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ORIGINAL ARTICLE

Effects of Long-Time Exposure to Lipopolysaccharide on Intestinal Lymph Node Immune Cells and Antibodies Level in Mice

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ABSTRACT

Background: Endotoxin, widely present in the living environment of humans and animals, leads to endotoxemia during a short period. However, the long-term effects of endotoxin on immune function are unclear. **Objective:** To determine the significance of long-term endotoxin treatment on the body's immune function. **Methods:** The mice were treated with different doses of lipopolysaccharide (LPS) for a month; the collected samples were then analyzed in terms of value changes in hematological parameters, lymphocyte subtypes, and immunoglobulins level. **Results:** The number of monocytes (MONO) and neutrophils (NEU) in the three treatment groups was significantly lower than the control after 30 days. However, the proportion of CD8+ T lymphocytes showed a rising trend in the mesenteric lymph nodes (MLNs) and Peyer's patches (PPs) while the CD4+ T cell was reduced. At the same time, a decrease was observed in the percentage of CD19+CD38+ B lymphocytes in plasma cells. Interestingly, the change of lymphocytes in PPs was more significant than that in MLNs, suggesting that immune response in the PPs occurred before the MLNs. Consistent with the changes in plasma cells, the content of IgA and IgG showed a downward trend. **Conclusion:** Long-term exposure to low-dose endotoxin had little or no effect on the immune function of the body, suggesting that the endotoxin can be rapidly eliminated by the immune system. Nonetheless, the number of immune cells was reduced in the high-dose group. Unfortunately, T-, B-lymphocytes and plasma cells were significantly reduced, resulting in a decrease in immunoglobulin level, and showing a significant immune suppression state.

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Keywords: Blood Cells, B Lymphocyte, IgA, IgG, Lipopolysaccharide, T Lymphocyte

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INTRODUCTION

Lipopolysaccharide (LPS, endotoxin) produced by the bacterial lysis of the Gram-negative bacteria can have various pathological effects on both humans and animals; among these effects, mention can be made of endotoxemia, shock, and inflammation, which can be life-threatening in severe cases. Although LPS has many negative impacts on the host, it can offer some upsides. For instance, in small amounts, LPS is able to activate the host's active immune response, thereby keeping the body in an excited state (1). Studies have shown that LPS may play an active role given its ability to proliferate and differentiate the T and B lymphocytes and initiate the activation of macrophages (2). It is a known fact that over different periods, lymphocytes have different molecular markers and play diverse roles. Firstly, T lymphocytes importantly contribute to the host through playing a central role in cell-mediated immunity. In reality, the molecule CD3 is used to distinguish T lymphocytes from other cells. Furthermore, it can be divided into two different subtypes of T cells, namely CD4+ and CD8+ T cell. On the one hand, CD4+ T-cells, commonly known as T-helper (Th) cells, can help to detect and fight off infections (3). On the other hand, the second classification of the CD3+ cells can eliminate infections, hence the name cytotoxic T (Tc) cells marked with CD8+ biomarker (4). More importantly, the response of B cells to protein antigens and the production of antibodies require the assistance and participation of T cells. In other words, Th cells are the second messengers of B cell activation and differentiation (5). Besides, LPS can induce the polyclonal activation of B cells and macrophages, and antigen-presenting cells (APCs). In the environment, there are large amounts of endotoxin that can cause bacterial death, lysis, or autolysis. When the massive endotoxin enters the blood, it exceeds the ability of the body's defense system, leading to varying degrees of endotoxemia and severely affecting the function of immune organs. Nevertheless, how the long-term treatment of endotoxin affects immune function is a question that remains to be answered. Therefore, in this study, mice were administered with different doses of LPS for a month to observe the effect of endotoxin on their immune function and provide a scientific basis for studying the pathogenesis of endotoxin on animal pathogenicity.

