Polysaccharide of *Atractylodes Macrocephala* Koidz(PAMK) Enhance the Immunomodulatory Activity of RAW264.7 Through TLR4/MyD88/NF- κ B Pathway

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Abstract

The Polysaccharide of *Atractylodes macrocephala* Koidz (PAMK) has been reported to play an important role in improving the immune function of mammals and poultry. But the molecular mechanism in cell is still not unclear. For investigating the mechanism in mice, RAW264.7 were cultured and divided into six groups (cell, NC, siTLR4, NC + PAMK, PAMK, siTLR4 + PAMK). The serum cytokines, mRNA relative expression level of cytokines and TLR4 signal pathway, the protein expression level of TLR4 signal pathway were measured. The results showed PAMK could increase the level of IL-4, IL-6, TNF- α , IFN- γ , and increase the mRNA relative expression level and protein expression level of TLR4, MyD88, IKK, NF- κ B. In conclusion, PAMK may regulate the immune activity of RAW264.7 through the TLR4/MyD88/NF- κ B pathway.

1. Introduction

Polysaccharide of *Atractylodes macrocephala* Koidz (PAMK) as one of the main extracts of *Atractylodes macrocephala*, which is a traditional medicine and has been utilized for at least 2000 years. PAMK was often used to treat diarrhea, spleen and stomach disease, respiratory infection etc¹. PAMK has anti-inflammatory, anti-cancer activities, maintains the balance of gastrointestinal microbiome and regulates the organism's immunity balance¹. In addition, PAMK promotes the proliferation of lymphocytes which in spleen and thymus of human, chicken, and mice, cause it could induce T lymphocytes enter S and G2/M phases, and effectively elevates the percentage of CD4 + and CD8 + T cells⁴. PAMK also increases the index of spleen and thymus in geese, upregulates the level of interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interferon- γ (IFN- γ), Immunoglobulin G (IgG) in serum of mice and geese^{5; 6}. However, Xu⁷ indicated that PAMK could decrease the high level of Tumor Necrosis Factor- α (TNF- α), interleukin-1 (IL-1), H₂O₂ caused by heat stress (HS) in geese. Jeong D⁸. suggested that PAMK down regulated the high level of pro-inflammatory cytokines (IL-1 β , IL-6 and TNF- α) caused by Lipopolysaccharides (LPS). In our previous study, mice spleen was used to detect the mechanism of PAMK, and find that PAMK could enhance cytokines secretion by stimulating the TLR4/MyD88/NF- κ B signaling pathway⁸. However, the mechanism of PAMK in regulating the immune function of macrophages was still not clear. Therefore, RAW264.7 was used in this experiment.

TLR4 plays an important role in immunomodulatory, which could recognize the pathogen-related molecular patterns, transmit signal molecules to the cytoplasm, and promote the production of pro-inflammatory factors¹⁰. There are two ways to activate the TLR4/NF- κ B signal pathway: the dependent of myeloid differentiation factor 8 (MyD88) signal pathway and independent of MyD88 signal pathway. When TLR4 was active, the intracellular TIR region binds to the carboxyl group of MyD88, and its amino group binds to IL-1 amino group to activate IL-1 receptor-associated kinase (IRAK), IRAK activates TNF-receptor-associated factor 6 (TRAF6), TRAF6 activates inhibitor of NF- κ B kinases (IKKs), and the NF- κ B inhibitor phosphorylates and degrades under the action of IKKs compound to activate NF- κ B, then induces the expression of specific cytokines (IL-1, IL-6, IL-8, IL-12) in the nucleus¹¹. As some article suggested that

polysaccharide regulate the immune function through TLR4 signal pathway. Some researchers indicated that a novel polysaccharide obtained from *Craterellus cornucopioides*, polysaccharides from *Cordyceps gunnii mycelia*, polysaccharide from *wheat bran* could induce lymphocyte proliferation, promote macrophage activation and phagocytic activity, promote cytokines IL-2, IL-6, IL-1 β , TNF- α and IFN- γ secretion and increase immune function through TLR4 signal pathway¹²⁻¹⁴.

