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Original article

Detection of IL-1β, IL-6 and TNF-α in Sprague-Dawely rats' atrophic thymus induced by lipopolysaccharide

Y.B. Zhong[1,2], X.L. Zhang[1], M.Y. Lv[1], X.F. Hu[1], Y. Li[1,3]

[1] College of Animal Science and Technology, Jiangxi Agricultural University, No. 1101 Zhimin Avenue, Economic and Technological Development District, Nanchang 330045, Jiangxi, P. R. China
[2] Technology Center of Experimental Animal, Jiangxi University of Traditional Chinese Medicine, No. 1688 Meiling Avenue, Wanli District, Nanchang 330004, Jiangxi, P. R. China
[3] Jiangxi Provincial Key Laboratory for Animal Health, College of Animal Science and Technology, Jiangxi Agricultural University, No. 1101 Zhimin Avenue, Economic and Technological Development District, Nanchang 330045, Jiangxi, P. R. China

Abstract

Objective: This study aimed to investigate developmental changes of the thymus and intrathymic IL-1β, IL-6 and TNF-α expression in weaned Sprague-Dawley rats induced by lipopolysaccharide.

Methods: Forty healthy weaned rats aged 26 days and weighing 83±4 g were randomly and equally divided into two groups. The lipopolysaccharide group was treated daily with a single injection of lipopolysaccharide for 10 consecutive days, and the saline group was treated with an equal volume of sterilized saline. On the 1st, 4th, 7th and 10th day, histological changes and distribution of IL-1β-, IL-6- and TNF-α-positive cells were detected in the thymus by hematoxylin-eosin and immunohistochemistry staining, respectively. Subsequently, the expression levels of IL-1β, IL-6 and TNF-α were evaluated in the thymus by the ELISA method.

Results: Thymus weight and index were significantly smaller in lipopolysaccharide-treated rats than in saline-treated rats ($p<0.05$), but no substantial changes were found in the thymus microstructure after lipopolysaccharide induction. Moreover, a large number of IL-1β-, IL-6- and TNF-α-positive cells were observed with brownish-yellow color and mainly distributed in the thymus parenchyma, both integrated optical density and average optical density increased significantly in lipopolysaccharide-treated rats than those in saline-treated rats. Compared with the saline group, most of the thymic homogenates had higher levels of IL-1β, IL-6 and TNF-α in the lipopolysaccharide group on different days.

Conclusion: These findings indicate that the thymus atrophied after lipopolysaccharide induction in weaned Sprague-Dawley rats, and excessive production of intrathymic IL-1β, IL-6 and TNF-α was probably involved in the atrophic process.

Key words: lipopolysaccharide, pro-inflammatory cytokines, thymus atrophy, rat

Correspondence to: Y. Li, e-mail: liyong2912@163.com

Introduction

Lipopolysaccharide (LPS) located in the outer cytoderm of gram-negative bacteria can be specifically recognized by antigen-presenting cells and induces a series of inflammation and immune responses (Forster et al. 2007). As a blood-borne antigen, LPS from the host's bacterium is released into the cardiovascular system through the bloodstream and then generates a new complex with the LPS binding protein. Subsequently, the interaction of the new complex with toll-like receptor 4 on lymphocytes activates the nuclear factor-κB (NF-κB) transcription factor signalling pathway and induces an acute phase response (Hotchkiss et al. 2005, Pinheiro-da-Silva et al. 2006, Peri et al. 2010). Binding of NF-κB to the promoters of responsive genes ultimately promotes gene transcription of inflammatory mediators such as TNF-α, IL-1, IL-6, COX-2, PGs, TXs and LTs (Hotchkiss et al. 2005, Verstrepen et al. 2008, Peri et al. 2010). It is known that over-production of these inflammatory mediators leads to organ injury and dysfunction, and even causes septic shock and mortality in severe cases (Kim et al. 2014). During the inflammatory response, the transcription and translation activity of pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6, are strictly regulated by the neuro-endocrine-immune system. When pro-inflammatory cytokines are abnormally elevated in tissues and blood, nutrient metabolism and requirements are regulated to enhance animal immunity. Many nutriments, which are normally used for organ development and protein deposition, are diverted to strengthen the immune response and advance immunity (Dritz et al. 1996). Although a moderate inflammatory response can improve the survival rates of animals and humans, long-term immunostimulation can restrain the growth of the animal (Coleman and Sartin 1996) and even leads to hypoplasia of internal organs.

