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Lycium barbarum polysaccharide antagonizes LPS-induced inflammation by altering the glycolysis and differentiation of macrophages by triggering the degradation of PKM2

Huan Ding[1], Jing-jing Wang[2], Xiao-Ya Zhang[1], Lei Yin[1], Tao Feng[1]*

[1]Intensive care unit (ICU), Department of Critical care unit, General Hospital of Ningxia Medical University, Yinchuan 750004, Ningxia, China

[2]Coronary care unit (CCU), Department of Cardiology, General Hospital of Ningxia Medical University, Yinchuan 750004, Ningxia, China

Corresponding author: Tao Feng (fengtao80@126.com)

Abstract

Lipopolysaccharide (LPS)-induced inflammation is the leading cause of multiple organ failure in sepsis. Pyruvate kinase 2 (PKM2) is a protein kinase and transcriptional coactivator that plays an important role in glycolysis. Recent studies have confirmed that glycolysis maintains the M1 differentiation and induces immune activation in macrophages. Lycium barbarum polysaccharide (LBP), the main bioactive component of Chinese wolfberry, suppresses glycolysis and inflammation. Here, RAW264.7 macrophages were treated with LBP for evaluating its effects against LPS-induced inflammation. The differentiation of M1/M2 macrophages was assessed by flow cytometry for assessing the cell surface markers, CD86 and CD206. The enrichment of HIF-1α and ubiquitin in the PKM2 protein complex was determined by co-immunoprecipitation. LBP suppressed LPS-induced glycolysis, differentiation of M1 macrophages, and the production of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and high mobility group (HMG) 1 proteins. The suppressive effects of LBP were similar to those of PKM2 knockdown, but were abolished by the overexpression of PKM2. LPS elevated the mRNA and protein levels of PKM2. LBP reduced the LPS-induced expression of PKM2 protein, but had no effects on the expression of PKM2 mRNA. LPS inhibited the ubiquitination of PKM2, probably by downregulating the expression of ubiquitin ligases, including Nedd4L, Nedd4, and Gnb2. LBP interfered with the inhibition of PKM2 ubiquitination by upregulating the expression of Nedd4L, Nedd4, and Gnb2. In conclusion, LBP suppressed the LPS-induced inflammation by altering glycolysis and the M1 differentiation of macrophages. The effects of LBP were mediated by the downregulation of PKM2 via enhanced ubiquitination.

Keywords: Lycium barbarum polysaccharide; PKM2; Inflammation; Glycolysis; Macrophage differentiation; Ubiquitination

Introduction

Sepsis is a systemic immune disorder that is caused by infections. More than 50.90 million people are annually diagnosed with sepsis worldwide [1-3]. Sepsis can cause multiple organ failure, including lung, liver, and kidney failure, as well as cognitive impairment (4). Individuals with sepsis have a high mortality rate, and the mortality rate can be as high as a 40-50% following the development of septic shock [3]. This is due to the fact that patients with septic shock suffer cardiovascular collapse and are often unresponsive to fluid resuscitation and treatment with vasopressors [4].

Macrophages are the most commonly occurring leukocytes that are closely related to the development of sepsis [5]. The M1 differentiation of macrophages is enhanced in the early stage of sepsis, probably due to stimulation by cytokines, including interferon-γ and granulocyte macrophage colony stimulating factor, and exogenous bacterial toxins, such as lipopolysaccharide (LPS). M1 macrophages are "classically activated" macrophages that are characterized by an increased ability to secrete pro-inflammatory cytokines, including interleukin (IL)-1β, tumor necrosis factor (TNF)-α, high mobility group 1 (HMGB1) protein, IL-12, and IL-18 [6]. These cytokines improve the ability of the immune system to eliminate microorganisms, however, they also cause tissue damage. Moreover, the overproduction of cytokines in the early hyperinflammatory stage induces immunosuppression in the later stages of sepsis, although the mechanism underlying this phenomenon is yet to be fully understood. A moderate inhibition of the immune response is therefore recommended during the hyperinflammatory phase.

Recent studies have demonstrated that the rate of glycolysis in macrophages is enhanced during the stage of immune activation, however, it is attenuated during the stage of immune tolerance, which suggests a correlation between glycolysis and the immune function [7]. The activated immune cells, including macrophages, dendritic cells, and T cells, also have the ability to switch from oxidative phosphorylation to aerobic glycolysis even when oxygen is abundantly available. LPS has been reported to promote the expression of genes related to glycolysis via activation of the hypoxia inducible factor (HIF) and mTOR signaling pathways [8]. It has been demonstrated

that the inhibition of glycolysis impairs the LPS-induced production of IL-1β by macrophages. This indicates that glycolysis mediates the LPS-induced activation of the immune system [9]. Pyruvate kinase 2 (PKM2) plays an important role in glycolysis by acting as a protein kinase and transcriptional coactivator. PKM2 catalyzes the conversion of phosphoenolpyruvic acid (PEP) and ADP to pyruvate and ATP, respectively, in the last step of the glycolytic pathway [10]. PKM2 can also function as a transcriptional coactivator of HIF-1α for stimulating the expression of the glycolytic enzymes and proinflammatory cytokines [11].

Lycium barbarum polysaccharide (LBP) is the main bioactive component of Chinese wolfberry that has several properties, including anti-inflammatory, anti-oxidative, anti-aging, and hypolipidemic activities [12]. The anti-inflammatory effects of LPB have been reported in various diseases [13-15]. For instance, LBP protects the kidneys from inflammatory injury in septic rats by regulating the Keap1-Nrf2/ARE signaling pathway [13]. Additionally, LBP attenuates the LPS-induced acute respiratory distress syndrome by inhibiting inflammation, apoptosis, and oxidative stress in pulmonary endothelial cells [14]. LBP also alleviates the inflammation induced by IL-1β in chondrogenic ATDC5 cells by modulating miR-124 [15].

LBP has been reported to suppress the expression of genes related to glycolysis and gluconeogenesis, in a model of insulin resistance induced by the long-term administration of a high-fat diet [12]. We therefore investigated whether LBP suppresses the LPS-induced inflammatory response in macrophages by suppressing glycolysis. This study confirmed that LBP inhibited the LPS-induced glycolysis in macrophages by reducing the levels of the PKM2 protein. The reduction in the levels of PKM2 by LBP was associated with the ubiquitination and degradation of PKM2. The results of this study elucidated a novel anti-inflammatory effect of LBP on macrophages.

Material and methods

Cell culture and treatment

Murine RAW264.7 macrophages were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The RAW264.7 macrophages were initially cultured in LPS-free Dulbecco's Modified Eagle Medium (DMEM, Gibco Company, Grand Island, NY), supplemented with 10% fetal bovine serum (Gibco), 100 U/mL streptomycin (Invitrogen, Carlsbad, CA, USA), and 100 U/mL penicillin (Invitrogen), in an incubator with 5% CO_2 and saturated humidity.

The RAW264.7 macrophages were treated with 10, 50, or 200 μg/mL LBP (No. 107-43-7; Guidechem Chemical Company, Shaanxi, China, https://china.guidechem.com/trade/pdetail1649419.html), alone or in combination with 100 ng/mL LPS (Sigma-Aldrich, St. Louis, MO, USA), for 24 h. Following treatment, the cells were subjected to several analyses that are described hereafter.