Based on previous research, we preliminary confirmed that PAMK promote cytokines secretion by stimulating TLR4/MyD88/NF- κ B signal pathway in mice spleen. Thus, we chose RAW264.7 as immune cell model in vitro, and measured serum cytokines of cellular supernatant, mRNA relative expression of cytokines and TLR4 signal pathway, protein expression level of TLR4 signal pathway by ELISA, qPCR, and western blot to further confirm that PAMK play an important role in promote the cytokines through TLR4 signal pathway in RAW264.7.

2. Results

2.1 The effects of different TLR4-siRNAs on TLR4 mRNA relative expression

Total RNA was extracted from RAW 264.7 to measure the mRNA relative expression of TLR4. As presented in Fig. 1, the results showed that si-TLR4-2 at 100 nM has the best inhibitory effect and efficiency of inhibiting TLR4 expression was up to 72 %.

PAMK promoted the cytokines production of RAW264.7 cellular supernatant

The cellular supernatant of six groups was collected, and then measure cellular supernatant cytokines level. The results in Fig. 2A-Figure2D revealed that all the cytokines were significantly ($P < 0.05$) down regulated by siTLR4 when compared with NC group. cellular supernatant cytokines levels in cell group and NC group showed no significant difference, and PAMK group and NC + PAMK group showed no significant difference ($P > 0.05$), which means that lip 3000 has no toxic side effect to RAW264.7. However, the cellular supernatant of all cytokines treated with PAMK were significantly ($P < 0.05$) upregulated when compared with siTLR4 group, which indicated that PAMK could promote cellular supernatant cytokines production.

2.2 PAMK promoted the mRNA relative expression of cytokines

Total RNA was extracted from RAW 264.7 to measure the mRNA relative expression of cytokines. Four genes were tested by qPCR. As showed in the Fig. 2E-Figure 2H, the results indicated that the relative mRNA of IL-2, IL-4, TNF- α , and IFN- γ in siTLR4 was significantly ($P < 0.05$) decreased when compared with NC group. The relative mRNA expressions of TNF- α , and IFN- γ were significantly ($P < 0.05$) upregulated and IL-2, IL-4 had the uptrend in siTLR4 + PAMK group compared to siTLR4 group. Compared with cell

group and NC + PAMK group respectively, the relative mRNA expression showed no significant ($P > 0.05$) difference in NC group and PAMK group. All the results indicated that PAMK could promote relative mRNA of cytokines expression production.

2.3 PAMK promoted the relative mRNA expression of TLR4 signaling pathway.

Total RNA was extracted from RAW 264.7 to measure the relative mRNA expression of TLR4 signal pathway. The results were showed in Fig. 3. The relative mRNA expressions of TLR4, MyD88, NF- κ B, IKK were significantly ($P < 0.05$) down regulated in siTLR4 group compared with NC group. However, when treating with PAMK, NF- κ B significant ($P < 0.05$) increased and TLR4, MyD88 and IKK had the uptrend. The difference between cell group and NC group, NC + PAMK group and PAMK group has no significant difference ($P > 0.05$). Thus, the results indicated that PAMK could promote relative mRNA expression of TLR4 signal pathway.

2.4 PAMK regulated the relative protein expression in the TLR4 signaling pathway

Proteins were extracted from RAW 264.7 to measure the relative protein level of NF- κ B, TLR4, MyD88, IKK, GAPDH. The relative protein expression level of the genes were showed in Fig. 4. The results indicated that in siTLR4 group, the relative protein expression level of all genes were significantly ($P < 0.05$) increased than NC group. The relative protein expression of TLR4 was significant upregulated ($P < 0.05$) and NF- κ B, MyD88, IKK has the uptrend in siTLR4 + PAMK group compared with siTLR4 group. The relative protein expression level has the same trend with the relative mRNA expression level. The above results suggested that PAMK could regulate the protein expression level in the TLR4 signaling pathway.

