# Study on the Antagonistic Effects of Lentinan on Palmitic Acid-Induced Lipid Accumulation and Inflammatory Cytokine Secretion in Macrophages

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## Abstract

[Objective]: To investigate the antagonistic effects of Lentinan (LNT) on palmitic acid (PA)-induced lipid accumulation and inflammatory cytokine secretion in macrophages, providing an experimental basis for the application of LNT in metabolic inflammation-related diseases.

[Methods]: RAW264.7 macrophages were divided into a blank control group, a PA model group, and LNT low/medium/high-dose intervention groups. RAW264.7 cells were treated with different concentrations of PA to construct lipid accumulation and inflammation models, and the optimal modeling concentration was screened; in subsequent experiments, the LNT intervention groups were pre-treated with corresponding concentrations of LNT before being co-cultured with the optimal concentration of PA. The secretion levels of interleukin-1$\beta$ (IL-1$\beta$) and interleukin-6 (IL-6) in the cell supernatant were detected by enzyme-linked immunosorbent assay (ELISA). Intracellular triglyceride (TG) and total cholesterol (TC) contents were detected using biochemical kits. The expression levels of key proteins in lipid metabolism and inflammation-related signaling pathways (PPAR$\gamma$, NF-$\kappa$B p65) were detected by Western blot.

[Results]: Compared with the blank control group, the intracellular lipid droplets in the PA model group increased significantly, the TG and TC contents were significantly elevated ($P < 0.05$), the secretion of IL-1$\beta$ and IL-6 in the cell supernatant increased significantly ($P < 0.05$), the expression level of PPAR$\gamma$ was downregulated, and the protein expression level of p-NF-$\kappa$B p65 was significantly upregulated ($P < 0.05$). Compared with the PA model group, the intracellular TG and TC contents in the LNT low-dose or high-dose intervention groups were significantly reduced, the secretion of IL-1$\beta$ and

IL-6 decreased significantly ($P < 0.05$), the expression level of PPAR$\gamma$ was significantly upregulated while the expression level of p-NF-$\kappa$B p65 was downregulated ($P < 0.05$), showing a certain dose-dependency.

## Full Text

# Antagonistic Effects of Lentinan Against Palmitic Acid-Induced Lipid Accumulation and Inflammatory Cytokine Secretion in Macrophages

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**Abstract Objective:** To investigate the antagonistic effects of lentinan (LNT) on lipid accumulation and inflammatory cytokine secretion in macrophages induced by palmitic acid (PA), and to provide experimental evidence for the application of LNT in metabolic inflammation-related diseases. **Methods:** RAW264.7 macrophages were divided into a control group, a PA model group, and low/medium/high-dose LNT intervention groups. Lipid accumulation and inflammatory models were established by treating RAW264.7 cells with different concentrations of PA to determine the optimal modeling concentration. In subsequent experiments, LNT intervention groups were pretreated with corresponding concentrations of LNT before co-incubation with the optimal concentration of PA. Secretion levels of interleukin-1$\beta$ (IL-1$\beta$) and interleukin-6 (IL-6) in the cell supernatant were detected by enzyme-linked immunosorbent assay (ELISA). Intracellular triglyceride (TG) and total cholesterol (TC) contents were measured using biochemical kits. Western blot was employed to detect the expression levels of key proteins in lipid metabolism and inflammation-related signaling pathways (PPAR$\gamma$ and NF-$\kappa$B p65). **Results:** Compared with the control group, the PA model group showed a significant increase in intracellular lipid droplets, significantly elevated TG and TC contents ($P < 0.05$), and significantly increased secretion of IL-1$\beta$ and IL-6 ($P < 0.05$). Furthermore, PPAR$\gamma$ expression was downregulated while p-NF-$\kappa$B p65 expression was significantly upregulated ($P < 0.05$). Compared with the PA model group, LNT intervention significantly reduced intracellular TG and TC contents and decreased IL-1$\beta$ and IL-6 secretion ($P < 0.05$). LNT also significantly upregulated PPAR$\gamma$ expression and downregulated p-NF-$\kappa$B p65 expression ($P < 0.05$) in a dose-dependent manner. **Conclusion:** LNT antagonizes PA-induced lipid accumulation and inflammatory cytokine secretion in RAW264.7 macrophages. The underlying mechanism may be related to the regulation of the PPAR$\gamma$/NF-$\kappa$B signaling pathway.