MATERIALS AND METHODS

Animals and Intervention. Forty C57BL/6 mice (female, 6 weeks, 22 g/body weight) were purchased from Air Force Medical University (Xi'an, China) and housed in a specific pathogen-free environment. Laboratory breeding conditions were suitable to ensure optimum growth. For instance, the basic operation setting had 22 ± 2°C temperature, 50 ± 15% humidity, and 12 h/12 h light/dark cycle. All mice had unlimited access to food and water. All experimental procedures in this study met the requirements of the Animal Ethics Committee of Northwest A&F University. To better comply with the animal welfare provisions, mice suffered little in the experiment. As for the health of the laboratory animals, these mice were fed 3-7 days to relieve stress reaction.

Forty mice were randomly divided into four groups (n=10), and three intervention groups were treated with different doses of LPS (Escherichia coli (O55: B5); Sigma, USA). The dosage regimens were 0 μg/kg (CT group, n=10), 200 μg/kg (LD group,

n=10), 600 μg/kg (MD group, n=10), and 1000 μg/kg (HD group, n=10) LPS mixed a total volume of 300 μL 0.01M PBS by oral gavage every 48 h. Whole blood samples and tissues of PPs and MLNs were freshly collected to analyze the subsequent experiments after LPS treatment for 15 and 30 days, respectively.

Hematological Parameters. The whole blood samples of each group were collected into EDTA-k2 anticoagulant tubes (BD, USA) before the mice were sacrificed. The changes in the basic physiological characteristics and the five-item analysis of the whole blood were immediately performed by the animal automated hematology analyzer (IDEXX, USA), especially in the complete blood count analysis. However, more attention was paid to the changes in the following type cells: white blood cell (WBC), lymphocyte (LYM), monocyte, neutrophil, eosinophil (EOS), and basophil (BASO).

Flow cytometry. In this detection process, the lymphocytes were freshly isolated from the MLNs and PPs. The MLNs were acquired directly after three times washing with PBS. The PPs were then isolated by flushing lumen contents away with PBS until they sparkled and removing the redundant tissues by micro-dissection. These lymph nodes were dissected and cell suspensions were obtained by use of a 200 mesh cell strainer. The operation method was as described previously (6). Briefly, the cell suspension was centrifuged and separated in a complete RPMI buffer. After taking counts, the cell cluster was centrifuged and 1 μg of the corresponding antibodies of CD3 (APC Hamster Anti-Mouse CD3e, Clone: 145-2C11), CD4 (PE Rat Anti-Mouse CD4, Clone: GK1.5), CD8 (FITC Rat Anti-Mouse CD8a, Clone: 53-6.7), CD19 (PE Rat Anti-Mouse CD19, Clone: 1D3), CD38 (FITC Rat Anti-Mouse CD38, Clone: 90/CD38) was added with micropipette. All antibodies were purchased from Becton, Dickinson and Company (NJ, USA). After staining for 30 min in the brown light-proof tube, the cells were washed with PBS and then fixed with 4% paraformaldehyde. Finally, the samples were detected by flow cytometer BD FACSAria™ III (NJ, USA). Data were analyzed using the relevant scientific software FlowJo.

IgA and IgG Level of Serum. The whole blood samples were collected in the test tubes with 100 μL heparin sodium solution and then centrifuged at 3000 rpm × 10 min. Afterwards, the supernatant was carefully absorbed into a new tube by micropipette. Subsequently, the contents of IgA and IgG were detected through the use of ELISA kit. Two factors were run three times at different times. To avoid repeated freeze-thaw cycles, samples were stored at -20°C when not in the test for a short period.

Statistical Analysis. Data were analyzed using the SPSS software (SPSS, USA) and expressed as Mean ± SD. Compared with the CT, *$p<0.05$ was considered as statistically significant, and **$p<0.01$ was considered as extremely significant, each treatment group versus the CT group at the same time.

RESULTS

Effect of endotoxin on hematological parameters in mice. Table 1 shows all the test results of blood parameters. Firstly, WBC decreased more significantly in the MD and HD groups than in the CT group after LPS exposure for 15 d, and the LD group had no significant changes. However, WBC in the HD, MD, and LD groups showed a downward trend after 30 days. Secondly, the NEU of the LD, MD, and HD groups was significantly different compared to the CT group after 15 days. Furthermore, NEU also showed a downward trend after 30 days, particularly in the HD group. Thirdly, LYM

was significantly reduced in the HD group, and there was no significant difference in the LD, MD, and CT group after 15 days of LPS exposure.