3. Discussion

PAMK has the function of promoting the lymphocytes active and proliferation, maintaining the stable cell morphology, increasing the secretion of cytokines such as TNF- α , IL-4, IL-6, IL-10, IL-17, IL-17A, IFN- γ , alleviating the immunosuppression caused by CTX, and increasing the index of immune organs, which was considered one of the characteristic of the immune level enhance^{15; 16}. We all know that cytokines play a very important role in body immunity^{15; 17; 18} and macrophages play an important role in defending the body against invading pathogens^{15; 17; 18}. Increased level of IL-4, IL-2, TNF- α and IFN- γ could enhance the macrophage activity and improve specific immunity. In our research, we found that PAMK could upregulated the mRNA and protein expression of IL-2, IL-4, TNF- α and IFN- γ caused by siTLR4. IL-2 can enhance the secretion of TNF- α , IL-1, NO and other cytokines from macrophages, enhance the cytotoxic effect of macrophages and increase the killing activity of macrophages. IL-4 can increase the tumor killing ability of bone marrow M ϕ and the expression of Fc(II) receptor, and promote the transformation of macrophages from M1 to M2 type. From our research, the results indicated that the relative mRNA expression level and cellular supernatant level of IL-2, IL-4 were significant decreased by

siTLR4, but it significantly increased or had an increase trend after PAMK treatment. Thus, the proliferative activity of macrophages is also increased, and the secretion levels of TNF- α and IFN- γ also increased when treated with PAMK, just as our research showed. According to our research, we suggested that PAMK could enhance macrophage activity and enhance the release of TNF- α and IFN- γ to increase macrophage immunomodulation. S, Li, Shi et al. found polysaccharide from *Auricularia auricula*, fungal polysaccharide could promote the activation of macrophages and induce the secretion of cytokines such as IL-1 β , IL-6, NO and TNF- α to enhance the phagocytic activity of macrophages and increase macrophage immunomodulation^{19; 21; 22}. However, Zeng, Ma, G, Wang et al. indicated that *Lonicerae Japonicae Flos* and *Lonicerae Flos*, polysaccharide from *Pleurotus eryngii* and *Procyanidin A2* could inhibit NO secretion in RAW264.7, down-regulate IL-1 β , IL-6 and TNF- α release, down-regulate iNOS protein and COX2, NF- κ B (p-IKK α / β , p-IkB α and p-p65) and MAPK (p-p38, p-JNK and p-ERK) protein levels caused by LPS²³⁻²⁵. These results were similar to our research. Consequently, we suggested that PAMK could maintain stable immune levels of macrophages and maintain the body's immune balance when the body is stimulated by either LPS or siTLR4, etc.

TLR4, an important member of the TLR family, was the first upstream gene for NF- κ B (NF- κ B) and has been shown to be involved in immune responses²⁶. Some research suggested that when TLR4 is activated, there are two conduction signal pathways: dependent myeloid differentiation factor 88 (MyD88) signaling pathway and non-dependent MyD88 signaling pathway. The activated TLR domain of TLR4 binds to PAMK in a complex that can bind to MyD88. Interleukin-1 receptor associated kinase 1 (IRAK1), IRAK4 and TNF receptor associated factor 6 (TRAF6) recruit to the TLR-MyD88 complex, resulting in phosphorylation of IRAK1 and TRAF6. The signal is propagated by the phosphorylated linker molecular complex to downstream MAP kinases (JNK, p38 MAPK), Ap-1 (AP1) and the nuclear factor κ -B kinase (IKK) complex NF- κ B. Activation of inhibitor of IKK α / β phosphorylates inhibitor of NF- κ B (I κ B), which is shed from NF- κ B and ubiquitinated, allowing NF- κ B to be activated by the inhibitory state leading to transcription factor activation^{12; 27; 28}. In our research, we indicated that PAMK could increase the relative mRNA expression level and protein expression level of TLR4, MyD88, IKK α / β , NF- κ B in siTLR4 + PAMK group when it compared with siTLR4 group, and we found that the trend of mRNA and protein level was the same. Thus, we suggested that PAMK activates IKK through a MyD88-dependent pathway, which phosphorylates I κ B and makes it off NF- κ B and allows NF- κ B to enter the nucleus to exert immunoregulatory effects. When NF- κ B signal pathway was activated, cells would secrete cytokines to maintain the immunity balance while the organism is stimulated, such as IL-2, IL-4, TNF- α and IFN- γ , just as our research showed. Li indicated that PAMK could increase the serum level of IL-2, IL-4, TNF- α and IFN- γ , enhance splenocytes immunomodulatory activity by TLR4/MyD88/NF- κ B signal pathway⁹. Some researchers showed that Polysaccharides from *Cordyceps gunnii* mycelia could increase the spleen thymus index, up-regulate the serum level of IL-2, IL-12, IFN- γ , IgG and other cytokines secretion levels, protect immunosuppressed mice through TLR4/TRAF6/NF- κ B signaling pathway²⁹. These findings were consistent with previous research. However, Zheng, He and Jun suggested that *Diethyl Blechnic Exhibits*, an inulin-type fructan from *Codonopsis pilosula*, a novel polysaccharide from *Acorus tatarinowii* could inhibit release of pro-inflammatory factors caused by LPS such as prostaglandin E2 (PGE2) and relative