**Keywords:** Lentinan; Palmitic acid; Macrophages; Lipid accumulation; Inflammatory cytokines

Non-alcoholic fatty liver disease (NAFLD) is a prevalent global chronic liver disease characterized by hepatic lipid accumulation and steatosis, with a prevalence of approximately 25% [**?**]. In the *Guidelines for the Prevention and Treatment of Metabolic Associated (Non-alcoholic) Fatty Liver Disease (2024 Edition)*, experts have renamed NAFLD to Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD) [**?**]. In China, the incidence of NAFLD is increasing exponentially alongside the aging population. Due to its long treatment cycles and poor prognosis, NAFLD significantly diminishes quality of life and increases economic burdens, becoming a major public health concern [**?**]. As the disease progresses, it can develop into non-alcoholic steatohepatitis (NASH), also known as metabolic dysfunction-associated steatohepatitis (MASH). MASH can further evolve into cirrhosis or hepatocellular carcinoma and is closely associated with cardiovascular diseases, chronic kidney disease, and extrahepatic malignancies [**?**].

Lentinan (LNT) is a natural $\beta$-glucan active ingredient extracted from the fruiting bodies of *Lentinula edodes*. It possesses various biological activities, including immunomodulation, anti-inflammation, and lipid-lowering effects. Studies have confirmed that LNT can improve hepatic lipid accumulation in MASLD and inhibit hepatic inflammation in MASH [**?**, **?**]. Macrophages in the liver are the primary cells responsible for secreting inflammatory cytokines; therefore, LNT' s regulation of hepatic inflammation is believed to be linked to the inflammatory immune activity of macrophages. Palmitic acid (PA), a common saturated fatty acid, shows increased cellular uptake in MASH patients due to upregulated transport protein expression. PA exhibits lipotoxicity and is a critical factor driving the progression of NASH toward end-stage liver disease [**?**]. Research indicates that excessive PA intake induces lipid accumulation and activates inflammatory responses in macrophages [**?**]. Currently, the antagonistic effects and specific mechanisms of LNT against PA-induced macrophage lipid accumulation and inflammation remain unclear. RAW264.7 macrophages serve as an ideal model for studying metabolic inflammation and pharmacological interventions.

This study utilizes PA-induced RAW264.7 macrophages to construct a lipid accumulation-inflammation model. We aim to investigate the effects of LNT on lipid content and inflammatory signaling in this model, providing an experimental and theoretical basis for developing LNT as a potential intervention for metabolic inflammation-related diseases.

### 1.1 Materials and Reagents

Mouse RAW264.7 macrophages were purchased from the Cell Bank of the Typical Culture Preservation Committee of the Chinese Academy of Sciences. Lentinan (LNT, purity $\geq 90\%$) was purchased from Sigma-Aldrich, and palmitic acid (PA, purity $\geq 99\%$) was obtained from Shanghai Yuanye Bio-Technology Co., Ltd. DMEM high-glucose medium and fetal bovine serum (FBS) were purchased from Invitrogen Gibco. Oil Red O staining kits, TG detection kits, and TC de-

tection kits were purchased from Nanjing Jiancheng Bioengineering Institute. ELISA kits for IL-1$\beta$ and IL-6 were obtained from Wuhan Boster Biological Engineering Co., Ltd. Antibodies for PPAR$\gamma$, p-NF-$\kappa$B p65, NF-$\kappa$B p65, $\beta$-actin, and secondary antibodies were purchased from Abcam. Trypsin, PBS buffer, and DMSO were purchased from Beijing Solarbio Science & Technology Co., Ltd.

### 1.2 Equipment

The primary equipment used included a $CO_2$ incubator (Thermo Fisher Scientific), a high-speed refrigerated centrifuge (Eppendorf), protein electrophoresis and transfer systems (Bio-Rad), a multi-functional microplate reader (BioTek), and a protein electrophoresis scanning imaging system (LI-COR).

### 1.3 Cell Culture and Grouping

RAW264.7 macrophages were cultured in DMEM high-glucose medium containing 10% FBS, 100 U/mL penicillin, and 100 $\mu$g/mL streptomycin at 37°C and 5% $CO_2$. The medium was changed daily. Upon reaching 80%–90% confluence, cells were passaged using 0.25% trypsin. Cells in the logarithmic growth phase were selected for experiments and divided into four groups: 1) Control group (normal medium for 48 h); 2) PA model group (optimal concentration of PA for 48 h); 3) Low-dose LNT + PA group (20.0 $\mu$mol/L LNT pretreatment for 2 h, followed by PA co-culture); and 4) High-dose LNT + PA group (40.0 $\mu$mol/L LNT pretreatment for 2 h, followed by PA co-culture).

