

Relationship between lymphocyte apoptosis and endotoxin translocation after thermal injury in rats

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Abstract

AIM: To investigate the relationship between lymphocyte apoptosis in peripheral blood, spleen and mesenteric lymph nodes (MLN) and endotoxin translocation after thermal injury in rats.

METHODS: In a Wistar rat model inflicted with 30% TBSA III degree scalding, serum LPS levels in portal vein and vena cava were quantified by tachypleus amebocyte lysate (TAL) technique. The analysis of peripheral blood lymphocyte was employed in *in situ* Cell Death Detection Kit and evaluated by flow cytometry. Apoptotic lymphocytes in paraffin-embedded spleen and MLN sections were examined by histologic analysis, *in situ* deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) and peroxidase (POD) staining. The images were taken by Coolcdd camera system, and the count and optical density value (transmission light) of apoptotic lymphocytes were analyzed with software Spot and Imagine proplus 4.10a(IPP4.10a).

RESULTS: In the period of 3 to 48 postburn hours (PBHs) serum LPS level ($\times 10^3$ EU·L⁻¹) in portal vein (2.11 ± 0.02 , 5.66 ± 0.20 , 3.70 ± 0.22 , 2.56 ± 0.28 , 0.90 ± 0.11) was higher than that in vena cava (0.63 ± 0.01 , 1.53 ± 0.18 , 0.83 ± 0.32 , 0.52 ± 0.12 , 0.23 ± 0.02 , $P < 0.01$), but both increased sharply in postburn rats ($P < 0.01$) and reached a peak at 6 PBH. Analysis of apoptotic lymphocytes showed that the proportion (%) of postburn apoptotic cells was much higher than that in healthy rats (8.34 ± 1.53 , 8.13 ± 1.81 , 20.77 ± 3.94 , 23.90 ± 3.92 , 11.23 ± 1.35 and 13.26 ± 2.09 at 3, 6, 12, 24, 48 and 72 PBH, respectively, *vs* 3.99 ± 1.72 , $P < 0.01$), especially after 6 PBH. The concentrations of lymphocytic apoptosis at 12 and 24 PBH were markedly higher than that at other time points. Meantime, few apoptotic lymphocytes were found in normal MLN, but increased postburn obviously (3 ± 1 *vs* 546 ± 83 , 285 ± 39 , 149 ± 30 , 58 ± 10 , 36 ± 11 and 33 ± 9 in turn, $P < 0.01$), especially at 3 PBH, whereas apoptotic lymphocytes were concentrated in splenic cortex before the burn and decreased obviously during 72 PBHs (499 ± 186 *vs* 12 ± 8 , 19 ± 15 , 12 ± 7 , 100 ± 15 , 123 ± 25 and 226 ± 26 in turn, $P < 0.01$) though a slight rise was found in the medulla after 24 PBH. Optical density of apoptotic lymphocytes was significantly reduced in spleen in the 24 PBHs and raised in MLN during 48 PBHs than that prior to the burn, respectively.

CONCLUSION: Gut-origin LPS is a major cause of endotoxemia taken place early in rats following severe thermal injury and could induce extensive lymphocyte apoptosis in blood and MLN, which suggests an immunosuppression state could follow the initial injury and favors a septic state based on apoptotic mechanism.

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INTRODUCTION

Endotoxin or lipopolysaccharide (LPS) is a major cause of the local inflammation and septic shock and has been shown to impair host immune defense^[1-10]. With the experimental endotoxemia or sepsis in C3H/HeN (endotoxin-sensitive) mice, it was demonstrated in recent studies that LPS could cause thymic atrophy and bring about the increased apoptotic lymphocytes in thymus, spleen and gut-associated lymphatic tissue^[11-13]. Since the lymphocytes appear essential to both competent immune function and to the control of inflammatory response^[14,15], the lymphocyte apoptosis induced by LPS may play important roles in the development and regulation of the immune system, especially in the gut-dysfunction situations induced by sepsis or severe injuries. Endotoxemia in the patients with severe injury has been suggested to be an important factor in the systemic inflammatory response syndrome (SIRS)^[16,17]. It was found in the previous *in-vivo* study that gut-origin endotoxemia following the intestinal mucosal barrier dysfunction could occur in early postburn period and lead to the injury of systemic organs such as lungs, liver etc^[18,19]. Although the exact routes by which translocating LPS reach the blood and systemic organs are not known with certainty, most researchers believed that LPS was capable of translocating from the gut via both the mesenteric lymphatics and the portal blood^[20]. Because the mesenteric lymph nodes (MLN) receives its lymphatic drainage from the small intestine, cecum and proximal colon, we believed that MLN should be a major site and pathway of translocated gut-origin LPS. However, little has been known about lymphocyte apoptosis in circulating blood and MLN induced by endotoxin translocation after thermal injury and the relationship between them.