The thymus is the major site of T cell differentiation and maturation. After stem cells migrate into the thymus from the bone marrow, these cells gradually divide and differentiate into various types of T cells upon thymosin induction (Haynes and Hale 1998). Subsequently, mature lymphocytes are exported to peripheral lymphoid organs through the bloodstream (Rezzani et al. 2008). It is a normal physiological phenomenon that thymus weight and size are associated with age; thymus weight and size begin to increase after birth, reach their maximums in adolescence, and thereafter decrease gradually with age (Steinmann et al. 1985). There are far fewer thymocytes produced in the aged thymus than in the young thymus (Henson et al. 2004). At the same time, thymus atrophy leads to developmental retardation of T cells and less efficient thymopoiesis (Sempowski et al. 2002, Goronzy et al. 2007). The immune system efficiency gradually reduces with age, which results in an increased risk of diseases such as bacterial infections, allergies and cancers (Chen et al. 2003). However, age is not the only factor leading to thymus atrophy, and abnormal pathophysiological states (inflammation, oxidation, bacterial and viral infection, mental stress and hypoxia, etc.) also alter the number of thymocytes and thymus size (Taub and Longo 2005). Several possible mechanisms for thymic involution have been reported in recent years, including blockage of T-cell receptor gene rearrangement, decreased self-peptide major histocompatibility complex molecules, and depletion of T-cell progenitors (Gruver et al. 2007). Intrathymic TNF-α, IL-1β and IL-6 and thymic developmental changes after LPS induction have not been evaluated in weaned Sprague-Dawley (SD) rats. Both the thymic histological changes and the distribution and expression of pro-inflammatory cytokines were analysed in weaned SD rats induced by LPS. The present study will further explain thymus aplasia and immunodeficiency disease during infection. It will also help to understand mechanisms of thymus atrophy and provide some information on how to enhance host resistance against a variety of harmful stressors, including mental and psychological pressure, bacterial and viral infections, toxic substance and so on.

Materials and Methods

Experimental animals and treatments

Forty healthy weaned SD rats aged 26 days and weighing 83±4 g were purchased from the Jiangxi University of Traditional Chinese Medicine and kept in the animal anatomy laboratory of the Jiangxi Agricultural University. SD rats were housed for 3 days to adapt to the new facility, where food and water were freely available during the experiment. All procedures throughout the experiment were performed in accordance with the Guidelines of Animal Experiments. The rats were randomly and equally divided into two groups. The LPS group was injected daily in the caudal vein with a single dose of LPS (100 μg/kg body weight) (*Escherichia coli* LPS Serotype O55:B5, Sigma) for 10 consecutive days, and the saline group was treated with an equal volume of sterilized saline. First, five individuals from each group were anaesthetized with ether on the 1st, 4th, 7th and 10th day. After necropsy, the thymus was immediately dissected from each rat and fixed in 4% buffered paraformaldehyde solution, and then, its weight and index were measured.