### 1.4 Screening for Optimal PA Concentration

RAW264.7 cells were seeded in 96-well plates ($1 \times 10^4$ cells/well). After 24 h, PA was added at final concentrations of 0, 10, 20, 40, and 80 $\mu$g/mL (in DMEM containing 1% BSA) for another 24 h. Cell viability was measured via MTT assay to identify a concentration that maintained 70%–80% viability while significantly inducing lipid accumulation.

### 1.6 Detection of Intracellular TG and TC Content

Following 48 h of treatment, cells were washed with PBS and lysed on ice for 30 min. After centrifugation (12,000 r/min, 4°C, 15 min), the supernatant was collected. Intracellular TG and TC levels were determined using biochemical kits according to the manufacturer' s instructions, with absorbance measured at 490 nm and 510 nm, respectively.

### 1.7 ELISA for IL-1$\beta$ and IL-6 Levels

Cell supernatants were collected after 48 h and centrifuged (1,000 r/min, 4°C, 10 min). IL-1$\beta$ and IL-6 secretion levels were measured using ELISA kits at

450 nm. This method allows for the direct detection of free-state inflammatory cytokines with high sensitivity.

### 1.8 Western Blot for PPAR$\gamma$ and NF-$\kappa$B p65 Expression

Total protein was extracted after 48 h, and concentrations were normalized using the BCA method. Equal amounts of protein were separated by SDS-PAGE and transferred to PVDF membranes. After blocking with 5% non-fat milk for 1 h, membranes were incubated with primary antibodies (PPAR$\gamma$, p-NF-$\kappa$B p65, NF-$\kappa$B p65, $\beta$-actin; 1:800) overnight at 4°C. After washing, membranes were incubated with secondary antibodies (1:1500) for 1 h at room temperature. Protein bands were visualized using ECL chemiluminescence and analyzed using ImageJ software, with $\beta$-actin as the internal control.

### 1.9 Statistical Analysis

Experiments were repeated three times. Data are expressed as mean ± standard deviation ($\bar{x} \pm s$). Statistical analysis was performed using SPSS 26.0. Comparisons between multiple groups were conducted using one-way ANOVA, followed by LSD-t tests for pairwise comparisons. $P < 0.05$ was considered statistically significant.

### 2.1 Screening for Optimal PA Concentration

MTT results showed that RAW264.7 cell viability decreased as PA concentration increased ($0.0, 10.0, 20.0, 40.0, 80.0$ $\mu$g/mL) ($P < 0.05$). At 40.0 $\mu$g/mL, viability was 75.32%±4.15%, which falls within the ideal 70%-80% range. At 80.0 $\mu$g/mL, viability dropped significantly. Thus, 40.0 $\mu$g/mL was selected as the optimal concentration for subsequent experiments.

### 2.2 Effect of LNT on PA-Induced Lipid Accumulation

Biochemical analysis [TABLE:1, FIGURE:1] revealed that TG and TC contents were significantly higher in the PA model group than in the control group ($P < 0.05$). Both low- and high-dose LNT interventions significantly reduced these levels compared to the PA model group ($P < 0.05$), with the high-dose group showing the most significant reduction. This indicates that LNT inhibits PA-induced lipid accumulation in a dose-dependent manner.

**Table 1. Effect of LNT on TG and TC content in PA-treated macrophages** | Group | TG (mmol/dL) | TC (mmol/dL) | | :–| :–| :–| | Control | $0.62 \pm 0.07$ | $0.85 \pm 0.09$ | | PA Model | $2.35 \pm 0.12^{*}$ | $2.81 \pm 0.15^{*}$ | | LNT Low + PA | $1.86 \pm 0.10^{*\#}$ | $2.24 \pm 0.11^{*\#}$ | | LNT High + PA | $1.01 \pm 0.06^{*\#\triangle}$ | $1.26 \pm 0.08^{*\#\triangle}$ | *Note:* $\bar{x} \pm s, n = 3;^{*} P < 0.05$ *vs. Control;* $^{\#}P < 0.05$ *vs. PA Model;* $^{\triangle}P < 0.05$ *vs. LNT Low + PA.*

[Figure 1: see original paper]

### 2.3 Effect of LNT on PA-Induced Inflammatory Cytokine Secretion

As shown in Figure 1 and Table 2 , PA treatment significantly increased the secretion of IL-1$\beta$ and IL-6 compared to the control group ($P < 0.05$). LNT intervention (both doses) significantly lowered these levels ($P < 0.05$), with the high-dose group exhibiting the lowest secretion levels. This suggests that LNT effectively suppresses the PA-induced inflammatory response in a dose-dependent manner.