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

The cell viability was evaluated by the MTT assay. The RAW264.7 macrophages were seeded into a 96-well plate at a density of 1×10^4 cells/well, and three parallel wells were set up for each group. The cells were incubated with 20 μL MTT solution (Sigma-Aldrich) for 4 h at 37°C. The MTT solution was subsequently removed and dimethyl sulfoxide (DMSO, 150 μL, Sigma-Aldrich) was added to each well. The values of optical density (OD) were determined for each well using an Infinite F500 microplate reader (TECAN) at a wavelength of 490 nm.

Measurement of glucose concentration in the culture medium

The culture medium was collected for measuring the concentration of extracellular glucose. The glucose concentration was determined using the conventional glucose oxidase/peroxidase method that has been previously described [16]. This method is based on the conversion of glucose and O_2 into gluconic acid and H_2O_2 by glucose oxidase. The H_2O_2 thus produced further reacts with 1,5-dimethyl-2-phenyl-4-aminopyrazoline and phenols, and generates colored compounds with a maximum absorption wavelength of 570 nm.

Measurement of lactic acid concentration

The concentration of lactic acid in the culture medium was determined using a lactic acid assay kit (Item number: K607-100, Biovision; Wuhan, China), according to the manufacturer's instructions. This kit consists of a highly sensitive probe that emits fluorescence (Ex/Em = 535/587 nm) after reacting with lactic acid. The emitted fluorescence was detected with an Infinite F500 microplate reader (TECAN).

Enzyme-linked immunosorbent assay (ELISA)

The RAW264.7 macrophages were cultured in 24-well plates at a density of 10^6 cells/well and treated with the aforementioned substances. The culture supernatants were collected and centrifuged in order to remove the particles for subsequent analyses. The cell supernatants were collected and stored at -20°C until further analysis. The quantities of IL-1β, TNF-α, and HMGB1 in the culture medium were assessed using a commercially available ELISA kit, according to the manufacturer's instructions (Sigma-Aldrich). The value of OD of each well was measured at a wavelength of 450 nm. The experiment was performed in triplicate, and the mean value was calculated. The IL-1β ELISA assay kit does not distinguish between the inactive 31-kDa precursor (pro-IL-1β) and the bioactive 17 kDa mature form, as is the case for all commercially available ELISA kits. The standard curves were used for measuring the concentrations of IL-1β in the samples (pg/mL).

Reverse transcription-quantitative polymerase chain reaction (PCR)

The total RNA was extracted using the single-step TRIzol method (Invitrogen) and reverse-transcribed to cDNA using the reverse transcriptase of avian myeloblastosis virus. The primers for PCR were designed and synthesized by Invitrogen, and are enlisted in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal reference. The following amplification conditions were used for PCR: pre-denaturation at 94°C for 5 min, denaturation at 94°C for 40 seconds, annealing at 60°C for 40 seconds, and extension of the DNA strands at 72°C for 1 min. This protocol was run for 40 cycles, followed by extension at 72°C for 10 minutes. The

$2^{-\Delta\Delta Ct}$ method was used for calculating the relative expression of the target genes using to the following formula: ΔΔCt= (Ct[target gene] -Ct[reference gene])experimental group - LRB - Ct[target gene] - Ct[reference gene])control group.

Analysis by western blotting

The proteins that were extracted from the cells were mixed with the loading buffer and boiled for 10 min at 95°C. The protein samples were loaded onto a 10% polyacrylamide gel and separated by electrophoresis (Boster Biological Technology, Wuhan, China). The proteins were transferred onto a polyvinylidene fluoride membrane and the membrane was blocked with 5% bovine serum albumin for 1 h at room temperature. The membrane was incubated overnight at 4°C with the primary antibodies against PKM2, phosphorylated (p)-IRAK4, IRAK4, p-IRAK1, IRAK4, Nedd4L, Nedd4, Gnb2, Smurf1, HIF-1α, and GAPDH (1:1000, Abcam Inc., Cambridge, MA). The membrane was subsequently washed with Tris-buffered saline in Tween-20, and subsequently treated with the secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h. The images of the gels were captured using a Bio-Rad Gel Doc EZ Imager (Bio-Rad). The target protein bands were analyzed in terms of the gray values using the Image J software.

Gene knockdown and overexpression

Three sets of siRNAs against PKM2 were designed by GenePharma (Shanghai, China) and the efficiency of PKM2 knockdown was initially determined. The siRNA with the highest PKM2 knockdown efficiency was used for further studies. For inducing the ectopic expression of PKM2 in RAW264.7 macrophages, the cells were transfected with the pEGFP-C1-PKM2 vector (Genephama Biotech) using Lipofectamine 2000 (Thermo Fisher Scientific), according to the manufacturer's instructions.

Flow cytometry

CD86 (BioLegend, San Diego, CA, USA) is a surface marker of M1 macrophages,

while CD206 (BioLegend) is a surface marker of the M2 macrophages. The cells were harvested by centrifugation, and the supernatant was discarded. The cells were washed with pre-cooled PBS buffer at 4°C, and subsequently centrifuged twice at 1000 rpm, for 5 min each. The cell density was adjusted to 1×10^6 cells/mL. The cell suspension was subsequently washed twice with pre-cooled PBS and centrifuged. The cells were suspended in 300 μL binding buffer and incubated with anti-IgG1 labeled with phycoerythrin (PE), PE-cyanine 5 (Cy5), or fluorescein isothiocyanate (FITC), which were used as the negative controls, or with PE-Cy5-conjugated anti-CD86 or FITC-conjugated anti-CD206. After 30 min of incubation, approximate 30,000 cells were selected from each sample and suspended in 300 μL of PBS. The cells were subsequently analyzed by flow cytometry using a BD LSRFortessa flow cytometer within 24 h. The FlowJo software was used for data analysis.

Co-immunoprecipitation (Co-IP) assay

The whole cell extracts were prepared using IP lysis buffer containing 10 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, and 1% Triton X 100 supplemented with the complete protease inhibitor cocktail and PhosStop tablets (Roche). The lysates (0.5 -1.5 mg) were incubated with 2 μg of PKM2 antibody and Protein G-Sepharose (GE Healthcare). The PKM2-protein complexes were subjected to western blotting for determining the enrichment of HIF-1α and ubiquitin in the protein complex.

Statistical analyses

The experiments were repeated thrice, and the mean values were calculated. All the data were analyzed with SPSS software, version 21.0 (SPSS Inc., Chicago, IL). The quantitative data are expressed as the mean ± standard deviation. The normally distributed quantitative data were compared using t-tests, while one-way analysis of variance was performed for comparing the multiple groups. Statistical significance was defined at $p < 0.05$.

