strain name, FVB/N-TgN (MMTVneu)-202Mul] was obtained from the Jackson Laboratory (Bar Harbor, ME). TLR2-/- mice was originally obtained from Dr. Shizuo Akira (Osaka University, Japan). All of the procedures were performed in compliance with the University of Washington Institutional Animal Care and Committee.
- **Tumor growth inhibition experiment**: For the effect of PSK on tumor growth, PSK, or control PBS treatments started at the onset of palpable tumors (<5mm3). PSK (from Kureha Inc., 100mg/kg) was given three times a week via oral gavage. 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 x width x height x 0.5236.
- **Bone marrow-derived dendritic cell (BMDC) culture and activation**: BMDC was cultured using standard protocol. In brief, bone marrow cells were collected from the marrow of femurs of mice and cultured in 24-well plates in complete RPMI medium (RPMI with 10% FBS, 50mM beta- mercaptoethanol, and penicillin/streptomycin). After 6 day of culture with recombinant murine (m) GM-CSF and mll4-4, the cells were split and cultured in the presence of PSK (200ug/ml), LPS (100ng/ml, positive control), or PBS (negative control). After 48h treatment, culture supernatant from each well was collected for ELISA analysis. The adherence cells were detached and stained with anti-CD11c-APC, anti-CD80-PE, anti-CD86-PerCP for FACS analysis.
- **Cell activation**: Splenocytes from neu transgenic mice, TLR2 knockout mice, or WT BL/6 mice were cultured in complete RPMI in the presence of PSK (10-200ug/ml) or control PBS. After incubation at 37°C for 48 hours, [3H]thymidine was added to measure cell proliferation. The culture supernatant from each culture well was transferred to another 96-well plate and 160ul Quanti-BLUE(InvivoGen) was added to each well. OD650 were measured after either 3 hour or overnight incubation at 37°C.
- **Statistical analysis**: Statistical analysis was performed using GraphPad (GraphPad Software, San Diego, CA). Data were analyzed using the Student's t test or single factor ANOVA. A p value of less than 0.05 was considered statistically significant.

**HEK-TLR assay**: HEK cells transfected with different TLR2, 3, 4, 7, 8, or 9 were purchased from InvivoGen. Ninety-six well plates were seeded with 50,000 cells per well and transfected with pNlItf-SEAP plasmid overnight. Then fresh medium with PSK (0.01-1000ug/ml, 1:5 dilution) or positive control ligand for each TLR (Pam3CSK4 for TLR2, poly I:C for TLR3, LPS for TLR4, imiquimod for TLR7, resiquimod for TLR8, Cpg2006 for TLR9) was added. After overnight culture at 37°C, twenty ul of supernatant from each culture well was transferred to another 96-well plate and 10μl of SEAP Quanti-Blu (InvivoGen) was added to each well. OD450 were measured after either 3 hour or overnight incubation at 37°C.

**Cytokine ELISA**: ELISA was used to measure the cytokine levels (TNFα, IL12p40, IL12p70) in cell culture supernatant using kits from eBiosciences following the manufacturer recommended procedures. In some experiment, a multiplex mouse TH-1 cytokine kit from Mesa Scale Discovery (Gaithersburg, MA) was used to measure multiple cytokines simultaneously.

- **Statistical analysis**: Statistical analysis was performed using GraphPad (GraphPad Software, San Diego, CA). Data were analyzed using the Student's t test or single factor ANOVA. A p value of less than 0.05 was considered statistically significant.