Table 1. Effect of LPS treatment on hematological parameters of whole blood in mice.

	t / d	CT group	Low group	Middle group	High group
WBC/(10^9•L^{-1})	15d	11.76 ± 1.21	9.89 ± 0.98	7.95 ± 1.02*	7.87 ± 1.12*
	30d	12.01 ± 1.55	8.46 ± 1.11*	6.01 ± 1.02*	4.61 ± 0.85**
NEU/(10^9•L^{-1})	15d	3.34 ± 0.55	1.91 ± 0.32*	1.88 ± 0.21*	1.59 ± 0.52**
	30d	3.14 ± 0.22	1.57 ± 0.21*	1.23 ± 0.25*	1.05 ± 0.14**
LYM/(10^9•L^{-1})	15d	6.20 ± 0.88	5.49 ± 0.95	5.44 ± 0.87	4.04 ± 0.91*
	30d	6.10 ± 0.65	4.83 ± 0.74*	4.45 ± 0.83*	3.11 ± 0.52*
MONO/(10^9•L^{-1})	15d	2.11 ± 0.53	0.86 ± 0.15**	0.61 ± 0.08**	0.30 ± 0.04**
	30d	2.08 ± 0.62	0.50 ± 0.14**	0.43 ± 0.09**	0.22 ± 0.05**

*p<0.05 each group versus the CT group at the same time. **p<0.01 each group versus the CT group at the same time.

Nevertheless, at the next monitoring time, the LYM value in the LD, MD, and HD groups significantly decreased compared with the CT. Ultimately MONO in the LD, MD, and HD groups was significantly lower than in the CT group after 15 and 30 days of LPS exposure. However, regarding other cell types (EOS and BASO), there was no significant difference between 15 and 30 days of exposure (data not shown).

Effect of endotoxin on the subtypes of T cell and B cell in MLNs and PPs of mice.

Figures 1A and 1B show the percentage of T cell subtypes in MLNs and PPs respectively. Moreover, to graphically show the data of CD4+ and CD8+, changing trends are shown in Figure 2 A-D. As observed in Figure 2 A and B, the CD4+ and CD8+ T cell proportions of the MLNs in the LD and HD group were not significantly different from the CT after LPS exposure for 15 days. However, after 30 days of LPS exposure, the proportion of CD8+ T lymphocytes of the MD and HD group significantly increased compared with the CT. Accordingly, the number of CD4+ T cells in the HD group was significantly lower after 30 days. Based on Figure 2 C and D, after LPS treatment for 15 days, there was no significant difference between the proportions of CD4+ and CD8+ T lymphocytes in the LD, HD, and CT in PPs. Meanwhile in the MD group, CD4+ was lower and CD8+ cells had an opposite trend after 15 days. After 30 days of LPS treatment, LD and CT groups had no significant difference regarding the percentage of CD4+ T cells. However, in the MD and, particularly, HD group, the percentage of CD4+ T lymphocyte was significantly lower than the CT. In the meantime, the percentage of CD8+ T cell had an upward trend. In other words, the percentage of CD8+ T cell in the MD group increased compared to the CT, and it was significantly higher in the HD group. The percentage of B cell subtypes in MLNs and PPs was respectively depicted in Figures 1C and 1D; Figure 2 E and F illustrate the graphical data. Figure 2E shows that the percentage of CD19+CD38+ B lymphocytes in the LD and MD groups was significantly higher than the CT whereas in the HD group, it was significantly lower after LPS treatment for 15 days. Interestingly, after 15 days of LPS exposure, the percentage of CD19+CD38+ B lymphocytes in all three groups was higher than after 30 days. However, the LD, MD, and CT groups did not significantly change while the HD group had a downward trend after 30 days of LPS exposure.

