The influence of Th1/Th2 and CD4$^+$ regulatory t cells of mesenteric lymph nodes on systemic lipopolysaccharide

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Our aims were to study the influence of the mesenteric lymph nodes (MLN) of rats on systemic lipopolysaccharide and to identify the factor that affects the intestinal endotoxin translocation.

Ninety-six male Wistar rats were randomly divided into a sham-operation group (S group) and a cecal ligation and perforation group (CLP group). Twenty-four hours after modeling, we tested the Th1/Th2 ratio and percentage of CD4$^+$ CD25$^+$Foxp3$^+$ Treg in the MLN. At the same time, the lipopolysaccharide (LPS) in the abdominal aortic blood was detected.

In the CLP group, the Th1/Th2 ratio was obviously lower than in the S group. Otherwise, the percentage of CD4$^+$CD25$^+$Foxp3$^+$ Treg of the CLP group was significantly higher than the S group. In the abdominal aortic blood, the LPS level of the CLP group was also higher than in the S group. Through correlation analysis, we found that the level of LPS was positively correlated with the percentage of CD4$^+$CD25$^+$Foxp3$^+$ Treg, and negatively correlated with the Th1/Th2 ratio.

This model reveals that the immune suppression of the MLN might affect the level of LPS in the abdominal aortic blood, which might play a certain role in affecting the endotoxin translocation.

Key words: lipopolysaccharide (LPS), mesenteric lymph node, percentage of CD4$^+$CD25$^+$Foxp3$^+$ Treg, Th1/Th2 ratio.

Introduction

Intestinal endotoxemia caused by perforation peritonitis, severe acute pancreatitis, abdominal abscess, and other serious intra-abdominal infections is the main cause of sepsis and multiple organ dysfunction syndrome (MODS) [1]. Studies have shown that there are three translocation pathways for intestinal endotoxin into blood: 1) intestinal lymphatic channels, 2) the portal vein system, and 3) direct infiltration through collateral circulation. However, the lymphatic system is the most important translocation pathway for intestinal endotoxin [2]. In severe intra-abdominal infections, intestinal endotoxin firstly affects the inflammatory and immune system, which leads to disordered inflammatory and immune function [3, 4]. Systemic inflammatory and immune dysfunction can further aggravate the intestinal mucosal barrier injury, which increases the intestinal endotoxin in the blood. Repeatedly, a vicious circle forms [5]. Therefore, it is important to study the function of the intestinal lymphatic immune system in sepsis. According to known literature data, changes in the number of T lymphocytes in mesenteric lymph nodes (MLN) accompanying an entrance of lipopolysaccharide (LPS) to the circulation have not been documented as yet. In order to know this, we assayed the percentage of CD4$^+$ CD25$^+$Foxp3$^+$ Treg, and the Th1/Th2 ratio in MLN

and detected the LPS level in the abdominal aortic blood in a rat model of severe intra-abdominal infection.

Material and methods

The rat models

All experiments were approved by the Ethics Committee at Tianjin Medical University. Male Wistar-Hannover rats (8 weeks old, from the Military Medical Academy, animal no. DD20013, weighing 200-250 g) were maintained in a clean room with 12-hour day/night cycles and room temperature of 21°C. Cecal ligation and puncture (CLP) was performed as a clinically relevant animal model of severe intra-abdominal infection [6]. After anesthesia with ketamine (80 mg/kg body weight) and xylazine (15 mg/kg), the cecum was carefully ligated below the ileocecal valve through a 1-cm abdominal midline incision to avoid obstruction of the ileum or colon. The cecum was subjected to a "through-and-through" perforation (18 G needle), and 0.1 ml of intestinal contents were drained. The abdominal incision was closed in layers. Sham-operated rats underwent the same procedure except for ligation and perforation of the cecum. All procedures were performed under sterile conditions.

Before surgery, 96 rats were evenly divided into two groups (sham-operated group and CLP group); among 48 rats in each group, 24 rats were for operation and 24 served as a negative control. After surgery, they were immediately injected with 50 ml/kg of Ringer's lactate. Twenty-four hours later, the remaining 24 rats in each group were selected. 2.5 ml of abdominal aortic blood and 0.2 g of MLN tissue were collected from each animal. MLN were isolated in sterile conditions, minced into small fragments, and washed in cold phosphate-buffered solution (PBS) to clear blood. Fragments were used to prepare single-cell suspensions, and they were passed through a 230-mm steel mesh. We resuspended the cells with RPMI-1640 and adjusted the cell concentration to 2×10^6/ml after being counted under a microscope at a low magnification.