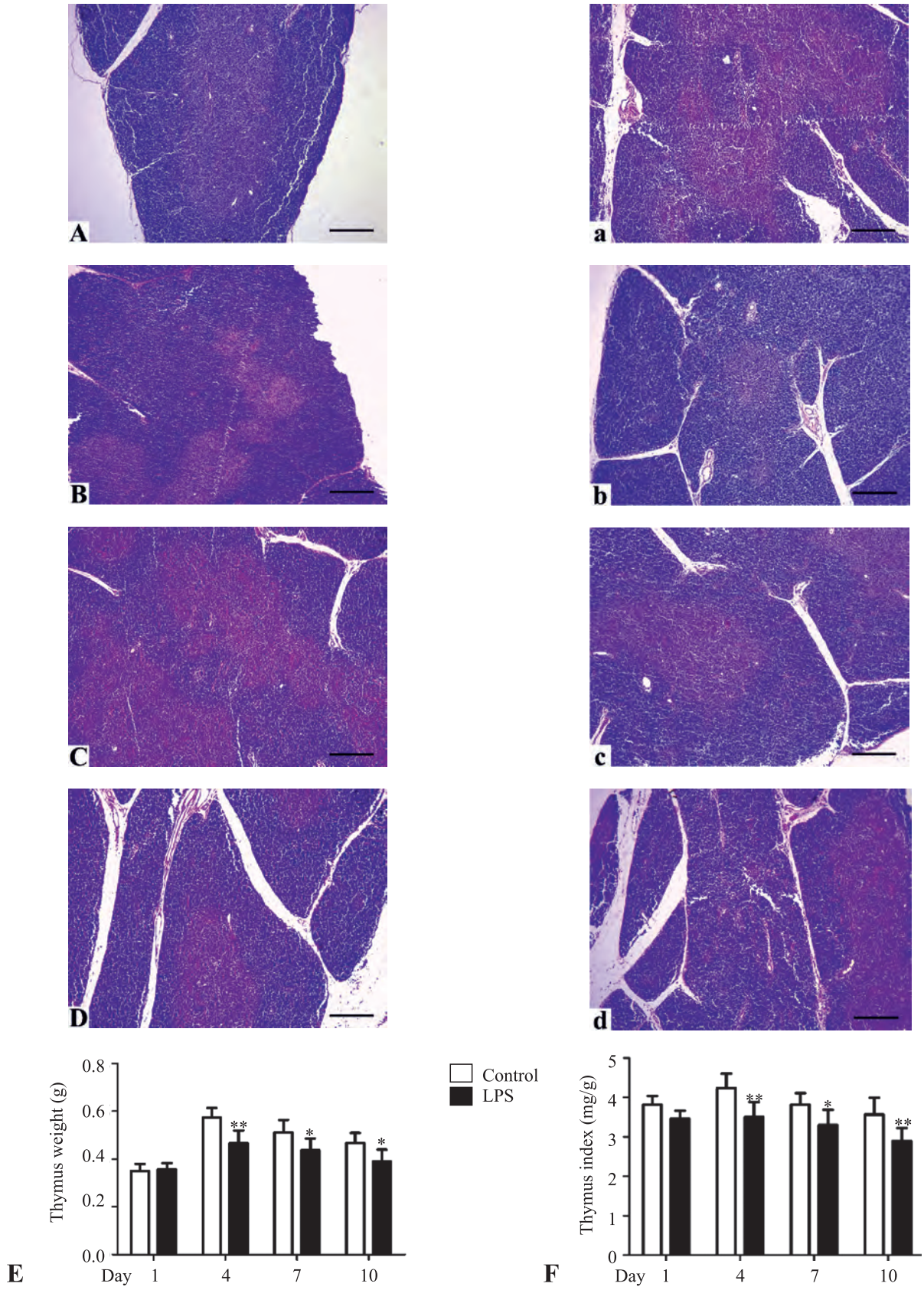

Fig. 1. Thymus microstructure, weight and index in lipopolysaccharide (LPS)- and saline-induced Sprague-Dawely (SD) rats (Scale bars=200 μm; *p< 0.05, **p< 0.01). **A:** 29-day-old rats treated with saline; **B:** 32-day-old rats treated with saline; **C:** 35-day-old rats treated with saline; **D:** 38-day-old rats treated with saline; **a:** 29-day-old rats treated with LPS; **b:** 32-day-old rats treated with LPS; **c:** 35-day-old rats treated with LPS; **d:** 38-day-old rats treated with LPS; **E:** Thymus weight; **F:** Thymus index.

