

Effect Long-Term Exposure to Different Doses of Lipopolysaccharides on the Intestinal Mucosal Immune Barrier

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Abstract

The small intestinal villus is covered with a thick layer of mucus that is secreted by goblet cells and functions primarily to first barrier from damage by toxic substance. Recent studies showed that goblet cells and mucins involved in complex immune function. Lipopolysaccharide (LPS) is widespread in the housing of livestock, which can induce bacterial infection symptoms and immunological stress within a short of time. Therefore, we aimed to study the effects of long-term exposure to different doses of LPS on intestinal mucus layer and immune barrier. The result showed that mucus layer thickness and goblet cell functions were significantly increased after low doses of LPS. The intestinal mucosal barrier can block the bacteria of the lumen, but LPS can penetrate this barrier into the blood, putting the body in a state of chronic low-grade inflammation and reducing the body’s immune function. However, after long-term exposure to high doses of LPS, a large number of lysosomes in goblet cells caused loss of function, and mucus layer thickness was significantly decreased. A large amount of LPS stuck to the mucus, leading to normal LPS and inflammatory cytokines level of plasma. The intestinal tissue morphology was damaged, and a number of immune cells were necrosis in the intestine. Collectively, long-term exposure to low doses of LPS lead to chronic low-grade inflammation in the body. Long-term exposure to high doses of LPS can be directly linked to the severity of the immunosuppression in the body.

Keyword: lipopolysaccharides; mucin; ileum; MUC2; mucosal barrier; mouse

1. Introduction

The intestinal tract is an important part of the digestive system. The integrity of the intestinal mucosal barrier is very important for maintaining the normal function (Schroeder, 2019). When mucous membrane penetrability increased to a certain degree, the intestines of macro-molecular substances, such as bacteria and toxins, through the damaged intestinal mucosa to peripheral tissue,

the liver, lymph, and blood, which occurred bacterial translocation and endogenous infection (Hu Y-Y, 2013; Yang X-Y, 2013; Nakarai, 2012). The gastrointestinal tract is resided by a wide variety of microorganism, which are called the gut microbiota. These microorganisms live in their host, at the same time it help the host to complete a variety of physiological and biochemical functions (Formosa-Dague,2018). Not only intestinal is the digestion and absorption of place, it is also the biggest immune organ, and plays an extremely important role in maintaining normal immune defense function. The first line of defense of the intestinal barrier is a dense layer of mucus to prevent translocation of the gut microbiota into underlying tissues. Intestinal mucus is an organized glycoprotein network with a host-specific glycan structure (Johansson,2014). The goblet cells can generate to mucins through regulate secretion pathway and update the intestinal mucous layer. Gut microbiota is the crucial factor about the composition of mucus, thickness and mucous barrier permeability (Chassaing,2017). Intestinal mucous can prevent to bacteria contact epithelium, resistance to infection and adjust to balance between immune function and external stimuli (Gill, 2010). Recent studies have found that long-term exposure to low doses of LPS can lead to low-grade inflammatory syndrome (Nakarai,2012). LPS, sometimes called endotoxin, is an amphiphilic glycolipid of the outer membrane of Gram-negative bacteria. Upon infection, LPS is associated with toxicity and immunogenicity (Zhang,2013). The accumulation of LPS in the body mainly occurs in the intestine. Previous studies have shown that the ileum is injured after exposure to low doses of LPS in the small intestine (Li, 2019). Therefore, in this study, we aimed to investigate the mechanistic connections between long-term exposure to different dose LPS on microbiota composition and inflammatory syndrome as well as the resulting effects on the mucus barrier. We examined changes in intestine mucous layer, inflammatory factors, and immune cells in long-term exposure to different dose LPS. Understanding the molecular details of this different dose LPS may effect on immune function through intestinal mucous layer, these result may contribute to the development of novel treatment options for diseases involving a dysfunctional mucus layer.

2.Materials and methods

2.1 Animals and experimental design

One hundred twenty Kunming mice (female, 6 weeks old, 25 g/body weight) were acquired from Xi'an Jiaotong University (Xi'an, China), and they were housed in a specific environment of pathogen-free. All experimental procedures in this study were met the requirements of the animal ethics committee of Northwest A&F University and complied with the animal welfare provisions formulated by the Institutional Animal Care and Use Committee. These mice were fed for three days to relieve the stress reaction prior to the experiment.

Mice randomly equally divided into four groups (n=30) were housed and absolutely fasted for 12 h to ensure a physiological situation of gastric emptying and then were gavaged with LPS

(Escherichia coli [O55:B5]; Sigma, USA). The different dosage regimens were 0 mg/kg (control group, n=30), 1 mg/kg (low group, n=30), 1.5 mg/kg (middle group, n=30), and 3 mg/kg LPS (high group, n=30) mixed a total volume of 300 μ L 0.01M of phosphate-buffered saline (PBS) by oral gavage every 48 h. On the purpose of decreasing gavage-related stress response, the tools of the gavage needle were dipped in a 30% sucrose solution. After that, the mice had unrestricted access to water, but each mouse was fed 3 g per day. The samples of the whole blood, Peyer's patches, mesenteric lymph nodes, ileum tissues, and the ileum contents were freshly collected to analyze subsequent experiment after LPS treatment for 16 and 32 d (15 mice were sacrificed in each group at the time of collection).