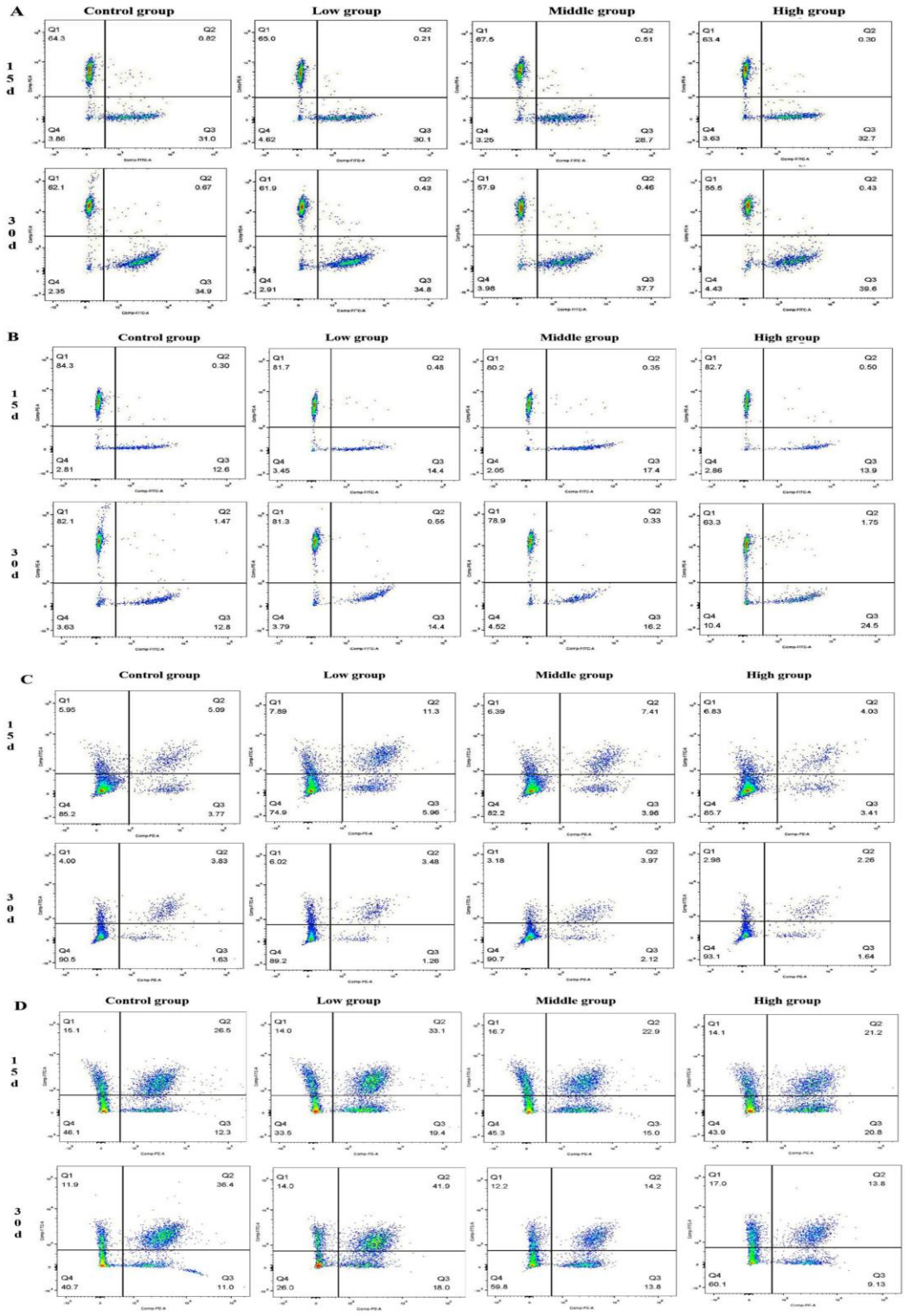

Figure 1. Effects of endotoxin on T cell subtypes and B cell subtypes in MLNs and PPs of mice. After LPS treatment for 15 and 30 days, the percentage of T and B lymphocyte subtypes had different trends in MLNs and PPs. Primarily, the percentage of T lymphocyte subtypes in MLNs and PPs was respectively shown on A and B. The value of CD4+CD8- subtype was shown on the first quadrant (Q1) and CD4-CD8+ on the third quadrant (Q3). Secondly, the percentage of B lymphocyte subtypes in MLNs and PPs was respectively shown on C and D. The value of the CD19+CD38+ subtype was shown in the second quadrant (Q2).

