Microstructural changes and immunohistological analysis of pro-inflammatory cytokines in spleens of lipopolysaccharide-induced rats


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ABSTRACT
This study investigated splenic status changes in weaned Sprague-Dawley rats induced by lipopolysaccharide. There were forty 26-day-old rats selected randomly and equally divided into two groups. The treatment group received daily single doses of lipopolysaccharide, and the control group was treated with normal saline. We conducted haematoxylin-eosin staining, immunohistochemical staining and semi-quantitative optical density analysis for both groups on the 29th, 32nd, 35th and 38th days after treatment. The results indicated that splenic marginal zone in the lipopolysaccharide group was thinner or disappeared compared to that of the saline group. However, the periarterial lymphoid sheath and the diameters of splenic lymphoid follicles appeared thicker and wider than those in the saline group (P<0.05). The expression of interleukin-1 beta, interleukin-6 and tumour necrosis factor alpha was mainly localized within the periarterial lymphoid sheath and splenic lymphoid follicles in the lipopolysaccharide treated rats. The integrated optical density and the average optical density in the lipopolysaccharide group were greater than those in the normal saline treated group (P<0.05). In conclusion, splenic immune function is probably strengthened by altering microstructures and releasing pro-inflammatory cytokines following lipopolysaccharide treatment.

Key words: Immunohistological expression, Lipopolysaccharide, Pro-inflammatory cytokine, Splenic microstructure, Weaned rat.

INTRODUCTION
Lipopolysaccharide (LPS) is an important activator of the innate immune system and causes macrophages to release a variety of bioactive substances including bioactive lipids, reactive oxygen species, and polypeptide mediators (tumour necrosis factor: TNF, interleukin-1: IL-1, interleukin-6: IL-6, interleukin-8: IL-8) (Peri et al., 2010; Verstrepen et al., 2008). These secondary protein-like parahormones have strong biological activity and produce biological effects in synergetic or antagonistic ways after activation. LPS is regarded as a major factor in acute infection and inflammation caused by Gram-negative bacteria (Rietschel and Brade, 1992; Meseguer et al., 2014). A dose of intravenous or intraperitoneal LPS can induce acute injury in spleen (Xiao et al., 2015), thymus (Huanget et al., 2016), liver, lung (Du et al., 2014), colon (Zong et al., 2016) and other organs, and also induce inflammation while clinically mimicking Gram-negative infections. LPS activates the monocyte-macrophage system and leads to the synthesis and release of excessive inflammatory mediators such as tumour necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β) and leukotriene, and even cause systemic inflammatory response syndrome, acute lung injury, septic shock and multiple organ dysfunction syndromes (Ikezoe et al., 2003). Weaned rats lack mature immunity, and investigations of immune organ responses helped to clarify disease resistance. However, splenic immune changes during the immature stage are unknown when mimicking infection. In this study, a model of weaned Sprague-Dawley (SD) rats was established by using a daily, single-dose injection of LPS for ten consecutive days. Splenic immune status was explored by analysing the histological changes and the immunohistological expression of pro-inflammatory cytokines.

MATERIALS AND METHODS
Experimental animals: Forty healthy weaned SD rats aged 26 days and weighing 83±4 g were purchased from Jiangxi University of Traditional Chinese Medicine and housed in the animal anatomy laboratory of Jiangxi Agricultural University. The SD rats had unrestricted access to food and water. The animals were moved to the facility 3 days prior to the experiment to relieve stress. All procedures were performed in accordance with the Guidelines of Animal Experiments. The rats were randomly and equally divided into either the LPS (Escherichia coli serotype O55:B5, Sigma, St.Louis, MO, USA) or the control group. The animals

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were treated from the 29th to 38th day with single daily injections of LPS (100 μg/kg body weight) or normal saline through the caudal vein each morning. Five SD rats in each group received deep anaesthesia on the 29th, 32nd, 35th and 38th day. The spleens were instantly taken from each LPS- and saline-treated rat.