Results

LBP had moderate effects on glycolysis and the production of inflammatory factors in macrophages

The effect of LBP on glycolysis has not been previously investigated in macrophages. In order to determine the effects of LBP on macrophages, we determined the concentrations of lactate and glucose in the culture medium of macrophages following treatment with LBP. We found that the concentrations of lactate and glucose in the culture medium were not significantly altered following treatment with 10, 50, and 200 μg/mL of LBP (Figure 1A). We observed that LBP significantly reduced cell viability at concentrations of 400 μg/mL, but not at lower concentrations (data not shown). We therefore performed the subsequent experiments using LBP at concentrations < 200 μg/mL. The concentrations of IL-1β, TNF-α, and HMGB1 in the culture medium remained unaltered following treatment with LBP (Figure 1B).

LBP suppressed glycolysis and the production of inflammatory factors in the macrophages in the presence of LPS

Co-treatment with LPS and LBP decreased the concentrations of lactate in the culture medium, compared to those after treatment with LPS alone ($p<0.05$ or $p<0.01$, Figure 1C). The inhibition induced by 50 μg/mL LBP was more significant than that of 200 μg/mL LBP. Treatment with LPS and LBP at all the tested concentrations increased the concentrations of glucose in the culture medium, compared to those induced by treatment with LPS alone ($p<0.05$ or $p<0.01$, Figure 1C). The results of ELISA indicated that LBP reduced the concentrations of IL-1β, TNF-α, and HMGB1 in the culture medium in the presence of LPS. The inhibitory potential of 50 μg/mL of LBP was significantly higher than that of LBP at all the other concentrations tested herein. We therefore performed further experiments with 50 μg/mL LBP.

The effects of LBP against LPS-induced glycolysis and inflammation are mediated via PKM2

In this study we performed PCR and western blotting for determining the mRNA and

protein levels of PKM2, respectively, in the macrophages following treatment with LPS alone or in combination with LBP. LPS increased the mRNA levels of PKM2 in the macrophages in the presence or absence of LBP ($p<0.05$, Figure 2A). The protein levels of PKM2 were markedly increased following treatment with LPS ($p<0.001$, Figure 2B), whereas treatment with LBP suppressed the increase in the protein levels of PKM2 that had been induced by LPS ($p<0.001$ vs. LPS group). In order to determine whether the effects of LBP on LPS-induced glycolysis and inflammation are mediated via PKM2, we transfected the macrophages with PKM2-siRNA or an overexpression vector prior to treatment with LPS and/or LBP. Transfection with the PKM2 overexpression vector dramatically increased the mRNA levels of PKM2 in the macrophages following treatment with LPS and LBP ($p<0.01$ vs. LPS group, Figure 2C). Transfection with PKM2-siRNA in turn reduced the mRNA levels of PKM2 in the macrophages following exposure to LPS ($p<0.001$ vs. LPS group, Figure 2C). LBP restrained the LPS-induced increase in the protein levels of PKM2, however, transfection with the PKM2 overexpression vector before these treatments increased the protein levels of PKM2 ($p<0.001$ vs. control group, Figure 2D). Transfection with PKM2-siRNA also abolished the LPS-induced increase in the protein levels of PKM2 ($p<0.001$ vs. LPS group).

As depicted in Figure 3A, LPS increased the concentration of lactate in the culture medium ($p<0.001$ vs. control group). The increase in the concentration of lactate was inhibited by LBP ($p<0.01$ vs. LPS group). However, the inhibitory effect of LBP was antagonized by the forced expression of PKM2 by transfection with the PKM2 overexpression vector ($p<0.01$ vs. LPS+LBP group). The silencing of PKM2 also suppressed the LPS-induced increase in the concentration of lactate, similar to the inhibitory effect of LBP ($p<0.01$ vs. LPS group). LPS decreased the concentration of glucose in the culture medium ($p<0.01$ vs. control group, Figure 3B), while LBP antagonized the LPS-induced reduction in the concentration of glucose ($p<0.01$ vs. LPS group). The overexpression of PKM2 abolished the inhibitory effect of LBP on the LPS-induced reduction in the concentration of glucose ($p<0.01$ vs. LPS+LBP group). PKM2 knockdown reversed the LPS-induced reduction in the concentration

of glucose ($p<0.001$ vs. LPS group). The results of ELISA demonstrated that the concentrations of IL-1β, TNF-α, and HMGB1 in the culture medium were elevated following treatment with LPS ($p<0.001$, $p<0.01$, and $p<0.001$, respectively, Figure 3C). LBP antagonized the increase in the concentrations of IL-1β, TNF-α, and HMGB1 that was stimulated by LPS ($p<0.01$, $p<0.05$, and $p<0.001$, respectively, vs. LPS group), however, the overexpression of PKM2 abrogated this effect of LBP ($p<0.01$, $p<0.01$and $p<0.001$, respectively, vs. LPS+LBP group). PKM2 knockdown also suppressed the increase in the concentrations of IL-1β, TNF-α, and HMGB1 that were induced by treatment with LPS ($p<0.001$, $p<0.05$, and $p<0.01$, respectively, vs. LPS group).

LBP disrupted the interaction between PKM2 and HIF-1α, and suppressed the differentiation of M1 macrophages

We performed western blotting and Co-IP assays for determining the interaction between PKM2 and HIF-1α in the macrophages following treatment with LPS and LBP. The results of western blotting revealed that LPS increased the levels of HIF-1α protein (input) in the macrophages ($p<0.001$, Figure 4A). The LPS-induced increase in the levels of HIF-1α protein were unaffected by treatment with LBP. Treatment with LPS increased the enrichment of HIF-1α in the PKM2 protein (IB, $p<0.001$), as revealed by the results of Co-IP. However, LBP suppressed the increase in the enrichment of HIF-1α in the PKM2 protein ($p<0.05$ vs. LPS group).

The M1/M2 differentiation of macrophages was assessed by flow cytometry for determining the cell surface markers of M1 (CD86) and M2 (CD206) macrophages. Treatment with LPS increased the percentage of macrophages with M1 phenotype ($p<0.001$, Figure 4B), but decreased the percentage of macrophages with M2 phenotype ($p<0.01$). LBP suppressed the effects of LPS on the M1/M2 differentiation of macrophages ($p<0.01$ vs. LPS group). The expression of IL-4 and IL-10 mRNA in the macrophages with M2 phenotype was additionally investigated by PCR. The expression levels of IL-4 and IL-10 mRNA reduced following stimulation with LPS ($p<0.001$, Figure 4C). LBP reversed the LPS-induced reduction in the expression of

IL-4 and IL-10 mRNA ($p<0.001$ vs. LPS group).