mRNA expression of TNF- α , IL-1 β , IL-6 and myeloperoxidase (MPO) through TLR4/MyD88/NF- κ B in RAW264.7^{30–33}. That also indicated that polysaccharide regulate RAW264.7 immune function by TLR4/MyD88/NF- κ B signal pathway.

In conclusion, our research indicated that PAMK could increase the relative mRNA expression level and serum cytokines level of IL-2, IL-4, TNF- α and IFN- γ , enhance the immunomodulatory activity of RAW264.7 by TLR4/MyD88/NF- κ B signal pathway

4. Methods

4.1 Culture, grouping and transfection of RAW264.7

PAMK (purity 95 %) was purchased from Yanglingciyuan Biotechnology company (Xi'an, China). PAMK was diluted to 12.5 μ g/mL with RAW264.7 culture medium (Procell Life Science Technology Co.,Ltd.,Wuhan, Chian). Mice mononuclear macrophage leukemia cells RAW264.7 (Zhongqiao Xinzhou Biotechnology Co., Ltd, Shanghai, China), adjusted to a density of 1×10^6 cells/mL, and cultured in a 12-well plate (Corning Incorporated, USA). After 4 h of culture, randomly devided into six groups (cell, NC, siTLR4, NC + PAMK, PAMK, siTLR4 + PAMK). Transfection reagent NC, siTLR4 (RiboBio Co., LTD, Guangzhou, China), (Table 1), and PAMK was added, and cells were collected 24 h later for mRNA relative expression detection.

Table 1
Sequences for siTLR4

cDNA	Primer sequences	cDNA	Primer sequences
si-TLR4-1	CAATTCTGTTGCTTGTATA	si-TLR4-3	CCATGAACTGACTCTAAGA
si-TLR4-2	GTCCCTGATGACATTCCT		

4.2 Quantitative reserve-transcription PCR analysis

Total RNA was extracted from the RAW264.7 with TRIzol reagent (Thermo Fisher, USA) and reverse-transcribed was performed by the Reverse Transcription Kit (Takara, Japan). The primers (Table 2) of related genes were designed by using Primer Premier 5.0 software (Premier Biosoft International, USA). An ABI PRISM 7500 detection system (Applied Biosystems, Foster City, CA) was used to measure the mRNA relative expression of genes.

Table 2
Primer sequences for qPCR

cDNA	Primer sequences	cDNA	Primer sequences
TLR4-F	ACCTGGAATGGGAGGACAA	TLR4-R	GTCCAAGTTGCCGTTTCTTG
β -actin-F	GCTTCTAGGCGGACTGTTAC	β -actin-R	CCATGCCAATGTTGTCTCTT
MyD88-F	GGCAACTAGAACAGACAGA	MyD88-R	GTGCAAGGGTTGGTATAGTC
IKK-F	TGCAGCTGATGCTTATGTG	IKK-R	TGCCTGTGACCATGTTCAA
NF κ B-F	GAACCAGGGTGTGTCCATG	NF κ B-R	TCCGCAATGGAGGAGAAGTC
IL2-F	CCTGAGCAGGATGGAGAAT	IL2-R	CGCAGAGGTCCAAGTTCAT
IL4-F	GAGATGGATGTGCCAAACG	IL4-R	AGCACCTTGGAAGCCCTACA
IFN- γ -F	GGCTTCCATCATGAACAAC	IFN- γ -R	TGTAGGTGAGGTTGATCTTTC
TNF- α -F	TCGAGGCTCCAGTGAATT	TNF- α -R	GCTTCTAGGCGGACTGTTAC

4.3 Assays of cell cytokines in cellular supernatant

The cellular supernatant of six groups was collected.. The levels of IL-2 (70-EK2022/2), IL-4 (70-EK2042/2), IFN- γ (70-EK2802/2), and TNF- α (70-EK2822/2) were measured by using ELISA kits (Hangzhou Lianke Biotechnology Co., Ltd., Hangzhou, China).