The present experiments were performed in severe scalded rats to determine the effects of gut-origin LPS translocation on the apoptosis of lymphocytes in circulating blood, spleen and MLN, and to investigate the relationship between them.

MATERIALS AND METHODS

Animals and reagents

Forty-two adult Wistar rats weighing 235-345g were randomly distributed in the normal control and 6 thermal injury groups, i.e. 3, 6, 12, 24, 48 and 72h after scalding. Each group contained 6 animals (half males and half females). Rats in the thermal injury groups were inflicted with 30% total burn surface area (TBSA) III degree scalding on their back after anesthetized with 30mg/kg of

intraperitoneal pentobarbital. Then under general anesthesia at the different time points after the thermal injury, the blood of rats' portal vein and vena cava were collected for LPS test and the isolation of peripheral blood lymphocyte, respectively. MLN and spleen were harvested by aseptic manipulation and fixed in 10mL·L⁻¹ paraformaldehyde solution (pH 7.4) for 24h. All chemicals used in this study were purchased from Sigma Chemical Co. (USA) unless specified otherwise. Endotoxin-free glassware and plasticware were prepared by baking at 250°C for 1.5h and radiating with ⁶⁰Co, respectively.

Determination of serum LPS levels in portal vein and vena cava

Sera were obtained from the blood samples of portal vein and vena cava by clotting for 60min on ice and then centrifuged at 2500g at 4°C for 5min respectively, filtered, aliquoted, and frozen at -70°C. After all serum samples were collected, the serum LPS levels were determined with a commercially available kit for tachypleus amebocyte lysate (TAL) technique (Zhanjiang A & C Biological Ltd, Zhanjiang, China) according to the manufacture's guidelines.

Isolation and preparation of lymphocyte

Lymphocyte in the heparinized blood from vena cava were isolated by density gradient centrifugation using ficoll-hypaque (d=1.077) followed by two washing steps in phosphate buffered saline (PBS) and lysis of residual erythrocytes using nine volumes of an ice-cold isotonic ammonium chloride solution (NH₄Cl 155mmol·L⁻¹, KHCO₃ 10mmol·L⁻¹, EDTA 0.1mmol·L⁻¹) to one volume of cell pellet at 4°C for 7min^[21]. Lymphocytes were greater than 98% as analyzed by microscopy using Hemacolor staining (Merk, Germany). Cell viability was greater than 97% as assessed by the trypan blue exclusion test. Isolated lymphocytes were maintained in a glass bottle in RPMI 1640-medium without fetal calf serum (endotoxin-free; Gibco BRL, Life Technologies, USA) at a concentration of 1×10⁹ cells·L⁻¹ in 5mL·L⁻¹ CO₂ at 37°C for 2h to exclude macrophage before the analysis of apoptosis. Then the cells were collected by centrifugation and washed twice in PBS.

Flow cytometric analysis of apoptotic lymphocyte

The analysis of apoptotic lymphocytes was performed by an in situ Cell Death Detection Kit, Fluorescein (Roche, Germany). The procedures in brief was according to the manufacture's guidelines as follows: lymphocyte were suspended and fixed with 40mL·L⁻¹ paraformaldehyde solution (pH 7.4), rinsed in PBS twice, and incubated in permeabilization solution (1 g·L⁻¹ Triton X-100 in 1 g·L⁻¹ sodium citrate) for 2min on ice. The cells were again rinsed in PBS, centrifugated and incubated in terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) reaction mixture for 1h at 37°C in a humidified atmosphere at dark. Then cells were rinsed in PBS again. TUNEL fluorescence of individual nuclei in a final volume of 500μL cells solution was analyzed by an FACS Calibur (Becton Dickinson, USA), while gating on physical parameters was enacted to exclude cell debris. A minimum of 10000 events were counted per sample. The results were reported as the percentage of hypodiploid (fragmented) nuclei reflecting the relative proportion of apoptotic cells.