LPS increased the expression of PKM2 protein by suppressing LBP-induced disruption of PKM2 ubiquitination

It was observed that LBP suppressed the LPS-induced increase in the expression of the PKM2 protein but not that of the PKM2 mRNA, which suggested that the modulatory effect of LBP on the PKM2 protein is mediated by a post-transcriptional mechanism. Bioinformatics analysis (http://ubibrowser.ncpsb.org/ubibrowser/) revealed that the PKM2 protein is ubiquitinated by a series of ubiquitin ligases, including Nedd4L, Nedd4, Gnb2, and Smurf1. Data from the GSE76562 dataset (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE76562) [17] revealed that LPS downregulates the expression of Nedd4L, Nedd4, and Gnb2, but has a moderate effect on the expression of Smurf1. Based on this information, we investigated whether LBP disrupts the effect of LPS on the expression of the ubiquitin ligases. The results of PCR revealed that LPS downregulated the expression of Nedd4L ($p<0.001$), Nedd4 ($p<0.001$), and Gnb2 ($p<0.01$) (Figure 5A). LBP reversed the LPS-induced increase in the expression of Nedd4L ($p<0.001$ vs. LPS group) and Nedd4 ($p<0.05$ vs. LPS group). The protein levels of Nedd4L ($p<0.001$), Nedd4 ($p<0.001$), Gnb2 ($p<0.01$), and Smurf1 ($p<0.05$) decreased following treatment with LPS (Figure 5B). LBP reversed the increase in the protein levels of Nedd4L ($p<0.001$ vs. LPS group), Nedd4 ($p<0.05$ vs. LPS group), and Gnb2 ($p<0.05$ vs. LPS group) that were induced by LPS. However, treatment with LBP alone, at least at the concentration of 50 μg/mL, had no effect on the expression level of NEDD4, NEDD4L, and Gnb2 (data not shown). Analysis of the results of Co-IP indicated that LPS enhanced the ubiquitination of PKM2; however, this effect of LPS was impaired following treatment with LBP (Figure 5C).

LBP had no effect on the activation of TLR4 by LPS

To determine whether LBP interacts with LPS and inhibits the effect of LPS on TLR4 activation, this study investigated the downstream signal of TLR4 after treatment with

LPS alone or in combination with LBP. Upon LPS stimulation, the intracellular TIR region of TLR4 binds to the carboxyl terminus of MAL and MyD88. MyD88 further recruits IRAK4 (IL-1 receptor-associated kinase-4), IRAK1, and IRAK2 and results in their phosphorylation [18]. IRAKs interact with TAK1, whereby influencing IKKα/IκB/NF-κB signal. Therefore, the phosphorylation status of IRAKs, such as IRAK4 and IRAK1, has been used to evaluate the activation of TLR4 signal by LPS. This study found that LPS induced the phosphorylation of IRAK4 and IRAK1 ($p<0.001$, Figure 6). LBP failed to reverse the phosphorylation of IRAK4 and IRAK1 induced by LPS. The protein levels of IRAK4 and IRAK1 were neither influenced by LPS nor LPS plus LBP. This result suggested that LBP had no effect on the stimulation of TLR4 by LPS.

Discussion

The anti-inflammatory effect of LBP has been reported in various studies [13-15]. The results of this study demonstrated that the treatment of macrophages with LBP alone had no effect on the secretion of the inflammatory factors, including IL-1β, TNF-α, and HMGB1. However, LBP suppressed the secretion of IL-1β, TNF-α, and HMGB1 in the presence of LPS. These data suggested that the anti-inflammatory effects of LBP depend on the cellular environment.

Accumulating evidence suggests the important role of glycolysis in the activation and polarization of macrophages. Zhong and coworkers demonstrated that 2-deoxyglucose (2-DG), an inhibitor of glycolysis, markedly attenuates the production of proinflammatory factors and lung tissue injury in a murine model of LPS-induced acute lung injury [19]. Using *in vitro* and *in vivo* experiments, Tannahill and coworkers also reported that 2-DG suppresses the LPS-induced production of IL-1β in macrophages [9]. These observations agree with the finding that the rate of glycolysis in macrophages with the M1 phenotype is higher than that of the macrophages with the M2 phenotype [20-22]. Glycolysis is essential for maintaining the M1 macrophages as the inhibition of glycolysis impairs the LPS-induced differentiation of M1 macrophages [21]. M1 macrophages are predominantly

pro-inflammatory immune cells that produce IL-1β, TNF-α, HMGB1, CXCL10, iNOS, and IL-6. M2 macrophages have anti-inflammatory properties, and primarily express IL-10, IL-4, and arginase-1. The activation of M1 macrophages by LPS and interferon-γ induces aerobic glycolysis, which involves an increased glucose uptake and the conversion of pyruvate to lactate. Lactic acid is a distinctive metabolic product of glycolysis, as oxidative phosphorylation does not produce lactic acid. The energy efficiency of glycolysis is lower than that of oxidative phosphorylation. Therefore, cells that rely on glycolysis need to consume more quantities of glucose in order to maintain energy equilibrium. The present study demonstrated that the concentrations of lactic acid in the culture medium increased after stimulation with LPS, while the concentrations of glucose decreased after stimulation with LPS. However, co-treatment with LBP suppressed the LPS-induced increase in glycolysis and the reduction in the concentrations of glucose, which suggested that LBP suppresses the LPS-induced glycolysis in macrophages.

PKM2 plays an important role in the glycolysis of cancer and immune cells. It has been reported that the expression of PKM2 is high in various types of cancer cells [23, 24]. The depletion of PKM2 diminishes the rate of glycolysis in cancer cells and also suppresses their proliferation and increases apoptosis, suggesting that the PKM2-mediated glycolysis promotes cancer progression [23, 24]. Xie and coworkers demonstrated that PKM2 promotes glycolysis in macrophages, resulting in the increased release of IL-1β, IL-18, and HMGB1 by macrophages [25]. Ouyang and coworkers elucidated the critical role of PKM2 in the development of early thymocytes, including $CD4^{-}CD8^{-}$ T cells [26]. Deoxyelephantopin is a naturally occurring sesquiterpene lactone from *Elephantopus scaber* that counteracts inflammation during fulminant hepatitis by regulating PKM2 and PKM2-mediated glycolysis [27]. The results of this study demonstrated that LPS increased the expression of PKM2 mRNA by 1.3-folds and increased the levels of PKM2 protein by 3-folds, suggesting that the upregulation of PKM2 by LPS primarily depends on post-transcriptional strategies. It was observed that silencing PKM2 dramatically suppressed the LPS-induced glycolysis and production of pro-inflammatory factors,

which suggested that PKM2 has a key role in the effects of LPS. LBP had no effects on the expression of PKM2 mRNA, however, LBP abolished the increase in the levels of PKM2 protein induced by LPS. In order to determine whether PKM2 mediated the effects of LBP against LPS-induced glycolysis and inflammation, we transfected macrophages with a PKM2 overexpression vector before treatment with LPS and LBP. LBP failed to suppress the increase in the expression of PKM2 in macrophages transfected with the PKM2 overexpression vector in the presence of LPS. Moreover, PKM2 overexpression abolished the effects of LBP against LPS-induced glycolysis and inflammation. These results suggested that LBP antagonized the effects of LPS by inhibiting the expression of PKM2.

The glycolysis induced by PKM2 induces the transcriptional transactivation of HIF-1α. It has been confirmed that PKM2 translocates to the nucleus, where it interacts with HIF-1α and regulates the expression of numerous glycolytic enzymes by enhancing the recruitment of p300 to the HRE sites of the target genes. Moreover, PKM2 forms a complex with HIF-1α and directly binds to the promoters of pro-inflammatory cytokines, and activates the transcription of IL-1β and HMGB1. As these pro-inflammatory cytokines are primarily secreted from M1 macrophages and seldom from M2 macrophages, this suggested PKM2 could have also affected the M1/M2 differentiation of macrophages by regulating the secretion of these pro-inflammatory cytokines. The results of this study demonstrated that LPS promoted the expression of HIF-1α and its interaction with PKM2. LBP had no effects on the expression of HIF-1α, but suppressed the interactions between PKM2 and HIF-1α. This suggested that the LPS-induced differentiation of M1 macrophages was disrupted by LBP.