2.2 Tissue collection and histomorphology

For histology, mice fresh tissues of ileum, Peyer's patches and mesenteric lymph nodes were harvested and fixed with 10% neutral formalin buffer solution prior to paraffin embedding. After shaping, the tissues were rinsed in tap water for removing formalin overnight, dehydrated with alcohol in gradient style, transparentized with dimethylbenzene, and embedded in paraffin. Sections were cut (3 μ m), deparaffinized, hydrated, and stained with a Hematoxylin-Eosin Staining Kit (H&E, Solarbio, China) according to standard protocols. The pictures were shot with a Nikon Eclipse Ni-U microscope equipped with a Nikon DS-Ri2 camera, using the NIS-Elements software (Nikon Instruments, Japan).

Mucins underwent histological staining. Sections were cut (3 μ m), deparaffinized, hydrated and stained with a Periodic Acid-Schiff Stain Kit (PAS, Solarbio, China) in the dark environment, which stains mucins a light red, then thoroughly rinsed in tap water. Sections were counter-stained with hematoxylin, dehydrated, and mounted with neutral balsam with using standard protocols. Sections were imaged by brightfield as described above.

For ultrastructure, ileum tissues, including Peyer's patches, were freshly harvested from mice, cut longitudinally, fixed with 2.5% glutaraldehyde at 4°C for 2-3 h, and then immersed in 1% osmic acid for 2-3 h. The embedded sections (70 nm) were stained with uranium acetate-lead citrate. The pictures were obtained with transmission electron microscopy (TEM, JEOL JEM-1230, 80 KV).

2.3 Immunohistochemistry (IHC)

Formalin-fixed paraffin-embedded sections were deparaffinized, rehydrated and manufactured with the SP (rabbit immunoglobulin G (IgG))-POD Kit (Solarbio, China). Endogenous peroxidase blocking was performed by incubation with 3% H₂O₂ at RT. Antigen in tissues was resurrected in boiling citrate buffer (pH6.0) and blocking nonspecific antibody binding with goat serum. Sections were respectively incubated with rabbit anti-MUC2 (GeneTex, USA) and anti-MUC1(Rabbit Anti-MUC1 antibody [EP1024Y] (ab45167), Abcam, USA) overnight at 4°C, and then stained with goat anti-rabbit IgG (with biotin) at 37°C 30 min, incubated with

Streptavidin-POD at 37°C 30 min, colored with DAB solution, counter-stained with hematoxylin, dehydrated, and mounted with neutral balsam with using standard protocols, and imaged by brightfield as above according to standard protocols.

2.4 Western blotting

The ileum tissues were washed with cold PBS and lysed with using a tissue protein extraction kit (Bestbio, China). The total protein concentrations were detected with the BCA Protein Assay Kit (TianGen, China). And then the total protein (50 ng/lane) were respectively electrophoresed with 12% (secretory immunoglobulin A, sIgA) and 15% (MUC1) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). According to color of the prestained protein marker, the protein of sIgA and MUC1 were transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were carefully blocked with 5% skim milk and respectively incubated with specific primary antibodies, MUC1 (1:1,000 in PBS buffer, a reagent is the same as the IHC), sIgA (1:2,000 in PBS buffer, Cloud-Clone, China), and β -actin (1:8,000 in PBS buffer, Cell Signaling Technology, USA) at 4°C overnight, and incubated with goat anti-rabbit IgG (horseradish peroxidase-conjugated, 1:20,000, Xi 'an Zhongtuan Biotechnology Co., Ltd., China) in washing solution. Both proteins were detected using the Electrophoresis Image Analysis System (Shanghai Peiqing Science & Technology Co., Ltd., China).

2.5 Enzyme-linked immunosorbent assay (ELISA) analysis

All mice were sacrificed, and their plasma were taken into test tubes containing sodium heparin for the measurements of biomarkers. The level of LPS (lipopolysaccharide), MUC2 (mucin2), TNF- α (tumor necrosis factor- α), IL-4 (interleukin-4), IL-6 (interleukin-6), IL-10 (interleukin-10), IL-2 (interleukin-2), and IFN- γ (interferon- γ) were respectively measured with special ELISA kits (Beijing Sinouk Institute of Biological Technology, China). All detection assays were performed according to the manufacturer's instructions, and samples were run in duplicate.