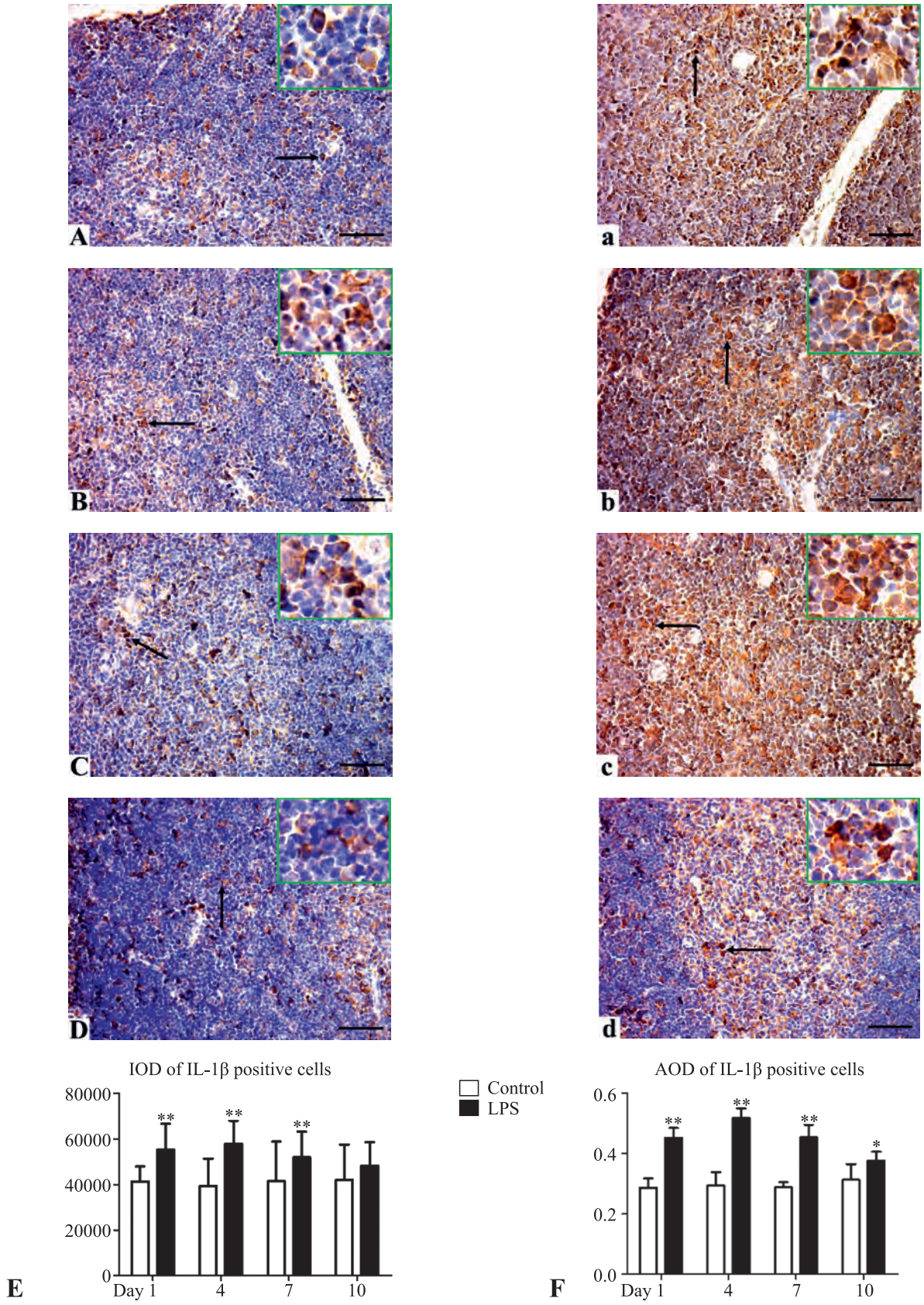

Fig. 2. Detection of IL-1β in the thymus of LPS- and saline-induced SD rats (the black arrow indicates IL-1β positive cells; Bars=100 μm; * $p<0.05$, ** $p<0.01$). **A:** 29-day-old rats treated with saline; **B:** 32-day-old rats treated with saline; **C:** 35-day-old rats treated with saline; **D:** 38-day-old rats treated with saline; **a:** 29-day-old rats treated with LPS; **b:** 32-day-old rats treated with LPS; **c:** 35-day-old rats treated with LPS; **d:** 38-day-old rats treated with LPS; **E:** integrated optical density (IOD) of IL-1β; **F:** average optical density (AOD) of IL-1β.