The results of previous data demonstrated that LPS downregulated the expression of the ubiquitin ligases, Nedd4L, Nedd4, and Gnb2, which mediate the ubiquitination of the PKM2 protein by triggering its ubiquitin-mediated degradation. These results were corroborated by the results of PCR and western blotting. However, LBP reversed the LPS-induced downregulation of Nedd4L, Nedd4, and Gnb2, and increased the ubiquitination of the PKM2 protein. The results of this study suggested

that LBP impaired the LPS-induced increase in the expression of the PKM2 protein that participates in restoring ubiquitin ligases for triggering the ubiquitination and degradation of PKM2. Zhang reported that the LPS-induced downregulation of the expression of Nedd4 is associated with the ALX/PI3K pathway [28]. LBP, at least at the concentration of 50 μg/mL, had no effect on the expression level of NEDD4, NEDD4L, and Gnb2. Therefore, LBP does not directly up-regulate NEDD4, NEDD4L, and Gnb2 to neutralize LPS-mediated inhibition in these proteins; LBP more likely disrupts LPS-induced signal that responsible for the suppression of NEDD4, NEDD4L, and Gnb2.

There is a possibility that LBP interacts with LPS directly to blocking LPS stimulating TLR4. However, this study found that LBP failed to suppress the phosphorylation of IRAK4 and IRAK1 (the up-stream effectors of TLR4 signal) after LPS stimulation. Therefore, LBP had no effect on the activation of TLR4 by LPS.

In summary, the results of this study demonstrated that LBP suppressed LPS-induced inflammation by affecting the glycolysis and the differentiation of M1 macrophages. The effects of LBP are associated with the downregulation of the expression of PKM2 mediated via the enhancement of PKM2 ubiquitination.

Conflict of Interest

The authors declare no conflict of interest

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Table 1. The information of primer in PCR assay

Genes	Direction	Sequence (5' -> 3')	Length	Tm	Location	Amplification Size
PKM2	Forward	ATGTCGAAGCCCCATAGTGAA	21	60.9	1-21	118
PKM2	Reverse	TGGGTGGTGAATCAATGTCCA	21	61.4	118-98	
IL-4	Forward	CCAACTGCTTCCCCCTCTG	19	62	15-33	150
IL-4	Reverse	TCTGTTACGGTCAACTCGGTG	21	61.7	164-144	
IL-10	Forward	GACTTTAAGGGTTACCTGGGTTG	23	60.5	217-239	112
IL-10	Reverse	TCACATGCGCCTTGATGTCTG	21	63	328-308	
NEDD4L	Forward	GACATGGAGCATGGATGGGAA	21	61.9	481-501	122
NEDD4L	Reverse	GTTCGGCCTAAATTGTCCACT	21	60.3	602-582	
NEDD4	Forward	TCAGGACAACCTAACAGATGCT	22	60.8	672-693	223
NEDD4	Reverse	TTCTGCAAGATGAGTTGGAACAT	23	60.2	897-875	
Gnb2	Forward	TGATGCCTCTATCAAGCTGTGG	22	61.8	612-633	76
Gnb2	Reverse	GATGTCGGATTCATGGCCGAT	21	62.3	687-667	
Smurf1	Forward	AGATCCGTCTGACAGTGTTATGT	23	60.8	41-63	92
Smurf1	Reverse	CCCATCCACGACAATCTTTGC	21	61.6	132-112	
GAPDH	Forward	GGAGCGAGATCCCTCCAAAAT	21	61.6	108-128	197
GAPDH	Reverse	GGCTGTTGTCATACTTCTCATGG	23	60.9	304-282	

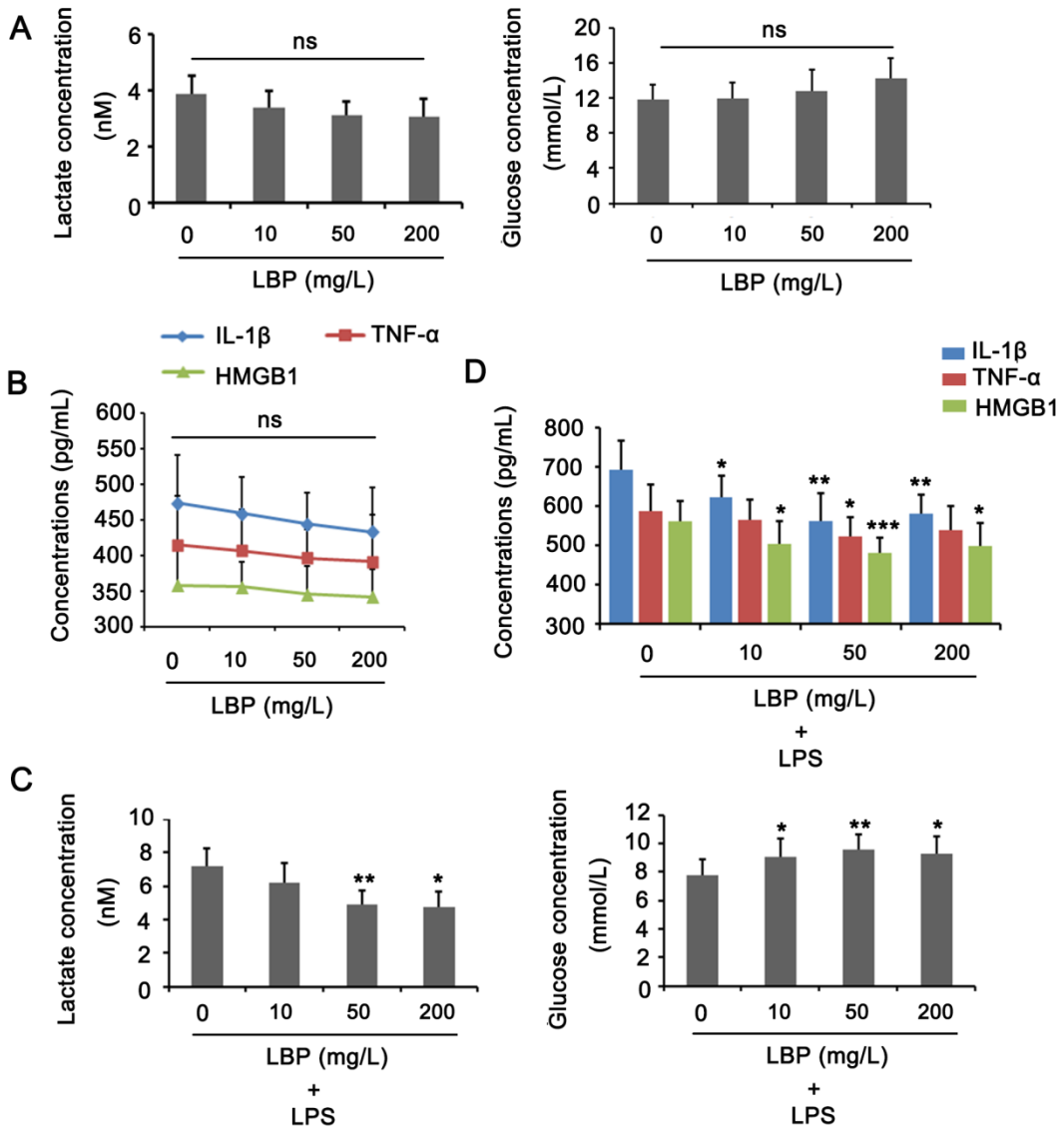

Figure 1. Effects of LBP on glycolysis and the production of inflammatory factors in macrophage in the presence or absence of LPS