Flow cytometric analysis

Mouse anti-rat monoclonal antibodies (mAb), including CD4, CD5, CD8a, CD8b, CD25, CD45RC, NKR-P1A, OX12, TCRab and TCRgd, labeled with fluorescein isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin-cyanine-5 (PE-Cy5) and peridinin chlorophyll protein (PerCP), were purchased from BD Pharmingen (San Diego, CA, USA) and Serotec (Oxford, UK). Four-color flow cytometric analysis was performed using a fluorescence-activated cell-sorting (FACS) Calibur (BD Biosciences, San Jose, CA, USA), as described earlier. For Foxp3+ cell staining, a previous cell membrane permeabilization step was performed following the manufacturer's instructions (Cell Fixation/Permeabilization Kit, eBioscience, San Diego, CA, USA).

Assay of the Th1/Th2 ratio

The ratio of Th1 and Th2 cells was measured by flow cytometry. T lymphocyte cell antibody against CD4 antigen (anti-CD4-FITC, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) was used to set a gate for helper T cells, and anti-IL-4 and anti-IFN-γ antigen antibodies (Becton Dickinson Immuno-cytometry Systems) were used to distinguish Th1 cells and Th2 cells. A live gate was set using forward and sideways scatter characteristics and the ratio of Th1 and Th2 cells was determined [7].

Assay of the percentage of $CD4^+CD25^+Foxp3^+$ Treg

Samples were subjected to flow cytometry to assess the proportions of lymphocyte subsets. Cells were washed and double-stained with indicated mAbs, anti-CD4-PerCP and anti-CD25-FITC (Becton Dickinson, San Jose, CA, USA). Finally, cells were washed and measured with a FACS Calibur cytometer (Becton Dickinson) and data were analyzed by Cell Quest software. For these analyses, lymphocytes were electronically gated based on their forward and sideways scatter characteristics, and at least 10,000 lymphocytes were detected to characterize double-positive cells. In all cases, negative controls of cell staining using isotype-matched irrelevant mAbs (Becton Dickinson) were run. For the detection of intracellular antigens, Foxp3 antibody (eBioscience, San Diego, CA, USA) and rat IgG2b (PCH101 clone; eBioscience) were used. Forward and lateral angle of flow cytometry (FACS Calibur; Becton Dickinson) was used to set the gate of T helper (Th), at least 10,000 cells counted. Antibodies of CD4-PerCP and CD25-FITC were used to set gates. The percentage of $CD4^+CD25^+Foxp3^+$ Treg among $CD4^+$ cells was calculated (percentage of $CD4^+CD25^+Foxp3^+$ Treg = $CD4^+CD25^+ \times Foxp3^+/CD4^+ \times 100\%$) [8-10].

Assay of lipopolysaccharide

Collected abdominal aortic blood samples from the S group and CLP group were diluted in endotoxin-free water to a ratio of 1 : 250 and 1 : 500 using a Limulus amebocyte lysate assay, and plasma LPS was quantified with a commercial assay kit (Limulus amebocyte lysate 071127, Xiamen, Fujian, China) according to the manufacturer's protocol. The results are expressed as pg/ml, and the limit of detection was 10 pg/ml. The endotoxin levels were measured in leishmanial lysate to exclude any possibility of cross

reaction between LPS and Leishmania-derived LPS-like molecules [11, 12].

Statistical analysis

SPSS v.16.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The results were expressed as median ± standard deviation (SD). Comparison of means was performed by Student's t-test, and $p<0.05$ was considered to be statistically significant.

Results

Th1/Th2 ratio and percentage of $CD4^+CD25^+Foxp3^+$ Treg of MLN

In the S group, the Th1/Th2 ratio of MLN lymphocytes did not change significantly after surgery. However, it was significantly different in the CLP group. Twenty-four hours after surgery, the Th1/Th2 ratio declined sharply compared with the initial level and was at a much lower level than in the S group (as shown in Table I). Unlike the ratio of Th1/Th2, the percentage of $CD4^+CD25^+Foxp3^+$ Treg in the CLP group increased significantly ($^bp < 0.05$) after surgery. Compared with the S group, we found that it was much higher ($^ap < 0.05$). However, the percentage in the S group decreased a little after surgery. These results are illustrated in Table II.