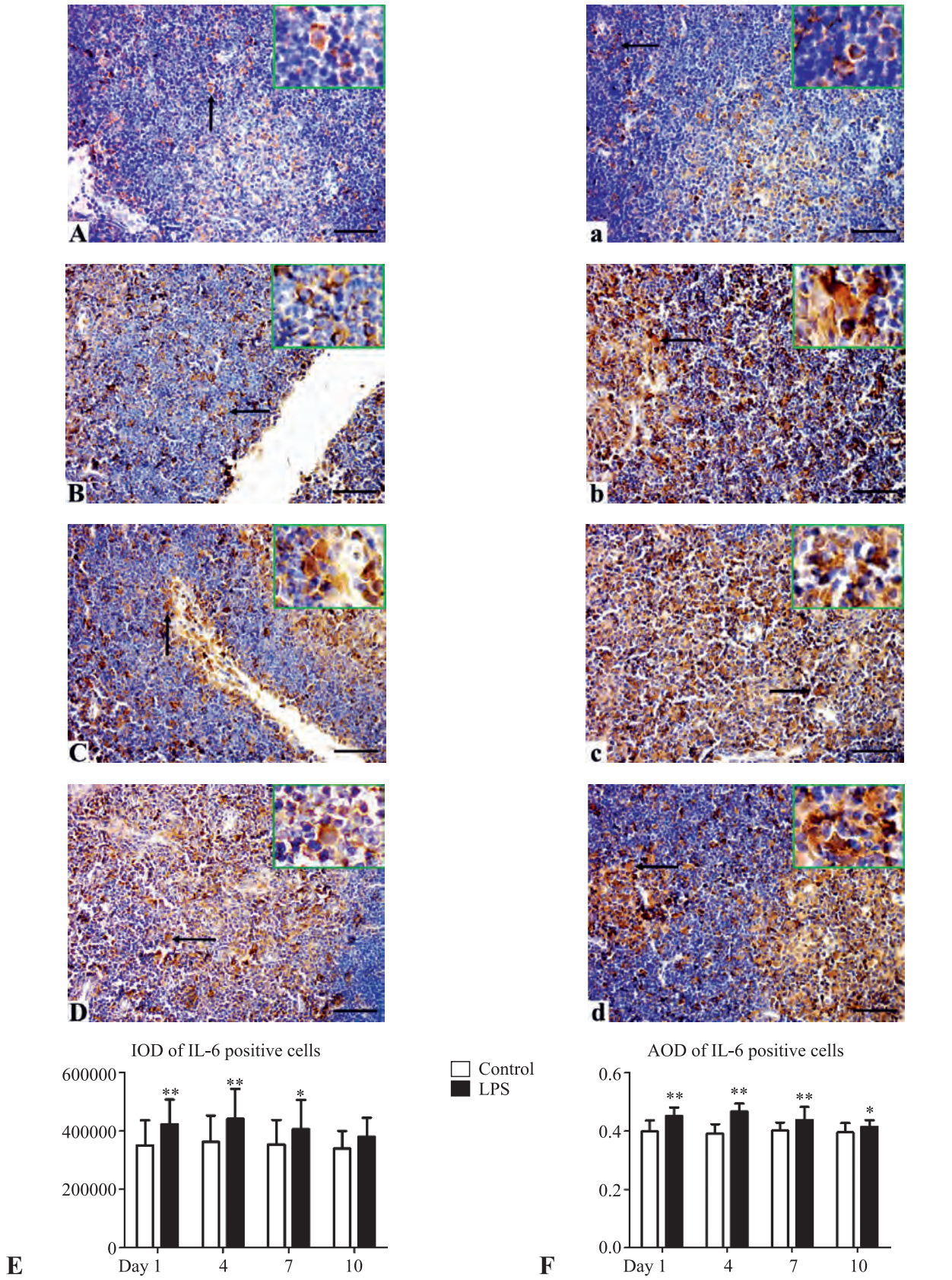

Fig. 3. Detection of IL-6 in the thymus of LPS- and saline-induced SD rats (the black arrow indicates IL-6 positive cells; Bars=100 μm; * $p<0.05$, ** $p<0.01$). **A:** 29-day-old rats treated with saline; **B:** 32-day-old rats treated with saline; **C:** 35-day-old rats treated with saline; **D:** 38-day-old rats treated with saline; **a:** 29-day-old rats treated with LPS; **b:** 32-day-old rats treated with LPS; **c:** 35-day-old rats treated with LPS; **d:** 38-day-old rats treated with LPS; **E:** IOD of IL-6; **F:** AOD of IL-6.

Hematoxylin-eosin staining

Thymi were fixed in 4% buffered paraformaldehyde solution for 24 hours and then routinely processed in alcohol-xylene and embedded in paraffin. Subsequently, 5-μm thymus sections were stained with hematoxylin-eosin for morphological analysis. Micrographs of thymus sections were taken under a biological microscope (BA210: Motic, Xiamen, China).

Immunohistochemistry staining

Paraffin-embedded thymi were cut into 5-μm sections, and then, these sections were deparaffinized in xylene and rehydrated in solutions with decreasing concentrations of ethanol. To eliminate endogenous peroxidase activity, the sections were incubated with 3% H_2O_2 in deionized water for 10 min, then washed three times with 0.01 M PBS. The sections were heated to 98°C in citrate buffer (pH=6.0) for 12 min, and then, the sections, together with the enamelled cup and citrate buffer, were cooled to room temperature (RT) with running water. After the samples were blocked for 15 min with normal goat serum in an immunohistochemical humidor, they were incubated overnight at 4°C with primary antibodies (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). After three washes with 0.01 M PBS, the sections were treated with horseradish peroxidase-conjugated goat anti-rabbit or mouse IgG for 15 min at RT. After three more washes with 0.01 M PBS, they were incubated with horseradish peroxidase conjugated-streptavidin (SP-9001/9002 link Detection Kits: ZSGB Biotechnology, Beijing, China) for 15 min at RT. The sections were washed three times with 0.01 M PBS, and then, diaminobenzidine (DAB kit: ZSGB Biotechnology, Beijing, China) was added to visualize the colour of reactants. The sections were put under running water for 10 min to clear the DAB raffinate and were counterstained with Mayor's haematoxylin. Finally, the samples were dehydrated in graded ethanol solution, treated two times in xylene, and then mounted with coverslips. The control sections were treated by the same procedure as described above, but the primary antibody was replaced by 0.01 M PBS. The distribution of positive cells was detected in non-overlapping fields from each section.