Macrophages were treated with 10, 50 and 200 μg/mL LBP for 24 hours. The concentrations of lactate and glucose in the culture medium of macrophages were assessed after treatment with LBP (A). The concentrations of IL-1β, TNF-α and HMGB1 in the culture medium were measured using Elisa assay (B). Macrophages were treated with 10, 50 and 200 μg/mL LBP in combination with 100 ng/mL LPS for 24 hours. The culture medium was collected for the measurements of lactate and glucose concentrations (C) as well as IL-1β, TNF-α and HMGB1 concentrations (D). ns: no significance. *P<0.05, **P<0.01, and ***P<0.01 vs. control. (Color figure can be accessed in the online version.)

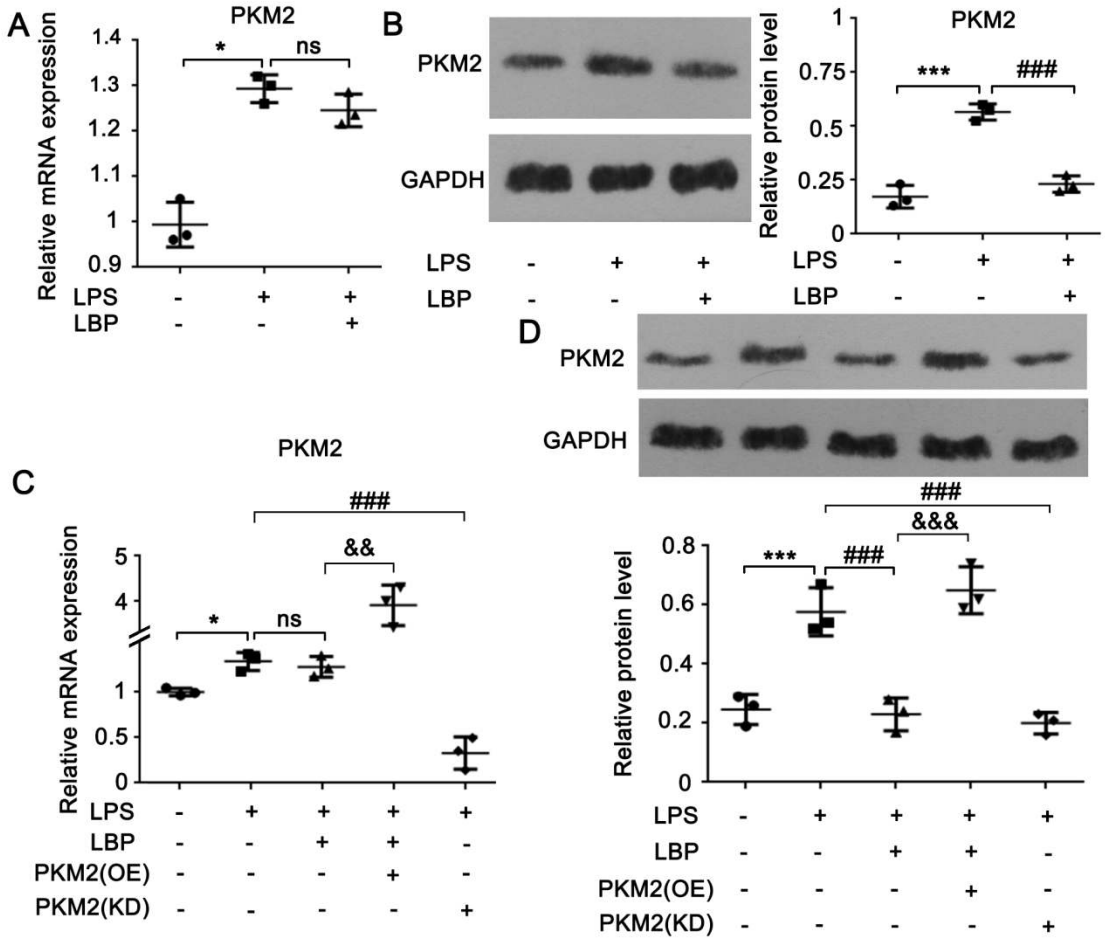

Figure 2. Knockdown or overexpression of PKM2 in macrophages before treatment with LPS and/or LBP

This study performed PCR (A) and western blot (B) assays to assess PKM2 mRNA and protein levels, respectively, in macrophages after treatments with LPS alone or in combination with LBP. PKM2-siRNA or over-expression vector was transfected into macrophages, before treatment with LPS and/or LBP. PCR (C) and western blot (D) assays was performed to assess PKM2 mRNA and protein levels. *P<0.05, and ***P<0.01 vs. control; $^{\#\#\#}P$<0.01 vs. LPS group. $^{\&\&}P$<0.01, and $^{\&\&\&}P$<0.01 vs. LPS+LBP group.