2.6 High-throughput sequencing and bioinformatics Analysis

Twenty-one ileum fecal samples (three samples from C, six samples from L1 and L2, six samples from M1 and M2, six samples from H1 and H2) were randomly collected. Total bacterial DNA were extracted using a Stool Genomic DNA Kit (CWBio, Beijing, China) and evaluated by the means of using 1% (w/v) agarose gel electrophoresis. Polymerase chain reaction (PCR) was performed with the following universal 16S rRNA primers (V3–V5 region): 338F (50-ACTCC TACGGGAGGC AGC-30) and 806R (50-GGACTACHVGGGTWTCT AAT-30). Before the sequencing with an Illumina Miseq PE300 system (MajorBio Co., Ltd., China), the PCR products were purified and the concentrations were adjusted for an appropriate value. The data were analyzed on MajorBio I-Sanger Cloud Platform (www. i-sanger.com).

2.7 Statistical analyses

Data was expressed as mean \pm SD. The statistical analyses used the Statistical Package for Social Sciences (SPSS, USA) software, including two related sample tests. Each group versus the control group at the same time. A probability (p)-value < 0.05 was considered statistically significant, $*P<0.05$.

3. Results

3.1 The body weight and pathologic autopsy of mouse after LPS treatment

One hundred twenty female mice aged 6 weeks were selected and randomly divided into four groups: control group (0 mg/kg LPS), low group (1 mg/kg LPS), middle group (1.5 mg/kg LPS), and high group (3 mg/kg LPS). The mice were gavaged every 48h and slaughtered 16 and 32 d after LPS treatment (15 mice per time in each group) (Figure-1A). The body weight of the mice was measured every 4 d during the whole test period, and the result showed that the mice were fed the same dose of diet (3 g/d/each mouse, Figure-1C), the body weight of the mice in the low and middle groups was significantly higher than that in the control group (Figure-1B) ($P<0.05$). At 16 d and 32 d of LPS treatment, the changes of intestinal pathological autopsy were observed. At 16 d of LPS treatment, the hyperemia in the mesenteric vessels of the mice were gradually increased in the high group, the middle group, and the low group, and the enlargement of the Peyer's patches in the ileum was clearly visible, but the changes of mesenteric lymph nodes were not obvious. After 32 d of LPS treatment, mice in the high, middle, and low groups showed enlargement of mesenteric lymph nodes and congestion of ileum segment (Figure-1D).

3.2 Effects of LPS on the pathological histology of the ileum

To understand the long-term effects of different LPS contents on the histology of the ileum, we evaluated the ileum tissue morphology. The small intestinal villi in the low group were not significantly different compared to control group after 16 d of LPS treatment; The small intestinal villi in the low group were irregular and dissolved after 32 d of LPS treatment. The small intestinal villi in the middle group were dissolved and epithelial shedding after 16 d of LPS treatment; The small intestinal villi in the middle group were shorted, dissolved and epithelial shedding after 32 d of LPS treatment. The small intestinal villi in the high group were irregular and dissolved after 16 d of LPS treatment; loss of intestinal villi occurred after LPS treatment for 32 d, and further fusion and necrosis of intestinal villi occurred in mice in the high group (Figure-2A).

After 32 d of LPS treatment, the villus height of the ileum in the middle and high groups was significantly lower than that in the control group ($P<0.05$) (Figure-2C). After 16 d of LPS treatment, the intestinal crypt depth of mice in the middle and high groups was significantly higher than that of the control group ($P<0.05$); After 32 d of LPS treatment, the intestinal crypt depth of mice in low, middle, and high groups were significantly higher than that of the control group

($P < 0.05$); After 16 d to 32 d of LPS treatment, the intestinal crypt depth of mice in low, middle, and high groups deepened (Figure-2D). After 16 d of LPS treatment, the number of lymphocytes in the lamina propria of small intestine increased in the low group after 32 d of LPS treatment, the number of lymphocytes in the lamina propria of the small intestine further increased in the low group, and neutrophils and plasma cells appeared. After 16 d of LPS treatment, the number of lymphocytes in the lamina propria of the small intestine increased, and hyperemia was observed in the middle group. After 32 d of LPS treatment, the number of lymphocytes and plasma cells in the lamina propria of the small intestinal villus in the middle group increased significantly. After 16 d of LPS treatment, the number of lymphocytes and neutrophils in the intestinal villus propria of the high group increased significantly. After 32 d of LPS treatment, the number of lymphocytes and plasma cells, especially plasma cells in the high group, increased significantly in the lamina propria of the small intestine (Figure-2B).

3.3 Effects of LPS on the morphology of the mucus layer and mucin expression in ileum

We speculated that LPS first damaged the intestinal mucous barrier, and second the morphology of the intestinal villus. Next, we determined whether the intestinal mucous barrier was altered in long-term LPS exposure. The changes of the mucus layer in the ileum of the four groups of mice at each stage of the experiment was determined by PAS staining and IHC.