ELISA detection

Concentrations of TNF-α, IL-1β and IL-6 were detected in the thymus tissue by using commercial quantitative ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. A microplate reader (Thermo Fisher Scientific; MA, USA) was used to measure the optical density (OD) of samples in the 450-nm wavelength. The regression equation was applied to OD values to calculate the corresponding concentration of the samples.

Data analysis

Statistical analysis was performed using SPSS 13.0 software (Chicago, IL, USA). Significant differences between the LPS and saline group were determined by one-way ANOVA. All data were presented as the mean±SD, and Graph Prism 5.0 software (GraphPad, San Diego, CA, USA) was used to generate the corresponding graphs.

Results

Thymus atrophy in LPS-induced SD rats

On day 4, LPS-treated rats showed up to a 30% reduction in thymus weight ($p<0.01$, 0.5733±0.030 vs. 0.467±0.053) and a 17.24% reduction in thymus index ($p<0.01$, 4.234±0.371 vs. 3.504±0.380) compared with saline-treated rats. On day 7, LPS-treated rats displayed a 15% reduction in thymus weight ($p<0.05$, 0.512±0.053 vs. 0.437±0.050) and a 14% reduction in thymus index ($p<0.05$, 3.815±0.297 vs. 3.297±0.392). Additionally, LPS-treated rats showed a 19% reduction in thymus index ($p<0.01$, 3.568±0.424 vs. 2.899±0.322) and a 16% reduction in thymus weight ($p<0.05$, 0.467±0.043 vs. 0.390±0.049) on day 10 (Figs. 1E, 1F). However, distinct areas of the cortex and medulla were visible in the thymus tissue with light microscopy, and damage to the histological structure was not obvious in LPS-treated rats (Figs. 1a, 1b, 1c, 1d).

Detection of IL-1β, IL-6 and TNF-α in thymus from LPS induced SD rats

According to immunohistochemistry staining, the immuno-positive signals for IL-1β, IL-6 and TNF-α were mainly localized in the cytoplasm with brown staining and were diffusely distributed in the thymus parenchyma. The distribution density was not consistent among three kinds of cells and was as follows from high to low: IL-6>IL-1β>TNF-α. An increase in the number of positive cells at the thymus parenchyma area was found in LPS-treated rats, especially on the 4th and 7th days of the experiment, in contrast to the results in saline-treated rats (Figs. 2, 3, 4). However, the positive signals (brown-yellow) for pro-inflammatory cytokines