4.4 Western blot analysis

The total protein was extracted by RIPA (Biyuntian Biological Technology Co., Ltd., Shanghai, China), adjusted the concentration with 5 × simple buffer (Biyuntian Biological Technology Co., Ltd., Shanghai, China), and denatured by heating at 100 °C for 10 min. We analyzed it by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditio 12 % gels were used to transfer to PVDF membranes using a tank transfer system at 200 mA in Tris-glycine buffer containing 20 % methanol for 70–90 min at 4°C. The membranes were then blocked with 5% skim milk at 37 °C for 60 min. Next, the membranes were incubated with diluted primary antibodies overnight; the diluted concentrations and antibody sources are shown in Table 3. We incubated peroxidase-conjugated secondary antibodies against rabbit IgG at room temperature for 1 h (1:10000, Beijing Boasens Biotechnology Co., Ltd, China). Chemiluminescence Detection Reagents (Applygen, Beijing) were incubated for 1 min to determine the ratios of target protein signals on a Chemiluminescence Imaging System (Tianneng, Shanghai) to signals of control proteins. GAPDH (Abcam) served as internal controls, and the relative expression levels of the proteins were expressed as the ratios of gray value of each of these proteins to that of GAPDH.

Table 3
Diluted concentrations and antibody sources

Antibody	Antibody sources	Antibody diluted
TLR4	AF7017	1:1000
MyD88	AF5195	1:1000
IKK	AF6014	1:2000
NF-κB	AF5006	1:2000
GAPDH	Abcam9484	1:1000

4.5 Statistical analysis

The data were analysed using SPSS for Windows (version 18, SPSS Inc, Chicago, IL, USA) and expressed as the means $\pm$ standard deviations (SD). All the qPCR assays were repeated in triplicate, and the relative expression levels were measured in terms of threshold cycle (Ct) values and normalized via the formula $2^{-\Delta\Delta C_t}$. Differences between groups were compared using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference test. $P < 0.05$ was considered statistically significant.

Declarations

Conflict of interest statement

There is no conflict of interest in this manuscript. The content has not been published or submitted for publication elsewhere. All authors are in agreement with the content of the manuscript and submission in this journal.

Acknowledgement

Wanyan Li contributed to the hypothesis generation. Yunbo Tian contributed to experimental design. Wenbin Chen contributed to data interpretation. Xumeng Zhang contributed to manuscript preparation. Xuelian Xiang conducted the experiments. Nan Cao contributed to the data interpretation. Danning Xu participated in the experiment. Wenjun Liu contributed to collection of experimental samples. Xinliang Fu was responsible for the purchase of related materials. Jia Liu contributed to data processing. All authors reviewed the manuscript.

Founding

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Figures

Figure 1

The effects of different TLR4-siRNAs on TLR4 mRNA relative expression. Data were expressed as the means $\pm$ SD, n = 3. *P < 0.05, **P < 0.01, compared with the NC group. .

Figure 2

Effects of PAMK on cellular supernatant and relative mRNA expression level of cytokines in mice RAW 264.7 treated with siTLR4. Cellular supernatant cytokines levels of (A) IL-2; (B) IL-4; (C) TNF- α ; (D) IFN- γ ; Relative mRNA expression of (E) IL-2; (F) IL-4; (G) TNF- α ; (H) IFN- γ ; Data were expressed as the means $\pm$

SD, n = 6. Means with different letters represent statistically significant difference ($P < 0.05$); the bars with the same letter are not significantly difference ($P > 0.05$).

Figure 3

Effects of PAMK on the TLR4 signal pathway in mice RAW264.7 treated with siTLR4. Relative mRNA expression of (A) TLR4; (B) MyD88; (C) NF-κB; (D) IKK; Data were expressed as the means ± SD, n = 6. Data were expressed as the means ± SD, n = 6. Means with different letters represent statistically significant difference ($P < 0.05$); the bars with the same letter are not significantly difference ($P > 0.05$).

Figure 4

Relative protein level. Data were presented as the means $\pm$ SD, $n = 3$. # $P < 0.05$, compared with the NC group; * $P < 0.05$, compared with the siTLR4 group.

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