In PPs (Figure 2F), the percentage of CD19+CD38+ B lymphocytes in the LD group was significantly higher than the CT whereas in the MD and HD groups, it was significantly lower than the CT group. Nonetheless, the percentage of CD19+CD38+ B lymphocytes in the MD and HD group decreased after LPS treatment for 30 days.

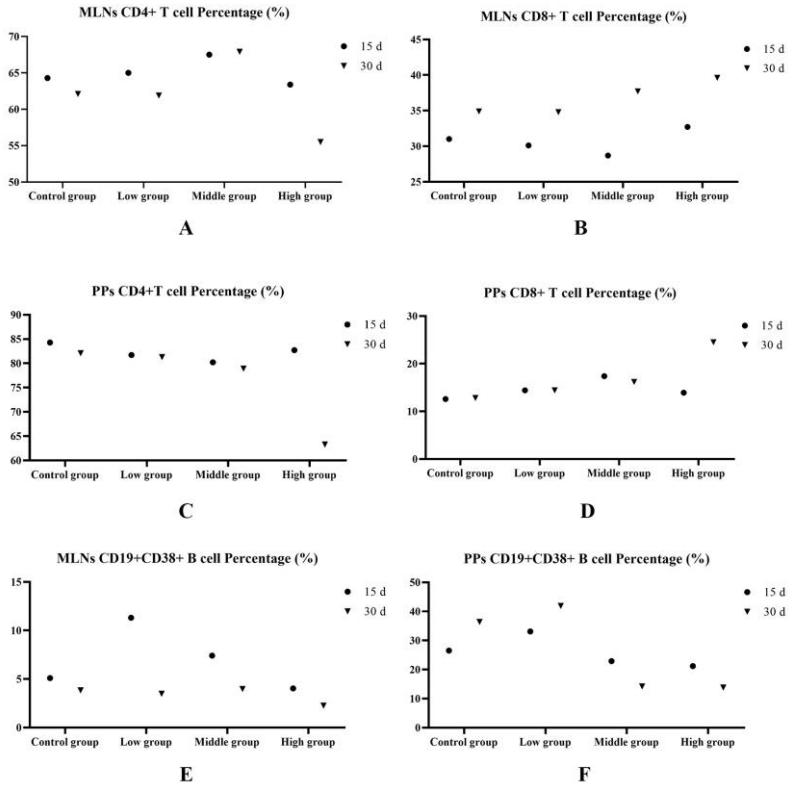

Figure 2. Graphical data of endotoxin affect T cell subtypes and B cell subtypes in MLNs and PPs of mice. After LPS treatment for 15 and 30 days, the changed percentage of T cell subtypes and B cell subtypes showed a different trend in MLNs and PPs. A and B: the percentage of CD4+CD8- and CD4-CD8+ T cell subtypes in MLNs. C and D: the percentage of CD4+CD8- and CD4-CD8+ T cell subtypes in PPs. E and F: the percentage of CD19+CD38+ B cell subtypes in MLNs and PPs.

Effect of endotoxin on IgA and IgG levels. The antibody levels of IgA and IgG are respectively shown in Figures 3 and 4. IgA did not significantly change in the HD and LD groups after LPS exposure for 15 days. However, it significantly increased in the MD group and significantly decreased in the HD group after 30 days. After LPS exposure for 15 days, the IgG content in the MD and HD groups significantly increased compared to the CT. It was significantly higher in the LD group, and no significant difference existed between the MD and CT after 30 days. On the other hand, the IgA and IgG levels showed a significantly downward trend after LPS treatment for 30 days.