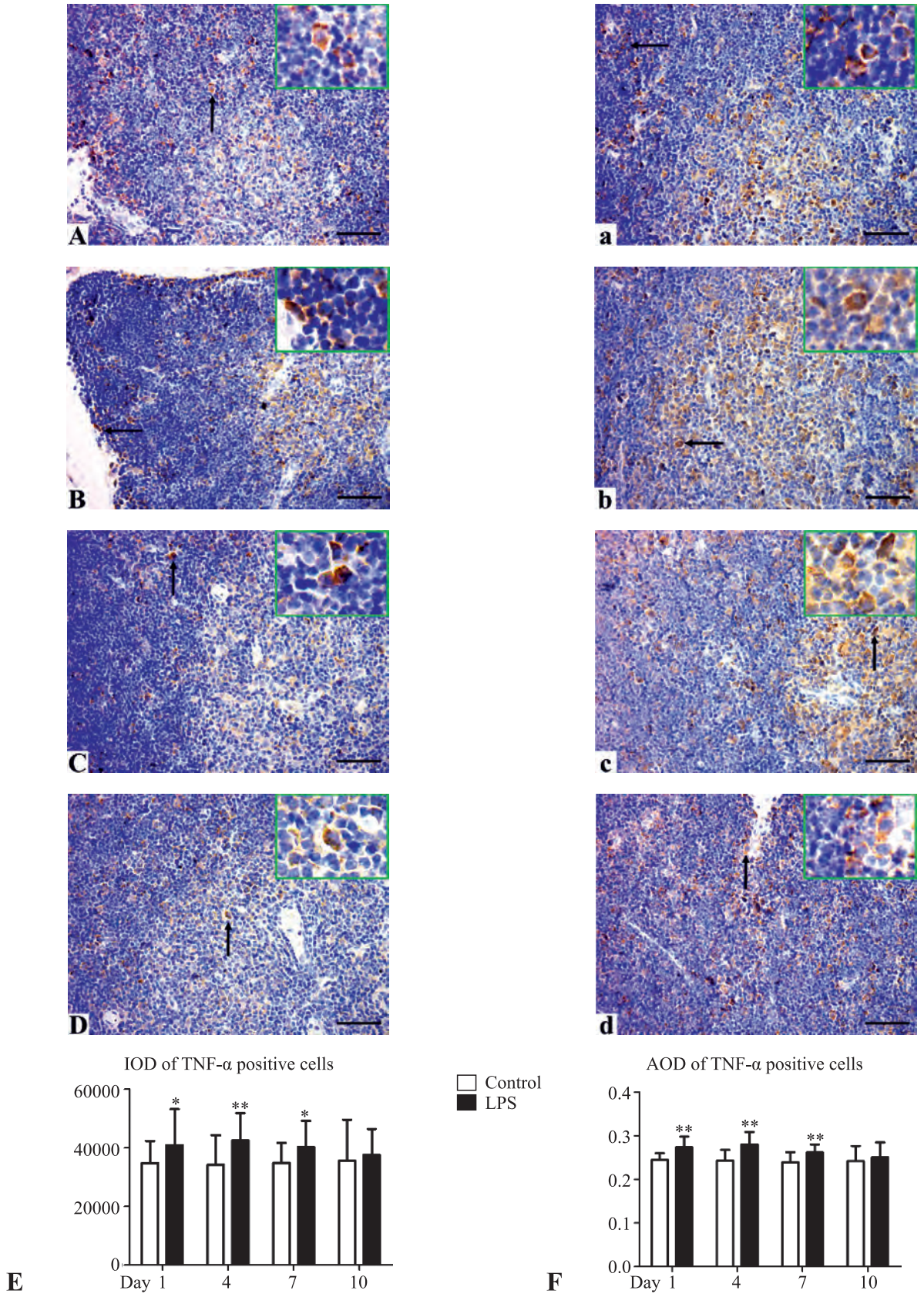

Fig. 4. Detection of TNF-α in the thymus of LPS- and saline-induced SD rats (the black arrow indicates TNF-α positive cells; Bars=100 μm; * $p<0.05$, ** $p<0.01$). **A:** 29-day-old rats treated with saline; **B:** 32-day-old rats treated with saline; **C:** 35-day-old rats treated with saline; **D:** 38-day-old rats treated with saline; **a:** 29-day-old rats treated with LPS; **b:** 32-day-old rats treated with LPS; **c:** 35-day-old rats treated with LPS; **d:** 38-day-old rats treated with LPS; **E:** IOD of TNF-α; **F:** AOD of TNF-α.

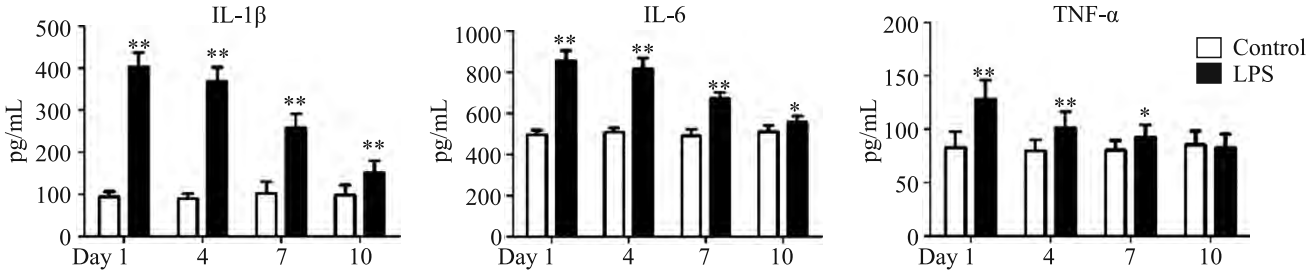

Fig. 5. IL-1β, IL-6 and TNF-α concentrations in the thymus measured by ELISA on the 1st, 4th, 7th and 10th days (data are presented as the mean±SD, *p<0.05 and **p<0.01).