Lipopolysaccharides in the abdominal aorta blood

In this study, we tested the level of LPS in abdominal aortic blood in each group. The results showed that the LPS in the S group after surgery was almost the same compared with the initial level. However, it increased in the CLP group ($p < 0.05$) after surgery, and it was also much higher than the S group (Table III).

Correlation between LPS and the Th1/Th2 ratio and percentage of $CD4^+CD25^+$ $Foxp3^+$ Treg of MLN

As shown in Fig. 1, the Th1/Th2 ratio of MLN lymphocytes decreased 24 hours after surgery; however, the LPS in abdominal aortic blood linearly increased ($R^2 = 0.821$). In other words, they were negatively correlated. Otherwise, the percentage of $CD4^+CD25^+Foxp3^+$ Treg of MLN increased accompanied by a linear increase of LPS in abdominal aortic blood ($R^2 = 0.521$) (Fig. 2).

Discussion

Deitch *et al.* [13] hypothesized that intestinal damage can be caused by shock, trauma and sepsis, which was named as the intestinal lymph hypothesis. Stress and inflammatory response can cause intestinal mucosal damage, which promotes bacterial translocation. In addition, gut-derived endotoxin could also enter the blood circulation through the MLN [14]. However, we did not know what was affecting the amount of gut-derived LPS in the blood circulation.

In this study, we first studied the influence of the immune status of MLN on intestinal bacteria or endotoxin translocation. As we know, lymphoid tissue plays an important role in sterilization and anti-endotoxin, and most of the bacteria or toxins are destroyed or removed before reaching the bloodstream [15]. Therefore, the translocation of bacteria or toxins must be associated with intestinal intraepithelial lymphocytes, scattered lamina propria lymphocytes, and Peyer's aggregated lymphoid nodules and MLN. The immune function of these tissues plays an important role in preventing endotoxin from accessing the abdominal aortic blood through the lymphatic pathway [16]. Based on the past research, this study found that the immune suppression of the local intestinal lymphatic system could result in leakage of endotoxin along the intestinal lymphatic pathway.

Many researchers have found that functional changes of Th, including Thl and Th2, could reflect the change of the body's immune function. To some degree, a change of Th1/Th2 ratio could reflect the imbalance of systemic inflammatory response syn-

Table I. Th1/Th2 ratios of MLN lymphocyte ($\bar{x} \pm s$)

Group	n	Before surgery	24 h after surgery
S group	24	0.49 ±0.11	0.51 ±0.17
CLP group	24	0.47 ±0.15	0.23 ±0.08[ab]

[a]p < 0.05, compared with the S group
[b]p < 0.05, compared with the level before surgery

Table II. Percentage of $CD4^+CD25^+Foxp3^+$ Treg in the MLN lymphocytes ($\bar{x} \pm s$)

Group	n	Before surgery	24 h after surgery
S group	24	7.99 ±2.57	7.45 ±3.67
CLP group	24	7.77 ±2.08	8.84 ±3.74[ab]

[a]p < 0.05, compared with the S group
[b]p < 0.05, compared with the level before surgery

Table III. LPS in abdominal aortic blood ($\bar{x} \pm s$)

Groups	n	Before surgery	24 h after surgery
S group	24	0.065 ±0.016	0.070 ±0.021
CLP group	24	0.071 ±0.018	0.180 ±0.017[ab]

[a]p < 0.05, compared with the S group
[b]p < 0.05, compared with the level before surgery

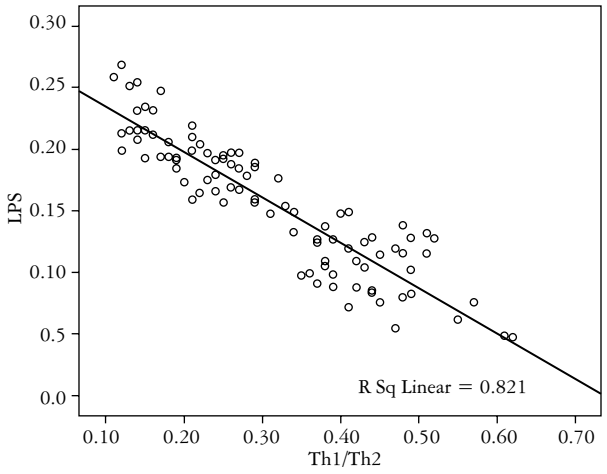

Fig. 1. The correlation between LPS in abdominal aortic blood and Th1/Th2 ratio of MLN