**Microstructure evaluation:** The spleens were fixed in 4% buffered paraformaldehyde solution for 18-24 h and then routinely processed using alcohol-xylene protocol and embedded in paraffin. Then, 5 μm sections of the spleens were prepared and stained with haematoxylin-eosin (HE kits: Sangon Biotech, Shanghai, China). Splenic microstructure changes were analysed and photographed. Image-pro plus 6.0 software was used to measure the diameters of splenic lymphoid follicles and the periarterial lymphoid sheath (PALS) area.

**Immunohistochemistry detection:** The spleen sections were processed as previously reported (Zhong et al., 2017), and then, the sections were incubated with the primary antibody (rabbit anti-IL-1β antibody: BA2782, BOSTER, Wuhan, China; rabbit anti-TNF-α antibody: BA0131, BOSTER, Wuhan, China; rabbit anti-IL-6 antibody: BS6309R, Bioss, Beijing, China) overnight at 4°C. The sections were then washed three times with 0.01 M PBS and treated for 15 min with HRP-conjugated goat anti-rabbit IgG at room temperature. The tissues were washed three times with 0.01 M PBS and incubated with HRP conjugated-streptavidin (SP-9001/9002 Link Detection Kits: ZSGB Biotechnology, Beijing, China) for 15 min at room temperature. The sections were then washed three times with 0.01 M PBS and stained with diaminobenzidine (DAB Kit: ZSGB Biotechnology, Beijing, China) to visualize the colour. The remaining procedures were conducted as per Zhong et al., 2017.

The distribution of positive cells was detected in non-overlapping fields from each spleen section. IL-1β, IL-6 and TNF-α expression were indirectly evaluated with area and integrated optical density (IOD). The data were collected with digital image analysis software (Image-Pro Plus 6.0: Media Cybernetics, Silver Spring, MD) with settings for colour and size identification. The average optical density (AOD)=IOD/area.

**Statistical analysis:** GraphPad Prism 5 software (GraphPad, San Diego, CA, USA) and SPSS (Version 13.0, Armonk, NY, USA) were used to perform all statistical analyses. All experimental data were tested by analysis of variance with significant differences between means.

**RESULTS AND DISCUSSION**

**Splenic proliferation is promoted in LPS-treated rats:** The spleens harvested from the day 29 LPS group showed a dense cluster of inflammatory cells that stained dark blue with H&E stain in the red pulp. In the day 32 LPS group, the red pulp was intensely congested with red blood cells, and the white pulp appeared diffuse and reduced in amount. In the day 35 LPS group, the marginal zone became thinner or disappeared, and the thickness of PALS was greater than that of the day 29 LPS group. There were also many splenic lymphoid follicles generated in the red pulp. In the day 38 LPS group, it was difficult to find the marginal zone around the white pulp under high magnification, and splenic lymphoid follicles were remarkably enlarged. The diameters of splenic lymphoid follicles were wider in LPS-treated rats than in saline-treated rats on the same day (P<0.05 or P<0.01) (Fig1A). The difference in PALS area was significant between the same-day LPS and control groups for all time points except day 29 (P<0.05 or P<0.01) (Fig 1B).

The results of present study indicated that the marginal zone was thinner in the LPS group. However, thicker PALS and more and larger splenic lymphoid follicles were observed in the LPS treated rats, especially at days 32 and 35. Additionally, the diameters of splenic lymphoid follicles and PALS area showed significant differences between the LPS and control groups. The flow into the marginal zone allows LPS to be captured and subsequent induction of specific immune responses. The lymphocytes are transferred to PALS and splenic lymphoid follicles through the marginal sinus blood. Activated T lymphocytes migrate into PALS and induce further proliferation (Tough et al., 1997). A large number of T cells then migrate from PALS to splenic lymphoid follicles. Certain B cells rapidly differentiate into plasmablasts after receiving signals.