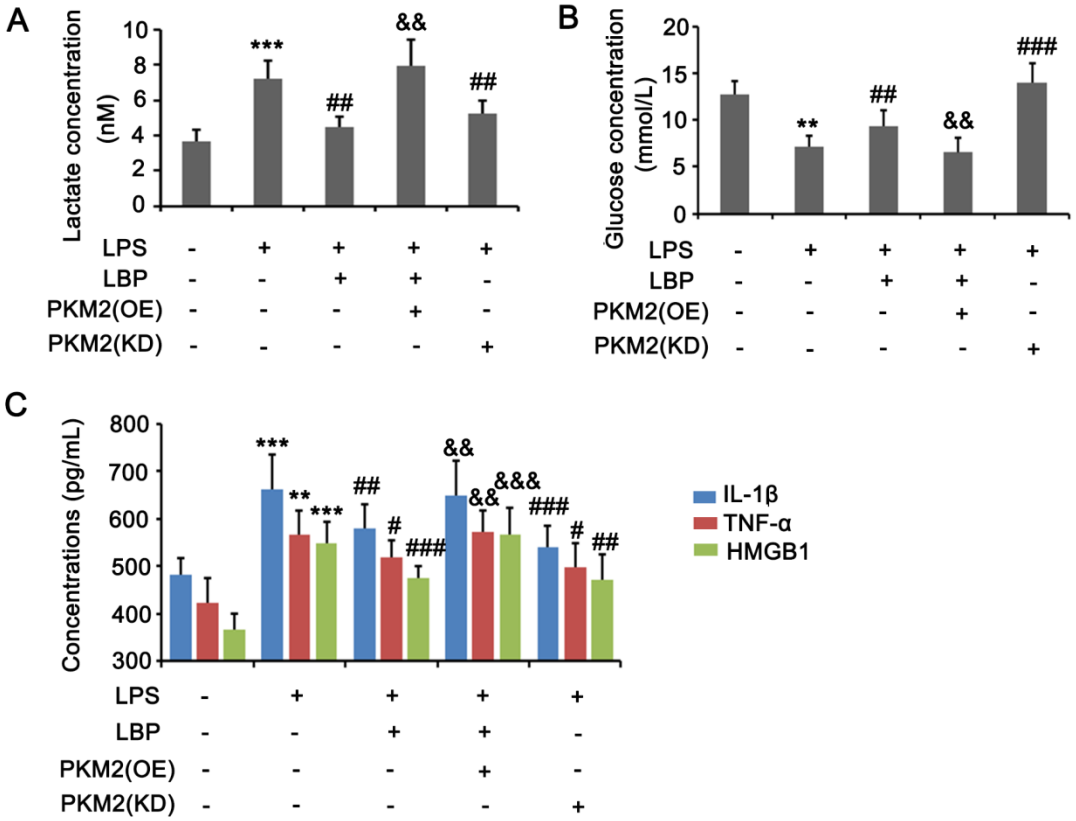

Figure 3. PKM2 was involved in the effects of LBP against LPS-induced glycolysis and inflammation

This study performed PCR (A) and western blot (B) assays to assess PKM2 mRNA and protein levels, respectively, in macrophages after treatments with LPS alone or in combination with LBP. PKM2-siRNA or over-expression vector was transfected into macrophages, before treatment with LPS and/or LBP. PCR (C) and western blot (D) assays was performed to assess PKM2 mRNA and protein levels. The culture medium was collected for the measurements of lactate (E) and glucose concentrations (F) as well as IL-1β, TNF-α and HMGB1 concentrations (G). ns: no significance. *P<0.05, **P<0.01, and ***P<0.01 vs. control; $^{\#}P$<0.05, $^{\#\#}P$<0.01, and $^{\#\#\#}P$<0.01 vs. LPS group. (Color figure can be accessed in the online version.)

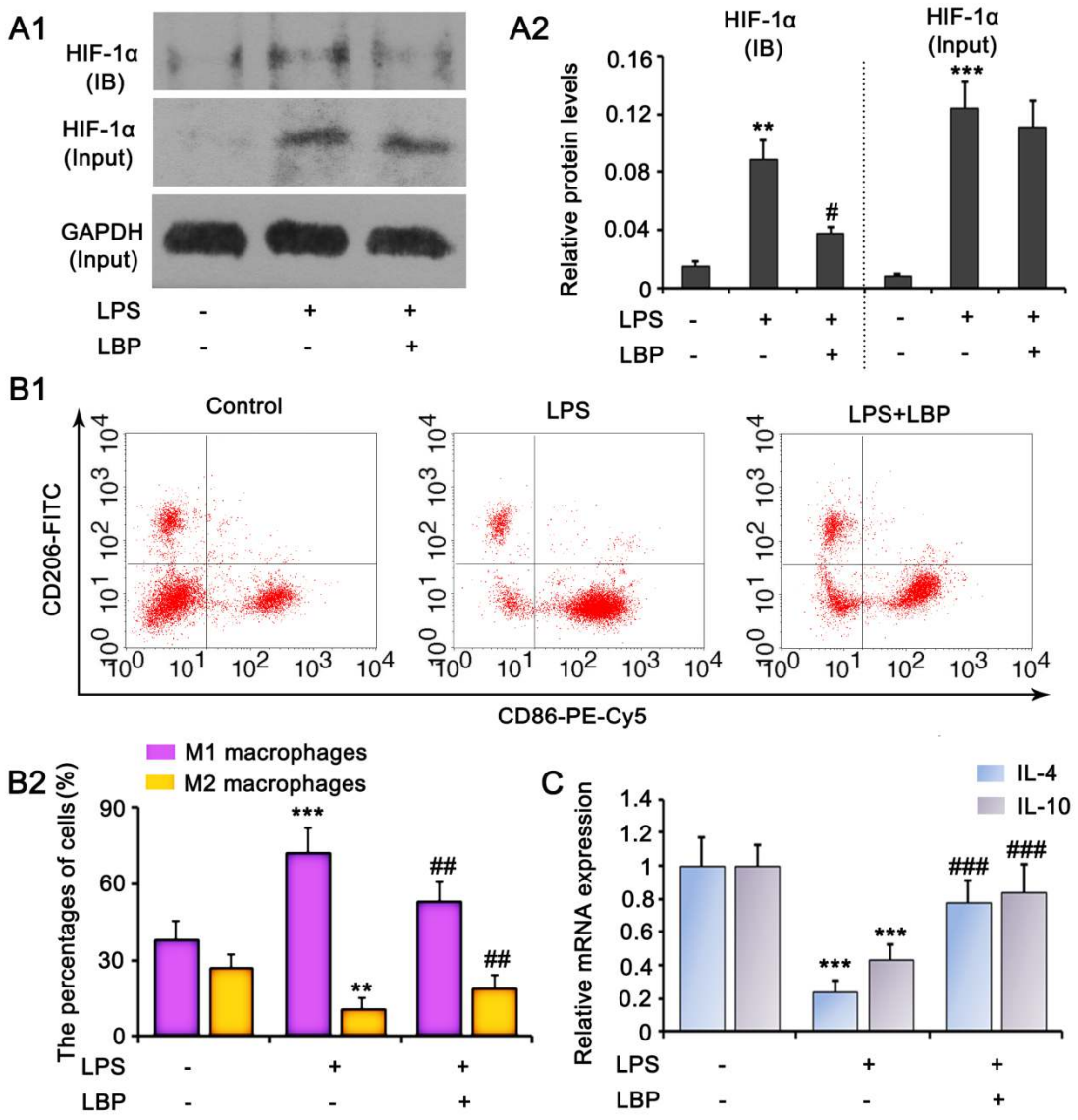

Figure 4. LBP disrupted the interaction between PKM2 and HIF-1α and suppressed M1 differentiation of macrophage

This study performed western blot and Co-IP assays to determine the interaction between PKM2 and HIF-1α in macrophages after treatment with LPS alone or in combination with LBP (A). The M1/M2 differentiation of macrophages was assessed through flow cytometry assay for the testing cell surface markers of M1 phenotype (CD86) and M2 phenotype (CD206) (B). PCR assay was performed to test IL-4 and IL-10 mRNA expression in macrophages (C). $^{**}P<0.01$, and $^{***}P<0.01$ vs. control; $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.01$ vs. LPS group. (Color figure can be accessed in the online version.)