The results showed that after 16 d of LPS treatment, compared with the control group, the number of goblet cells in the ileum of the low and middle groups increased significantly ($P < 0.05$), while the number of goblet cells in the ileum of the high group was not significantly different from that of the control group ($P > 0.05$). After 32 d of LPS treatment, the number of goblet cells in the ileum of mice in the low and middle groups was significantly higher than in the control group ($P < 0.05$), the number of goblet cells in the ileum of the high group was lower than the number of goblet cells after 16d of LPS treatment (Figure-3A, C). After 16 d of LPS treatment, the content of MUC2 in the ileum in the low and middle groups increased significantly ($P < 0.05$), and the content of MUC2 in the high group was higher than that in the control group ($P > 0.05$). After 32 d of LPS treatment, the content of MUC2 in the ileum in the low and middle groups was significantly higher than that in the control group ($P < 0.05$), but there was no significant difference between the high group and the control group ($P > 0.05$) (Figure-3B, D).

After 16 d of LPS treatment, the expression of MUC1 protein in the ileum in the low, middle, and high groups was higher than that in the control group ($P < 0.05$). After LPS treatment for 32 d, the expression of MUC1 protein in the ileum in the high group was significantly lower than that in the control group ($P < 0.05$), while there was no significant difference between the low and middle groups ($P > 0.05$) (Figure-3E, F).

3.4 Effects of LPS on the ultrastructure of the mucous layer in the ileum

To analyze the long-term effects of LPS on goblet cells and the mucus layer, we observed the

ultrastructure of the ileum of mice by TEM. The results showed that after 32 d of LPS treatment inhibited the secretion of mucin by goblet cells with the increase of LPS treatment. In the high group, the number of lysosomes increased and swelling was observed in the goblet cells (Figure-4A). Through further observation of intestinal mucus secretion, it was found that the production of mucus secreted in the control group was moderate, and the production of mucus secreted in the high group was higher than that in the middle and low groups (Figure-4B). Moreover, the more bacteria in the intestinal lumen of the low and middle groups, the more mucus secreted (Figure-4B1, B2). In the high group, the production of mucus in the intestinal lumen increased sharply, but the function barrier of the bacteria was not found, and no bacteria were found outside the mucous layer. However, a large number of black dots appeared in the mucus of the high group, so we suspected that LPS adhered to it (Figure-4 B3). The number of lymphocytes in the lamina propria of ileal villi increased with the dose of LPS (Figure-4C).

3.5 Effects of LPS on gut microbes of the ileum

The full complexity of the gut mucosa-associated bacteria is a barrier to deriving detailed conclusions because sequence-based approaches (16S rDNA gene) suffer from substantial functional uncertainty. We analyzed the gut microbiota by 16srDNA sequence.

After 16 d and 32 d of LPS treatment, phylum level difference analysis was presented based on the intestinal flora of the four groups of mice: After 16 d of LPS treatment, *Bacteroides* was dominant in the gut microbiota of low group mice. After 32 d of LPS treatment, *Bacteroides* abundance of low group mice decreased; instead, it was replaced by *Phylum Firmicutes*, but no significant difference was found between them ($P>0.05$) (Figure-5A,C). After 16 d of LPS treatment, *Phylum Firmicutes*, *Bacteroides*, and *Proteobacteria* were dominant in the gut microbes of mice in the middle group. After 32 d of LPS treatment, the abundance of *Phylum Firmicutes* and *Proteobacteria* in the gut microbes of mice in the middle group increased, but not significantly ($P>0.05$) (Figure-5A,D). *Phylum Firmicutes* were the dominant bacteria in the intestinal flora of mice treated with LPS for 16 d in the high group. When treated with LPS for 32 d, *Phylum Firmicutes*, *Bacteroides*, and *Proteobacteria* were the dominant bacteria in the ileum of mice in the high group, and the abundance of *Phylum Firmicutes* was not significantly decreased ($P>0.05$) (Figure-5A,E).

After 16 d and 32 d of LPS treatment, genus level difference analysis was presented based on the intestinal flora of the four groups of mice: after 16 d of LPS treatment, *Muribaculaceae* was the most abundant in the ileum of the low group mice, followed by *Romboutsia*. After 32 d of LPS treatment, the abundance of *Muribaculaceae* decreased in the ileum of the mice in the low group, and *Muribaculaceae*, *Romboutsia*, *Lactobacillus* were in high abundance ($P>0.05$). After 16 d of LPS treatment, *Muribaculaceae* and *Romboutsia* were the most abundant in the ileum of the middle dose group mice ($P>0.05$). After 32 d of LPS treatment, the abundance of *Muribaculaceae* and *Romboutsia* bacteria decreased, while the abundance of *Lactobacillus* bacteria reached the

highest level, becoming the dominant genus ($P>0.05$). After 16 d of LPS treatment, *Romboutsia* was the dominant genus in the ileum of mice in the high group, and its abundance was the highest ($P>0.05$). After 32 d of LPS treatment, the abundance of *Romboutsia* was significantly decreased in the ileum of the high group mice, and *Muribaculaceae* and *Lactobacillus* were the dominant bacteria ($P>0.05$) (Figure-5B).