**Table 2. Effect of LNT on IL-1$\beta$ and IL-6 secretion in PA-induced macrophages** | Group | IL-1$\beta$ (pg/mL) | IL-6 (pg/mL) | | :–| :–| :–| | Control | $28.63 \pm 3.21$ | $35.42 \pm 4.16$ | | PA Model | $112.57 \pm 8.45^{*}$ | $128.76 \pm 9.23^{*}$ | | LNT Low + PA | $85.34 \pm 6.62^{*\#}$ | $92.45 \pm 7.85^{*\#}$ | | LNT High + PA | $56.78 \pm 5.34^{*\#\triangle}$ | $68.20 \pm 6.54^{*\#\triangle}$ |

### 2.4 Effect of LNT on PPAR$\gamma$ and p-NF-$\kappa$B p65 Protein Expression

Western blot results showed that PA treatment significantly downregulated PPAR$\gamma$ expression and upregulated p-NF-$\kappa$B p65 expression ($P < 0.05$). LNT intervention significantly reversed these effects by upregulating PPAR$\gamma$ and downregulating p-NF-$\kappa$B p65 in a dose-dependent manner ($P < 0.05$). This suggests that LNT inhibits the activation of the NF-$\kappa$B signaling pathway by modulating PPAR$\gamma$ expression and NF-$\kappa$B p65 phosphorylation.

**Table 3. Effect of LNT on PPAR$\gamma$ and p-NF-$\kappa$B p65 expression** | Group | PPAR$\gamma$/$\beta$-actin | p-NF-$\kappa$B p65/NF-$\kappa$B p65 | | :–| :–| :–| | Control | $0.82 \pm 0.08$ | $0.28 \pm 0.03$ | | PA Model | $0.36 \pm 0.03^{*}$ | $0.92 \pm 0.08^{*}$ | | LNT Low + PA | $0.65 \pm 0.05^{*\#}$ | $0.71 \pm 0.06^{*\#}$ | | LNT High + PA | $0.87 \pm 0.07^{\#\triangle}$ | $0.35 \pm 0.04^{\#\triangle}$ |

## Discussion

The core feature of MASH is the mutual promotion of lipid metabolism disorders and inflammatory responses, where macrophage lipid accumulation acts as a key trigger for inflammation. As the most abundant saturated fatty acid in the body, excessive PA induces lipid overload and activates inflammatory pathways, promoting the secretion of cytokines like IL-1$\beta$ and IL-6 [**?**]. Our results confirm this, as PA treatment significantly increased TG/TC levels and IL-1$\beta$/IL-6 secretion in RAW264.7 cells. LNT, a natural polysaccharide with known antioxidant properties [**?**], was found to dose-dependently reduce lipid content and inhibit cytokine secretion, demonstrating its potent anti-inflammatory and lipid-lowering potential.

PPAR$\gamma$ is a nuclear receptor involved in regulating lipid metabolism and inflammation, while the NF-$\kappa$B pathway is central to the inflammatory response. PPAR$\gamma$ can inhibit NF-$\kappa$B-mediated inflammation by directly binding to the NF-$\kappa$B p65 subunit or by recruiting co-repressors [**?**]. In our PA-induced model, PPAR$\gamma$ was downregulated, leading to the over-activation of NF-$\kappa$B and subse-

quent cytokine release [?]. LNT intervention upregulated PPAR$\gamma$ and downregulated p-NF-$\kappa$B p65, suggesting that LNT inhibits NF-$\kappa$B p65 nuclear translocation. Given that IL-1$\beta$ can further exacerbate lipid accumulation by suppressing PPAR$\alpha$-dependent fatty acid oxidation [?], LNT' s ability to reduce inflammation through the PPAR$\gamma$/NF-$\kappa$B axis may be a primary mechanism for improving macrophage lipid metabolism.

This study has limitations, including the lack of animal model validation and a need for deeper exploration of the specific molecular targets of LNT. Future research will focus on *in vivo* validation and identifying the precise targets and optimal timing for LNT intervention.

## Conclusion

Lentinan (LNT) reduces TG and TC levels and inhibits IL-1$\beta$ and IL-6 secretion in PA-induced RAW264.7 macrophages. This antagonistic effect is likely mediated by the upregulation of PPAR$\gamma$ and the downregulation of NF-$\kappa$B p65 phosphorylation.

## References

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*Note: Figure translations are in progress. See original paper for figures.*