in the LPS group were similar to those observed in the saline group on day 10 (Figs. 2, 3, 4). Additionally, integrated optical density (IOD) and average optical density (AOD) were significantly different between the groups on different days of the experiment. On day 1 and 4, the IOD and AOD of IL-1β- (Figs. 2E, 2F), IL-6- (Figs. 3E, 3F) and TNF-α- (Figs. 4E, 4F) positive cells were almost dramatically higher ($p<0.01$) in LPS-treated rats than in the saline-treated rats. On day 7, the IOD and AOD of IL-1β- (Figs. 2E, 2F), IL-6- (Figs. 3E, 3F), TNF-α- (Figs. 4E, 4F) positive cells in the LPS group remained significantly higher than those in the saline group ($p<0.01$ or $p<0.05$). However, only the AOD of IL-1β- (Fig. 2F) and IL-6- (Fig. 3F) positive cells was obviously increased ($p<0.05$) in the LPS group compared to those in the saline group on day 10.

In the ELISA results, thymic homogenate concentrations of IL-1β, IL-6 and TNF-α were significantly greater ($p<0.01$ or $p<0.05$) in LPS-treated rats than in saline-treated rats on days 1, 4, and 7. However, on day 10, IL-1β and IL-6 concentrations were still greater in LPS-treated rats compared to saline-treated rats ($p<0.01$ or $p<0.05$), but there was no significant difference ($p>0.05$) in TNF-α concentrations (Fig. 5).

Discussion

Analyses of the immune status and pro-inflammatory cytokines have been focused in weaned animals recently (Obremski 2014, Lin et al. 2016, Valpotic et al. 2018), but the thymus is the key organ affecting development of peripheral immune organs. The thymi of rats and mice begin to grow and develop after birth, reach their maximum size by sexual maturity, and then gradually involute (Pearse 2006). The thymus can produce various subtypes of mature T lymphocytes for peripheral lymphoid tissues and organs (Pearse 2006, Rezzani et al. 2008). Physiological dysfunction of the thymus could result in decreased immune function, which can increase the risk of animal morbidity and mortality. In the present study, LPS-treated rats had the lighter and smaller thymus, and thymus development was obviously suppressed. Although there was not clear cell degeneration and necrosis in the thymus, thymocytes synthesized and secreted excessive amounts of pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α, after multiple LPS inductions. LPS immediately enters the circulatory system through the caudal vein and is quickly recognized by pattern-recognition receptors such as Toll-like receptor 4 (Dobrovolskaia and Vogel 2002, Biswas and Tergaonkar 2007). Subsequently, MyD88 protein is employed to activate the NF-κB signalling pathway and promote transcription of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α (Hayden et al. 2006, Akira 2009). Normally, monocyte and macrophage responsiveness is suppressed after multiple LPS challenges resulting in a reduced induction of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α (Biswas and Lopez-Collazo 2009, Yoza and McCall 2011). This phenomenon is called endotoxin tolerance. Macrophages and monocytes in the endotoxin tolerance stage are less efficient at forming the TLR4-MyD88 complex, and have impaired activation of IRAK-1, which leads to a block of the NF-κB cascades and suppression of IL-1β, IL-6 and TNF-α gene transcription (Fan and Cook 2004, Biswas and Tergaonkar 2007). Although SD rats received ten doses of LPS injection, pro-inflammatory cytokines quickly returned to normal levels. This finding suggests that weaned SD rats can resolve adverse reactions and maintain normal physiological functions through neurohumoral regulation.

Pro-inflammatory cytokines play a critical role in the acute stress response (Dhabhar et al. 2012) and inflammation process (Miller et al. 2009) and are involved in T cell and B cell proliferation and differentiation (Schiepers et al. 2005). Abnormal increases in pro-inflammatory cytokines could negatively feedback to the cerebral cortex and suppress appetite excitability in the central nervous system (Coleman and Sartin 1996), which would result in decreased nutrient intake and thymus development delay in puberty-stage rats. In the meantime, pro-inflammatory cytokines induce thymic progenitor cells to differentiate into mature T lymphocytes, and then all types of T cells are trans-

ported into peripheral tissues and organs (Rezzani et al. 2008). Thymic progenitors are greatly depleted in the stress-response process, which further exacerbates thymic involution.

In conclusion, this study has revealed that thymic atrophy and intrathymic IL-1β, IL-6 and TNF-α are probably involved in the process of thymic involution after LPS induction.

Acknowledgements

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