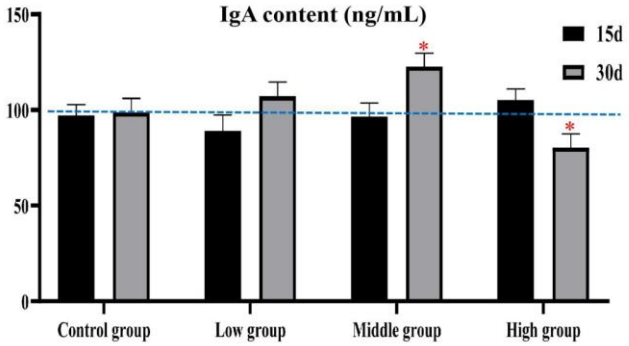

Figure 3. Effect of endotoxin on IgA levels. After LPS treatment for 15 and 30 days, the IgA content of serum antibody in the experimental groups had different trends than that in the CT group. The index in each group was run three times. *p<0.05 each group versus the CT group at the same time.

DISCUSSION

Bacterial endotoxin, also known as lipopolysaccharide, is the component of the cell wall in Gram-negative bacteria. LPS is a highly acylated glycolipid mainly composed of core polysaccharide lipids and antigen polysaccharides (7). Of note, endotoxin is abundant in our living environment, especially in the air, food, and medical services. For example, the release and accumulation of large amounts of endotoxin in the intestine is caused by the abuse of antibiotics and other sterilizing drugs.

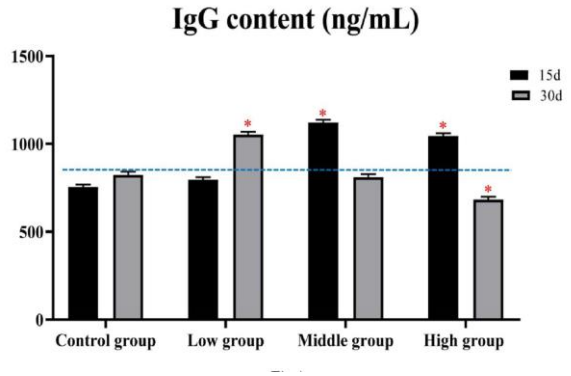

Figure 4. Effect of endotoxin on IgG level. After LPS treatment for 15 and 30 days, the IgG content of serum antibody in experimental groups had different trends than that in the CT group. The index in each group was run three times. *p<0.05 each group versus the CT group at the same time.

The effect of the long-term treatment of endotoxin on immune performance has yet to be clarified. In this study, mice were chosen and treated with different doses of LPS for a month. After LPS exposure for 15 and 30 days, the whole blood was collected, and the hematological indicators were tested. The results showed that the number of monocytes