Through the heat-map observations: After 32 d of LPS treatment, abundance of *Epsilonbacteraeota* and *Deferribacteres* bacteria in high group mice was higher than that in the control group ($P>0.05$). *Patescibacteria* abundance in the low group was higher than in the control group after treatment with LPS for 16 d and 32 d ($P>0.05$). The *Patescibacteria* abundance of the high and middle groups was higher than in the control group after treatment with LPS for 32 d ($P>0.05$). There was no significant difference between other bacteria treated with LPS at 16 d and 32 d and the control group ($P>0.05$) (Figure-5F). There were not different between Alpha analysis and Beta diversity analysis (Data not show).

3.6 Effects of LPS on the histopathology of lymph nodes in the ileum of mice

According to results shown in 3.1, we found that the damage of Peyer's patches occurred prior to mesenteric lymph nodes. We speculate that LPS first destroys the mucous-microbiota barrier, entering the intestine lamina propria into the mesenteric lymph nodes through the lymphatic circulation into the blood. After LPS treatment for 32 d, the germinal center of Peyer's patches of mice in the low group showed enlargement, star necrosis, and a small amount of neutrophil infiltration. The star necrosis of the Peyer patch nodal germinal center in the middle group increased further, and necrosis was more obvious. Neutrophil infiltration, necrotic cell fragments, neutrophil necrosis, and plasma cell number increases were observed in PP nodes of mice in the high group (Figure-6A). After LPS treatment for 32 d, lymphocyte and macrophage infiltration in the medullary sinuses of mesenteric lymph nodes (MLN) of mice in low group was observed. In the middle group, the germinal center of the MLN was found to be necrotic and to be infiltrated by a large number of inflammatory cells. In the high group, reticular endothelial cells of MLN were proliferated, with a large number of lymphocytes and macrophages infiltrating the medullary sinus and cortical necrosis and many cell fragments in the cytoplasm of reticular endothelial cells in MLN (Figure-6B).

3.7 Changes of LPS content in the blood and sIgA expression in the ileum

After LPS treatment for 16 d, LPS content in the plasma of mice in the high groups was significantly higher than that of the control group ($P<0.05$); LPS content in the plasma of mice in the low and middle groups were not difference compared with control group ($P>0.05$). After 32 d of LPS treatment, the LPS content in the plasma of mice in the low and middle groups was significantly higher than that of the control group ($P<0.05$). After 16 to 32 d of LPS treatment, the LPS content in the plasma of mice in the low and middle groups increased while that the in high

group decreased (Figure-7A).

After LPS treatment for 16 d, the expression of sIgA protein in the lumen contents of the high group was significantly higher than that of the control group ($P<0.05$). There was no significant difference in sIgA protein expression in the ileum between the middle group, the low group, and the control group ($P>0.05$). After LPS treatment for 32 d, the expression of sIgA protein in the ileum of the low, middle, and high groups was significantly higher than that of the control group ($P<0.05$). From 16 d to 32 d, the sIgA expression in the ileum of mice in the low and middle group showed a significant upward trend, while that in the high groups showed a downward trend in the test period (Figure-7B).

3.8 Effect of LPS on the content of inflammatory factors in plasma

After LPS treatment for 16 d, the content of IFN- γ in the plasma of mice in the low group was significantly higher than that in the control group, while the content of IFN- γ in the plasma of mice in the middle and high groups were significantly lower than that in the control group ($P<0.05$); After LPS treatment for 32 d, the IFN- γ content in the middle group was significantly higher than that in the control group ($P<0.05$); the IFN- γ content in the high group was significantly lower than that in the control group ($P<0.05$).

After LPS treatment for 16 d, the TNF- α content of plasma in the low and high groups were significantly higher than the control group ($P<0.05$); After LPS treatment for 32 d, the plasma TNF- α content of the low group mice were significantly lower than the control group ($P<0.05$).

After LPS treatment for 16 d, the IL-2 content of mice in the low, middle, and high groups was not significantly difference compared to the control group ($P>0.05$); After LPS treatment for 32 d, the IL-2 content of mice in the low and middle groups was significantly higher than that in the control group ($P<0.05$); There were not difference between high group and control group on the IL-2 level ($P>0.05$).

After LPS treatment for 16 d, the IL-10 content of mice in the middle groups was significantly higher than that in the control group ($P<0.05$). After LPS treatment for 32d, the plasma IL-10 content of mice in the middle and high group was significantly higher than that in the control group ($P<0.05$).