![Fig 1: Morphological index changes of spleens from LPS- and saline-induced rats. A: Diameter of splenic lymphoid follicle, B: PALS area. Compared with saline-treated rats at the same day: *P<0.05, **P<0.01.](image-url)
provided by T cells (Good-Jacobson et al., 2012; Zheng et al., 1996). These plasmablasts move to the red pulp and immediately differentiate into antibody-secreting cells. Furthermore, the activated B cells migrate from PALS and marginal zone to form a new germinal centre with the help of antigen-specific T lymphocytes.

**Splenic immune response is further activated by expressing high levels of pro-inflammatory cytokines:** The IL-1β positive signal in the saline group was weaker than that in the LPS group. The positive cells were mainly distributed in the marginal zone and red pulp (Fig. 2.A, B, C, D). In the normal saline treated group, there were no significant changes in both IOD and AOD of IL-1β-positive cells among the 29, 32, 35 and 38-day-old rats (P > 0.05) (Table 1). In the LPS-treated group, there were more IL-1β-positive cells located in splenic lymphoid follicles and PALS (Fig. 2.a, b, c, d). In addition, AOD and IOD of IL-1β-positive cells were observably greater than those in the saline-treated rats on the same day (P < 0.05) (Table 1). In the LPS group, IOD for the 35-day-old rats was obviously higher than that for the 29 and 38-day-old rats (P < 0.05). AOD differences of IL-1β positive cells were significant among different-day rats (P < 0.05) (Table 1). The cells secreting IL-6 were composed of T lymphocytes and a few macrophages. There were IL-6-positive cells densely distributed in the red pulp in the saline group (Fig. 3.A, B, C, D). However, the staining signal in the LPS group was much stronger than that in the normal saline treated group. There were many IL-6-positive cells among the 29, 32, 35 and 38-day-old rats (> 0.05) (Table 1). The cells secreting IL-6 in the LPS group were higher than those in saline group at days 29, 32, 35 and 38 (P < 0.05).

In addition, the changes in IOD values were consistent with AOD in the LPS treated rats (Table 1). IOD was highest in 32-day-old rats and decreased remarkably in the LPS group (P < 0.05) (Table 1). The analyses of TNF-α protein in spleens showed that there were a small number of TNF-α-positive cells sparsely localized in splenic PALS and red pulp in the saline-treated rats (Fig. 4.A, B, C, D). In the LPS-treated rats, there were more TNF-α-positive cells distributed in PALS and red pulp (Fig. 4.a, b, c, d). For the same-day rats, IOD and AOD of TNF-α protein in the LPS group were both evidently higher than those in the normal saline treated group (P < 0.05) (Table 1). In the LPS group, IOD of TNF-α-positive cells was also significantly larger in the 32- and 35-day-old rats than in the 29-day-old rats (P < 0.05).

Pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α are synthesized and secreted by immune cells (macrophages, neutrophils and dendritic cells) (Slavich and Irwin, 2014). The cytokines elicit the following physiological effects via Toll-like receptor 4 (Cen et al., 2016): improved differentiation of cytotoxic T cells, increased vascular permeability and blood adhesion (Dhabhar et al., 2012), activation of B cell proliferation and secretion of excessive antibodies, and increased IL-2 secretion (Bachmann and Oxenius, 2007). IL-1β is involved in the activation of lymphocytes, macrophages, neutrophils and endothelial cells and can enhance the synthesis of prostaglandins and IL-6 (Dantzer et al., 2008). IL-6 is a key mediator of acute stress proteins and increases the proliferation and differentiation of B and T cells while simultaneously down-regulating