significantly decreased. Monocytes, the predecessors of macrophages, have obvious movement and deformation. Therefore, they can devour and remove the injured and senescent cells along with their fragments (8). After the LPS component on the surface of the pathogenic microorganism enters the body from the mucosa of the gastrointestinal tract, the first immune cells encountered are macrophages located in the submucosal tissue. Macrophages are capable of expressing a variety of receptors, including CD14 and toll-like receptors (TLRs); they can also adhere to LPS to the surface, resulting in the absorption of LPS into the cells to form phagosomes; it is then cleared out of the cells by exocytosis (9,10). While the mice were exposed to LPS for 30 days, the number of neutrophils in the LD, MD, and HD groups was significantly reduced. Neutrophils account for 50-70% of peripheral white blood cells. Mature neutrophils are able to express pattern recognition receptors such as the CD14 receptor for LPS capture and the Fc receptor complement receptor (11). LPS binds to the TLR4 through pattern recognition on the membrane surface of the neutrophils to activate the cells. The activated neutrophils can release inflammatory factors, including IL-6, leucocyte triene B4, and reactive oxygen species (12). Neutrophils are in the first line of resistance to pathogenic microorganisms, especially suppurative bacteria. In case of infection, neutrophils will reach the inflammatory site at the first time, and the number will peak at 6 h (13). Neutrophils have a strong phagocytic capacity, thereby exhibiting phagocytic activity with the mononuclear phagocytic system. Lymphocytes produced by lymphoid organs are the smallest white blood cells mainly existing in the circulating lymphatic fluid in lymphatic vessels. Lymphocyte is a kind of cell line with immune recognition function; mature lymphocytes depend on antigen stimulation to differentiate and proliferate. Obviously, it is the frontline agent to fight against external infection and monitor the cell variation in the body (14,15). In the present study, after an LPS intervention of 30 days, the number of blood lymphocytes in the LD, MD, and HD groups was significantly lower than the CT. The T lymphocyte and B lymphocyte subtypes were further tested in the MLNs and PPs. Based on the results, the subtypes proportion of CD4+CD8- and CD4-CD8+ T cell in the three performed groups was not significantly different from those of the CT group in the MLNs and PPs after 15 days of LPS exposure. However, the percentage of CD4+CD8- T cell subtypes in MLNs and PPs in the MD and HD significantly decreased after LPS exposure for 30 days. CD4+ T cells can help detect and fight off infections, particularly in bacterial and viral infections. Similar to Th cells, CD4+ T-cells also are believed to be the second messengers of B cell activation and differentiation. Therefore, decreased CD4+ cells reduce the function of B cells. CD8 molecule is a co-receptor for MHCI-restricted T cells to recognize antigens; it can also bind to MHCI-type molecules to help TCRs recognize their presented antigens and enhance the interaction between T cells and APCs or target cells (16). Following LPS exposure for 30 days, the percentage of CD4-CD8+ T cell subtypes in MLNs and PPs in MD and HD group had a significant increase, which led to a rise in the clearance rate of pathogenic target cells. In this study, the percentage of B cell subtypes was separately tested in MLNs and PPs. The percentage of CD19+CD38+ B lymphocytes in MLNs increased in the LD and MD groups, and there was no change in the HD group after 15 days of LPS exposure. However, after 30 days, this percentage was significantly reduced in MLNs in the HD group. Additionally, the percentage of CD19+CD38+ B lymphocytes increased significantly in the LD group while it decreased significantly in the MD and HD group after 30 days of treatment with LPS. These results show that CD4+ T lymphocytes

recognize the processed antigen and activate it in peripheral lymphoid organs. Here, after B lymphocytes meet the antigen, Th cells induce B cell proliferation and differentiation (17,18). However, the reduced percentage of CD19+CD38+ B lymphocytes indicated that the direct binding of antigen to BCR transmits the antigen recognition signal to B cells. In the micro-environment of the germinal center, clonal expansion of B cells decreases, significantly reducing the number of differentiated plasma cells and memory B cells. Besides, with the significant decrease in the number of plasma cells, the IgA and IgG levels were reduced. Immunoglobulin is an effector immune molecule produced by the B cells into plasma cells proliferation and differentiation after the specific stimulation of B cell antigen epitope; this molecule is able to mediate fluid immunity. The secreted immunoglobulins are mainly present in blood and tissue fluids and play a role in anti-infection (19,20). IgG is the main antibody component in serum and body fluids, accounting for about 80% of the total serum immunoglobulins. It is also the main antibody for the second humoral immune response and has a central part in the immune response. This antibody has a wide distribution and a high affinity. In addition, it is second only to IgG in serum, comprising 10~20% of serum immunoglobulins. It exists in mucosal tissues such as the digestive, respiratory, and urogenital systems. Mucosal tissue has a mucosal layer of lymphatic tissue, generating IgA to prevent invasion by pathogens. In conclusion, exposure to the low-dose treatment of endotoxin after a long time increased the number of plasma cells and secreted higher levels of immunoglobulins. However, consistent with immunoglobulin levels and immunosuppression, high-dose ingestion of endotoxin decreased the number of plasma cells. Therefore, continuous exposure to high doses of endotoxin in the environment will eventually cause immunosuppressive diseases and reduce autoimmunity.

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