After LPS treatment for 16 d, the IL-4 content of mice in the low and high groups was significantly lower than that in the control group ($P<0.05$); After LPS treatment for 32 d, the IL-4 content of mice in the low and middle groups was significantly higher than that in the control group ($P<0.05$); There were not difference between high group and control group on the IL-4 level ($P>0.05$).

After LPS treatment for 16 d, the IL-6 content of mice in the low, middle and high groups was significantly lower than that in the control group ($P<0.05$); After LPS treatment for 32 d, the

IL-6 content of mice in the low and middle groups was significantly higher than that in the control group ($P<0.05$); There were not difference between high group and control group on the IL-6 level ($P>0.05$) (Figure-7C).

4. Discussion

The intestinal mucosal immune barrier is composed of mucus layer, epithelial and immune cells that participate with the resident microbiota which together form a barrier to harmful substances (Cornick, 2015). A thick layer of mucus covers the epithelial cells, which is secreted by goblet cells, and serves as the first line of innate host defense (Corfield, 2018). A protective physical barrier is formed to prevent micro-organisms and toxic substances from reaching the surface of the epithelium(Sisi feng, 2016). We observed that the number of goblet cells in the low and middle groups was dramatically higher than that in the control group after LPS treatment for 16 d and 32 d, but there was no difference in the number of goblet cells between the high group and the control group. The number of goblet cells after LPS treatment 16d were higher than that of LPS treatment 32d in high group. As a barrier, the mucous layer secreted by goblet cells consists of two layers, an inner mucus layer and an outer mucus layer. There are two different types of molecules called mucins. The classical gel-forming mucins (**MUC2**) form extremely large sugar O-glycan polymers. The second line, the transmembrane (TM) mucins (**MUC1**), cover the apical surface of the intestinal epithelial cells (glycocalyx) (Axelsson, 1998; Godl, 2002; Ambort, 2012). After LPS treatment for 32 d, the expression of MUC2 and MUC1 were analyzed by ELISA and Western blotting respectively. The MUC2 content in the low and middle groups was higher than that in the control group, while the MUC2 content in the high group was lower than that in the control group. After LPS treatment for 16 d, the MUC1 expression in the low, middle and high groups were higher than the control group. The expression of MUC1 was decreased, and the high group was significantly lower than the control group after LPS treatment for 32 d. Combined with the results of TEM, LPS can promote the secretion of mucus by goblet cells and increase the thickness of the mucous layer covering intestinal epithelial cells after long-term exposure to low-dose and middle-dose LPS, thus effectively protecting the intestine from the endotoxin. However, high-dose LPS destroyed the function of the goblet cells in the intestine (a large amount of swollen lysosomes appeared in the cytoplasm), which led to obvious decrease in the levels of MUC1 and MUC2. In return, the mucosa-associated bacteria are able to bind or degrade mucin glycan as a nutrient source (Celebioglu, 2018). The intestinal mucus layer is crucial line of defence between host and microbe, its breakdown leads to gut bacterial encroachment that can eventually cause inflammation and infection (Geerlings,2018). In our experiment, the abundance of the *Firmicutes* increased by analyzing intestinal microflora, but the intestinal flora have no distinct difference among the four groups of mice. The content of *Firmicutes* more than the *Bacteroides* in the intestinal tract can cause more effective absorption of calories in food and further lead to obesity (Meyer-Hoffert, 2008). Our results are consistent with the previous

conclusion that mice exposed to LPS have higher body weight than the control group (Bevins, 2011). A lot of pathogens cause disease pathogenesis is due to subvert and penetrate the intestinal mucus barrier (Loonen,2014). TEM pictures showed that the intestinal mucosal secretion of the low and middle group was thicker than the control groups. The content of MUC1 and MUC2 in high group were lower than the middle and low groups. However, the intestinal mucus layer of the low group can block the intestinal bacteria, and the middle group can adhere to the intestinal bacteria to prevent it. This found that the gut bacteria penetrate into the mucus layer may be due to increased bacterial glycan degradation, which it may also be caused by a host defect that leads to decreased production, secretion mucin. In the high group, the mucus layer loses its function due to adhesion of a large amount of LPS, which is consistent with MUC2-deficient mice. MUC2-deficient mice are incapable of preventing contact of the intestinal microbiota with the host epithelium (Forstner,1995; Celli, 2007; Harrington,2009). The above experimental results indicate that long-term exposure to high doses of LPS can damage both layers of mucus, which can shift bacterial toxins into intestinal epithelial cells. By observing the tissue structure of the intestinal villus, we found that the degree of dissolution of the small intestine villi gradually increased with the increase of the dose and time of exposure to LPS, and the damage of the small intestine villi and the depth of the crypt increased significantly. In summary, with long-term exposure to LPS, LPS first contacted the mucus layer of the intestine. Low-dose and middle-dose LPS can further promote the secretion of MUC2 by increasing the number of goblet cells, thereby protecting the structure of the mucus inner layer MUC1. Nevertheless, high doses of LPS decrease the secretion of MUC2 due to dysfunction of goblet cells, further disrupting the structure of MUC1. Therefore, to ensure the protective function of the intestinal mucus layer, we must first ensure the secretion of MUC2.