**Table 1:** Protein optical densities of pro-inflammatory cytokines in spleens.

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 29</th>
<th>Day 30</th>
<th>Day 35</th>
<th>Day 38</th>
</tr>
</thead>
<tbody>
<tr>
<td>IOD of IL-1β</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>131813.2±43562<strong>a</strong></td>
<td>128804.1±35836<strong>a</strong></td>
<td>137021.2±37426<strong>a</strong></td>
<td>134907.8±30114<strong>a</strong></td>
</tr>
<tr>
<td>LPS</td>
<td>164310.8±43890<strong>ab</strong></td>
<td>181577.9±26640<strong>ab</strong></td>
<td>198777.4±43311<strong>ab</strong></td>
<td>175988.2±30266<strong>ab</strong></td>
</tr>
<tr>
<td>AOD of IL-1β</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.3400±0.018<strong>a</strong></td>
<td>0.3358±0.021<strong>a</strong></td>
<td>0.3487±0.028<strong>a</strong></td>
<td>0.3401±0.028<strong>a</strong></td>
</tr>
<tr>
<td>LPS</td>
<td>0.5911±0.048<strong>b</strong></td>
<td>0.7249±0.087<strong>b</strong></td>
<td>0.6751±0.053<strong>b</strong></td>
<td>0.6289±0.055<strong>bc</strong></td>
</tr>
<tr>
<td>IOD of IL-6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>316357±107025<strong>a</strong></td>
<td>307420±72989.4<strong>a</strong></td>
<td>318726±65980.7<strong>a</strong></td>
<td>320673.8±80882<strong>a</strong></td>
</tr>
<tr>
<td>LPS</td>
<td>370022±83675.9<strong>b</strong></td>
<td>462794±81416.9<strong>b</strong></td>
<td>403980±84598.9<strong>b</strong></td>
<td>388760.8±87938<strong>b</strong></td>
</tr>
<tr>
<td>AOD of IL-6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.2696±0.011<strong>a</strong></td>
<td>0.2761±0.026<strong>a</strong></td>
<td>0.2722±0.025<strong>a</strong></td>
<td>0.2806±0.026<strong>a</strong></td>
</tr>
<tr>
<td>LPS</td>
<td>0.3715±0.021<strong>b</strong></td>
<td>0.4244±0.0325<strong>b</strong></td>
<td>0.3906±0.0249<strong>b</strong></td>
<td>0.3677±0.0187<strong>bc</strong></td>
</tr>
<tr>
<td>IOD of TNF-α</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>44266.1±6421.8<strong>a</strong></td>
<td>45787.3±49454.6<strong>a</strong></td>
<td>44378.6±9812.1<strong>a</strong></td>
<td>46073.4±6735.9<strong>a</strong></td>
</tr>
<tr>
<td>LPS</td>
<td>48395.9±7904.1<strong>b</strong></td>
<td>52833.9±7366.6<strong>b</strong></td>
<td>55071.3±5168.6<strong>b</strong></td>
<td>51647.0±6331.9<strong>b</strong></td>
</tr>
<tr>
<td>AOD of TNF-α</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.2176±0.022<strong>a</strong></td>
<td>0.2125±0.011<strong>a</strong></td>
<td>0.2205±0.019<strong>a</strong></td>
<td>0.2166±0.018<strong>a</strong></td>
</tr>
<tr>
<td>LPS</td>
<td>0.2438±0.022<strong>b</strong></td>
<td>0.2637±0.026<strong>b</strong></td>
<td>0.2581±0.021<strong>b</strong></td>
<td>0.2483±0.025<strong>bc</strong></td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± SD, n=5 SD rats per group. a, b, c, d Treatment means in the same row with different letter superscripts indicate significant difference (P < 0.05). A, B Treatment means in same column with different letter superscripts indicate significant difference (P < 0.05).

inflammation through the suppression of IL-1β and TNF-α secretion (Miller et al., 2009; Schiepers et al., 2005). TNF-α participates in the activation of neutrophils and endothelial cells and induces apoptosis (DellaGioia and Hannestad, 2010). Pro-inflammatory cytokines play a critical role in immune responses and the inflammation process. In this study, the cells positive for IL-1β, IL-6 and TNF-α were mainly distributed in splenic PALS and lymphoid follicles of the LPS-treated rats. We found both IOD and AOD increased significantly. Thus, LPS can activate specific immune responses and promote B and T cell proliferation and differentiation. The activated lymphocytes produce more pro-inflammatory cytokines and can enhance the synthesis and secretion levels of multiple cytokines.

ACKNOWLEDGEMENT
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