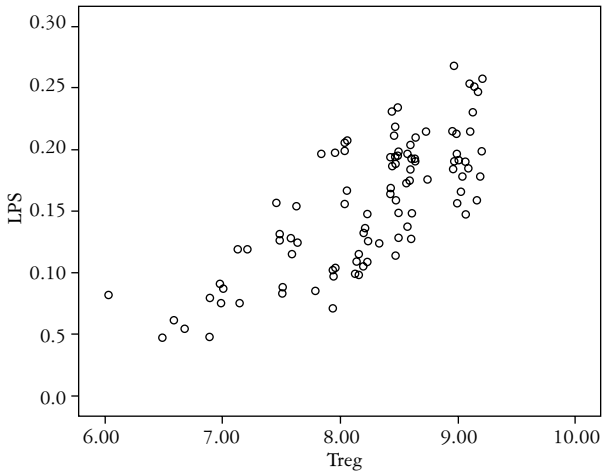

Fig. 2. The correlation between LPS in abdominal aortic blood and percentage of $CD4^+CD25^+Foxp3^+$ Treg of MLN

drome (SIRS) and compensatory anti-inflammatory response syndrome (CARS) [17]. In this study, we used Th1/Th2 balance in MLN to reflect the MLNs' immune status. Generally, in infection or trauma, endotoxin or tissue damage could activate macrophages. On one hand, the activation of macrophages leads to the release of the inflammatory cytokines interleukin-10 (IL-10) and interleukin-13 (IL-13) to produce a strong inflammatory response. On the other hand, the macrophages stimulate the release of endogenous prostaglandin hormone E2 (PGE2), which could strongly inhibit tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interferon-γ (IFN-γ), and interleukin-2 (IL-2) to inhibit the inflammatory response. These anti-inflammatory factors cause cell transformation between Th1 and Th2. Meanwhile, the T cell mitosis is inhibited, which leads to the paralysis of cellular immune function [18]. In addition, serious infections can activate the hypothalamus-pituitary-adrenal axis (HPA) to release glucocorticoids, and glucocorticoids inhibit the release of inflammatory cytokines and promote Th2 cell differentiation through promoting the nuclear ectopic NF-κB. As a result, the Thl-type immune response drifts to the Th2-type immune response, and then leads to early immune suppression after infection or trauma [19].

In this experiment, 24 h after surgery, immune suppression of MLN occurred in intra-abdominal infection, which led to impairment of endotoxin removal by the liver and inactivation of LPS to the systemic circulation, which resulted in a large number of LPS into the systemic circulation through the abdominal lymph vessels-thoracic duct. The elevated levels of endotoxin were further increased.

As an important regulator of the immune system, Treg plays a major inhibitory effect on cellular immunity in the complicated cellular immune regulation network [20]. The $CD4^+CD25^+$ Treg participates in the induction of lymphocyte anergy, which is an important part of immune suppression. Recent experimental data suggest that numbers of $CD4^+CD25^+$ Treg increase and their suppressive function is enhanced after stimulation by LPS [21]. Clearly, the increase/decrease of $CD4^+CD25^+$ Treg reflects the suppression/activation state of immunity in the septic host.

The results suggest that LPS was translocated to MLN in a rat model of intra-abdominal infection. LPS directly activates $CD4^+CD25^+Fox3^+$ Treg through TLR-4, and causes the proliferation of $CD4^+CD25$-$Foxp3^+$Treg, which results in an increased percentage of $CD4^+CD25^+Foxp3^+$Treg, and then immune suppression occurred in MLN. Immune suppression led to a decline in function of sterilization and anti-endotoxin of MLN and then resulted in the evaluated level of LPS indirectly.

In conclusion, we investigated the immune function of MLN in a rat model of intra-abdominal infection by evaluating the Th1/Th2 ratio of MLN, the percentage of $CD4^+CD25^+Foxp3^+$ Treg of MLN and LPS level. The decreased Th1/Th2 ratio, increased $CD4^+CD25^+Foxp3^+$ Treg percentage of MLN and LPS level indicate the immune suppression of MLN in abdominal infection. Meanwhile, these three indices have a certain positive/negative correlation. The present results strongly suggest that the local immune function of MLN has an impact on the level of LPS in the abdominal aortic blood, and that higher levels of LPS may lead to suppression of immune cells in the nodes by Treg, with subsequent impaired clearance of endotoxins.

The authors declare no conflict of interest.
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