Recent studies have showed that MUC2 and goblet cells closely linked to immune function, such as antigen sampling and tolerance (Boltin,2013; Quintana-Hayashi,2018;Formosa-Dague, 2018). At first, we measured sIgA expression of lumen content in ileum, the expression of sIgA in the low and middle groups was not significantly different from that in the control group at 16 d after LPS treatment, however, the expression of sIgA in the high groups was significantly increased compared with control group. After 32 d of LPS exposure, all three groups were higher than the control group (low group > middle group > high group>control group). sIgA can neutralize viruses, endotoxins, and exotoxins, allowing bacteria to aggregate (Gill,2010). sIgA is more protective than IgG and IgM in the blood when the body is infected by some bacteria, parasites, and viruses. Moreover, sIgA can combine with bacterial toxins to form a complex that prevents the toxin from binding to the villi of intestinal epithelial cells (Sheng,2011). Once secreted into the intestinal lumen, sIgA mixes with mucus and cover the epithelial cells of the small intestine, where it can directly interact with microorganisms or antigens to form antigen-antibody complexes for phagocytosis and clearance by macrophages (Martens Eric, 2018). Subsequently, we examined the content of LPS in the serum. After LPS treatment 16 d, the levels

of LPS in the high groups were significantly higher than those in the control group. There was not different between high and control group after LPS treatment 32 d, the levels of LPS in the low and middle groups were significantly higher than those in the control group after LPS treatment 32 d. We speculated that low dose of LPS could not adhere to the mucous layer secreted by goblet cells, it could enter the blood through lymphatic circulation. When high dose of LPS could adhere to the mucous layer in large quantities to destruction of goblet cells, so the level of LPS in blood was not significantly different from that in the control group. It is also unknown whether sIgA and mucin secreted by goblet cells can inhibit LPS level.

Damaged intestinal mucous barrier can subsequently cause to commensal and pathogenic microbes as well as microbial products to penetrate epithelial surface to intestinal lamina propria. This can promote immune cell activity and cytokine production leading to inflammation (Johansson, 2011). To further investigate the relationship between mucus layer and intestinal immune function in mice with long-term exposure to different doses of LPS, the immune cell status of the lamina propria and mesenteric lymph nodes in the ileum were imaged using H&E and TEM. This indicated that Peyer patch enlargement after LPS treatment for 16 d, and vascular congestion in ileum mesenteric vessels after LPS exposure for 16 d and 32 d. As the dose increased, the number of plasmacytes and neutrophils in the lamina propria of the small intestine increased significantly after long-term exposure to LPS (Figures-3 and Figure-4). Peyer's patch lesions first occurred, mainly stars necrosis, followed by reticulocyte hyperplasia in the mesenteric lymph nodes. It is indicated that LPS in the intestine first damages the Peyer's patches, then reticular cell hyperplasia in the mesenteric lymph nodes, and finally enters the circulatory system through lymphatic and blood circulation. Accumulation of LPS can cause to fever and inflammation reaction. To address this, we assessed the relationships between LPS exposure and inflammatory cytokines in serum of mice. In this study, the level of TNF- α of high group was higher than the control group mice after LPS treatment 16d. Several authors reported that the plasma concentration of pro-inflammatory cytokine TNF- α increased after injection of low-dose LPS (Charavaryamath, 2008). However, the level of TNF- α of low, middle and high group showed decline after LPS treatment 32d. We speculate that the TNF- α content decreased for long time exposure LPS. In addition, other studies have shown that repetitive endotoxin exposure could lead to LPS tolerance, which was indicated by decreased TNF- α expression (Roque,2016). The content of IL-10, IL-4, and IL-6 in low and middle group mice were significantly increased compared to those of the control group, and there were not difference between high group and control group on IL-10, IL-4, and IL-6 content after LPS treatment 32 d. IL-4 up-regulated MUC2 and MUC5AC expression in epithelial cells in vivo (Enss, 2000). Th-2 lymphocyte mainly secreted IL-4, 5, IL-6, IL-9, IL-10 and IL-13 cytokines to promote B cell proliferation, differentiation and the production of antibodies. Such as IL-4 and IL-13 are important induces to metaplasia of goblet cell (Grunig,1998; Dabbagh,1999; Shim, 2000). Inhibiting Th1 cells is the most important biological function of IL-4, which is the basis of cross-regulation of Th1 cells and

Th2 cells. The main role of Th2 cells is to enhance the humoral immune response of B cells (Roque, 2016; Hasnain, 2013). So while TH2 reactivity is dominant in the chronic phase. However, the TNF- α , IL-2, IL-10, IL-4 and IL-6 level of high group were not difference compared to control group mice after LPS treatment 32d. Combine with the histopathologic results, we showed that immune cells appeared severity of necrosis of lymph node, and the inflammatory cytokines level were decreased for long time exposure high dose of LPS. Finally, the body of mice appear immunosuppression state.