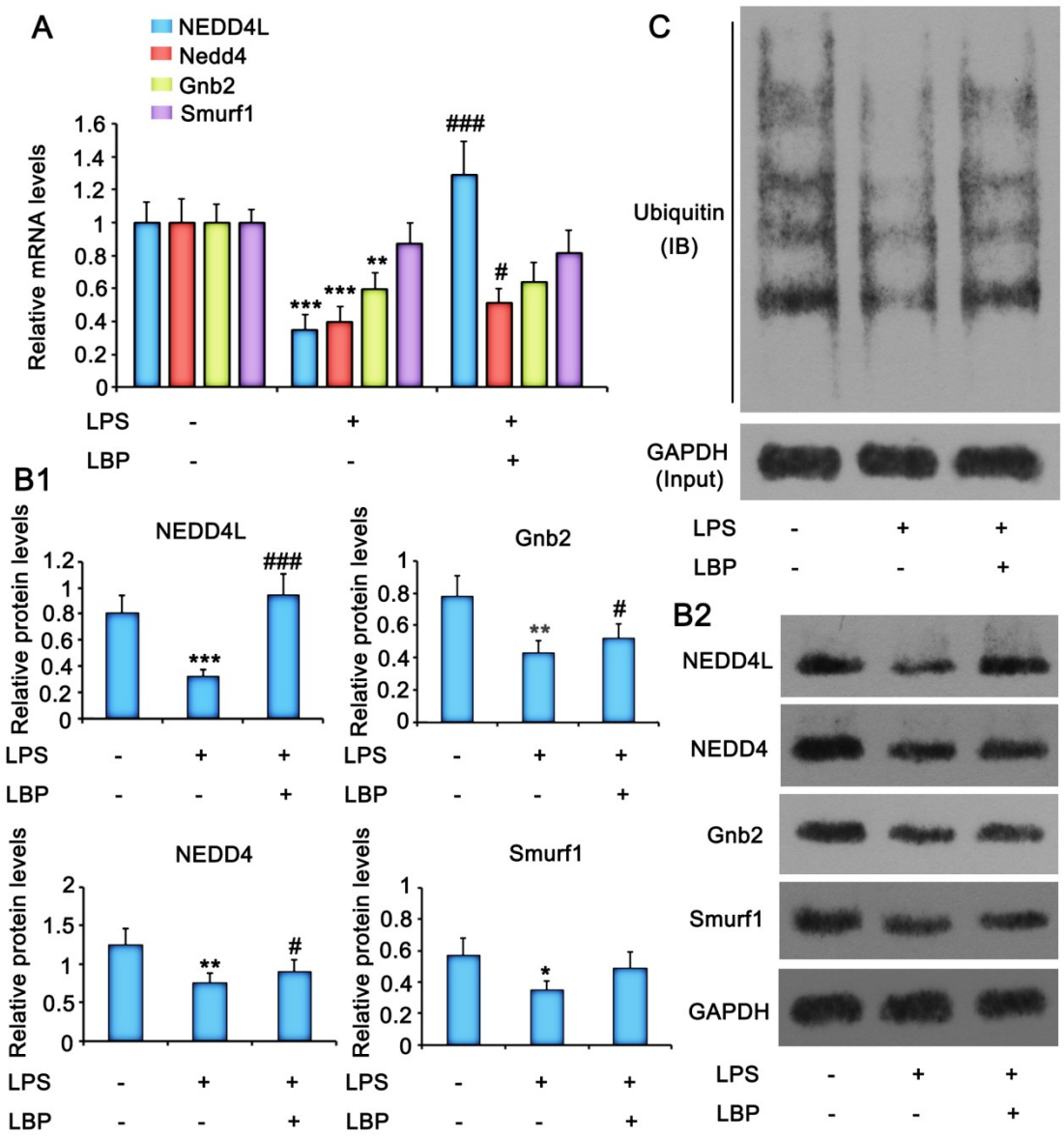

Figure 5. LPS increased PKM2 protein via suppressing ubiquitination which was disrupted by LBP

This study performed PCR (A) and western blot (B) assays to assess mRNA and protein levels of Nedd4L, Nedd4, Gnb2 and Smurf1 in macrophages after treatments with LPS alone or in combination with LBP. Co-IP analysis was conducted to determine the enrichment of ubiquitin in PKM2 protein. *P<0.05, **P<0.01, and ***P<0.01 vs. control; $^{\#}P$<0.05 and $^{\#\#\#}P$<0.01 vs. LPS group. (Color figure can be accessed in the online version.)

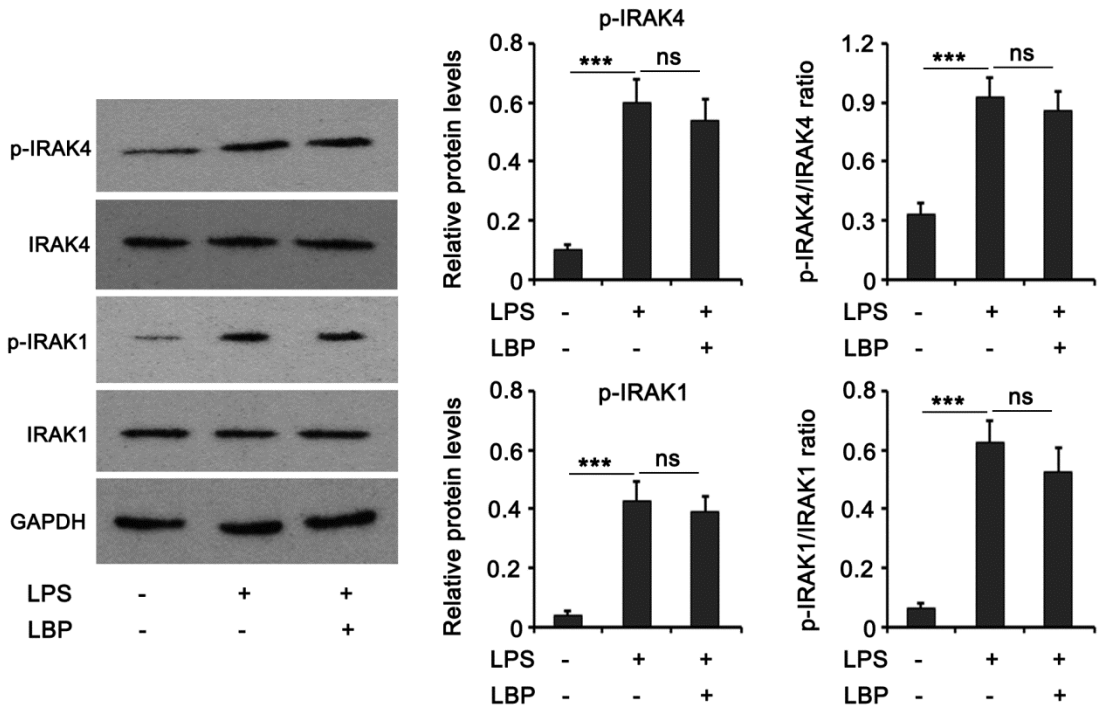

Figure 6. LBP had no effect on the activation of TLR4 by LPS

This study performed western blot assay to assess protein levels of p-IRAK4, IRAK4, p-IRAK1 and IRAK4 in macrophages after treatments with LPS alone or in combination with LBP. ***P<0.01 vs. control.