5.Conclusion

Accordance to this experiment, we established a mouse model for long-term exposure to LPS. In the high group, the mucus layer of the intestine adsorbed a large amount of LPS and lysosomes in goblet cells, so that the levels of LPS and inflammatory factors in the blood were not significantly different from those in the control group, but more serious in the damage of intestinal tissue. In the low and middle groups, a large amount of mucus was secreted in the intestine, and the goblet cells secreted well, which could prevent intestinal bacterial translocation. LPS accumulated in the intestine can enter the blood through the lymphatic circulation to stimulate the secretion of inflammatory factors, so that the body enters a chronic low-grade inflammatory state. Therefore, long-term exposure to low doses of LPS can cause chronic low-grade inflammation by altering the intestinal mucus barrier. Long-term exposure to high doses of LPS can be directly linked to the severity of the immunosuppression in the body.

Figure Legends

Figure-1 The changes of mice body weight and anatomy. (A) Experimental design. The four dosage group were 0 mg/kg (control group, n=30), 1 mg/kg (low group, n=30), 1.5 mg/kg (middle group, n=30), and 3 mg/kg LPS (high group, n=30). (B) Changing trends in body weight of mice after LPS treatment. (C) The feeding dose of mice diet every day. (D) Anatomical change of mice intestinal from Control group, Low group, Middle group and High group after LPS treatment 16d and 32d; In the first row are anatomical image of mice after LPS treatment 16 d, four figure from left to right in turn is control group, low group, middle group and high group respectively(white arrows point to Payer patch (PP)); In the second row are anatomical figure of mice after LPS treatment 32 d, four figure from left to right in turn is control group, low group, middle group and high group respectively(white arrows point to Mesenteric lymph nodes (MLN)).

Figure-2 HE stained transversal section of mice small intestine. (A) Effects of different doses LPS on mice ileum. (B) The pathologic changes of immune cells of the lamina propria in ileum (Yellow square). (C) Changes of villi height in the small intestine. (D) Changes of crypt depth. (×200)

Figure-3 After LPS treatment 16d and 32d, the structure of mucus layer and the expression of mucin in four groups. (A) The mucus layer of ileum was stained red using PAS staining. (B) Distribution of MUC2 were analyzed by IHC. (C) The number of globe cells in each 100 intestinal epithelial cells. (D) The content of MUC2 were measured by ELISA. (E) Distribution of MUC1 were analyzed by IHC. (F) The expression of MUC1 were measured by western-blot. (×200)

Figure-4 Ileum ultrastructure were measured by Transmission electron microscopy (TEM). (A) The ultrastructure of globe cells after LPS treatment 32d (×2500). (B) Mucus layer thickness of four group mice (×2500). The views of mucus layer and bacteria of mice in Low group (B1), Middle group (B2) and High group (B3) (×8000). (C) The lamina propria lymphocytes of ileum (×2500).

Figure-5 Effects of LPS on gut microbes of ileum. The analysis of mice intestinal flora about phylum (A) and genus (B) level difference analysis after LPS treatment 16d and 32d. The different genus of intestinal flora in Low group (C), Middle group (D) and High group (E) after LPS treatment 16d and 32d. (F) The heat map about intestinal flora of four group mice.

Figure-6 After treatment LPS 32d, the changes of histomorphology on Peyer's patches(A) and Mesenteric lymph nodes(B) from four group mice(×200).

Figure-7 The level of LPS in plasma (A) and the expression of sIgA in lumen contents (B) from four group mice ileum after LPS treatment 16d and 32d. The level of inflammatory factors in plasma from four group mice after LPS treatment 16d and 32d (C). The LPS and inflammatory factors were measured by ELISA, and the expression of sIgA in lumen contents were measured by WB. The first column of the result in each figure are value after LPS treatment 16d, and the second column of the result in each figure are value after LPS treatment 32d.

Figure-5S The sobs diversity index of the small intestine (A and B); The PCA (C), PCoA (D) and NMDS (E) plots based on treatment LPS in mice with different time. C1: control group; L1: Low group after LPS treatment 16d; L2: Low group after LPS treatment 32d; M1: Middle group after LPS treatment 16d; M2: Middle group after LPS treatment 32d; H1:High group after LPS treatment 16d; H2: High group after LPS treatment 32d.

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Not applicable.

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Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Chenchen Wu and Chao Li performed the experiment; Baoyu Zhao designed the experiments and analysed data; Chenchen Wu and Chao Li wrote the manuscript.

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