Analysis of apoptotic lymphocytes in spleen and MLN

Paraffin-embedded spleen and MLN tissue were cut into sections of 5μm and mounted on Vectabond Reagent slides, deparaffinized and rehydrated through xylene, graded ethanol to distilled water. Then the tissue sections were pre-treated with 20mg·L⁻¹ proteinase K for 30min, and analyzed with an in situ Cell Death Detection Kit, POD (Roche, USA) according to the manufacture's guidelines. Each

experiment set up by TUNEL reaction mixture without terminal transferase served as negative control. The images were taken by Coolcccd camera system, and the count and optical density (OD) value of apoptotic lymphocytes were analyzed with software Spot and Imagine proplus 4.10a (IPP 4.10). The counts and optical density values (transmission light) of TUNEL-POD positive lymphocytes in spleen and MLN were determined in three high-resolution fields selected randomly in the area concentrated positive apoptotic lymphocytes and two thousand cells per field were counted per slides.

Statistical analysis

All the data was analyzed by Student's *t* test and expressed as $\bar{x} \pm s$. The statistical difference $P < 0.05$ was considered as significantly and $P < 0.01$ as very significant.

RESULTS

Serum LPS in portal vein and vena cava increased after thermal injury

Serum LPS levels in portal vein and vena cava increased sharply postburn ($P < 0.01$) and reached to a peak level at 6 PBH and decreased thereafter. LPS level in portal vein was higher than that in the vena cava ($P < 0.01$) in the period of 3 to 48 PBHs, but both decreased to near control level at 72 PBH (Figure 1).

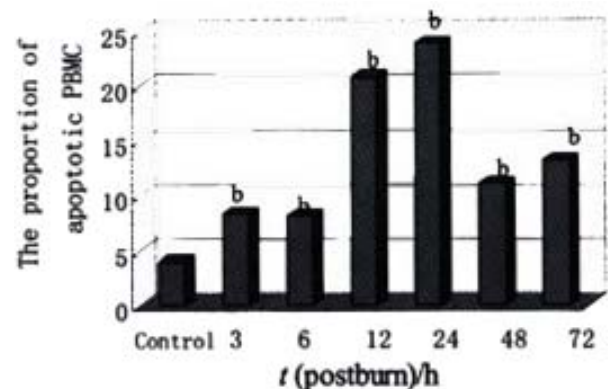


Figure 1 Comparison of the LPS levels postburn in portal vein and in vena cava. ^b $P < 0.01$, vs control; ^c $P < 0.01$, portal vein vs vena cava

Apoptosis of lymphocytes

Lymphocytes isolated from the circulation of healthy rats exhibited a low proportion of apoptotic cells at (3.99±1.72)%, but increased obviously during the whole postburn period of the experiment ($P < 0.01$), especially after 6 PBH. The concentrations of lymphocytic apoptosis at 12 and 24 PBH were markedly higher than that at other time points (Figure 2,3).

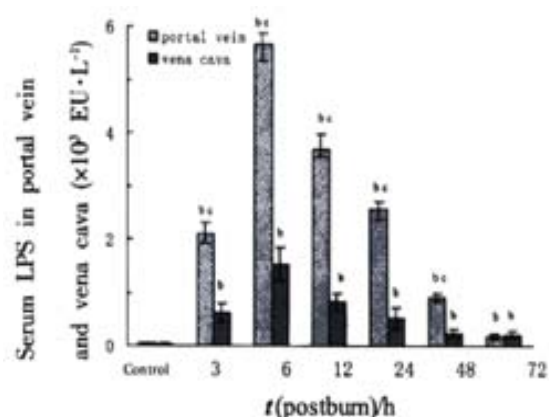


Figure 2 The relative proportions of apoptotic lymphocytes postburn. ^b $P < 0.01$, vs control

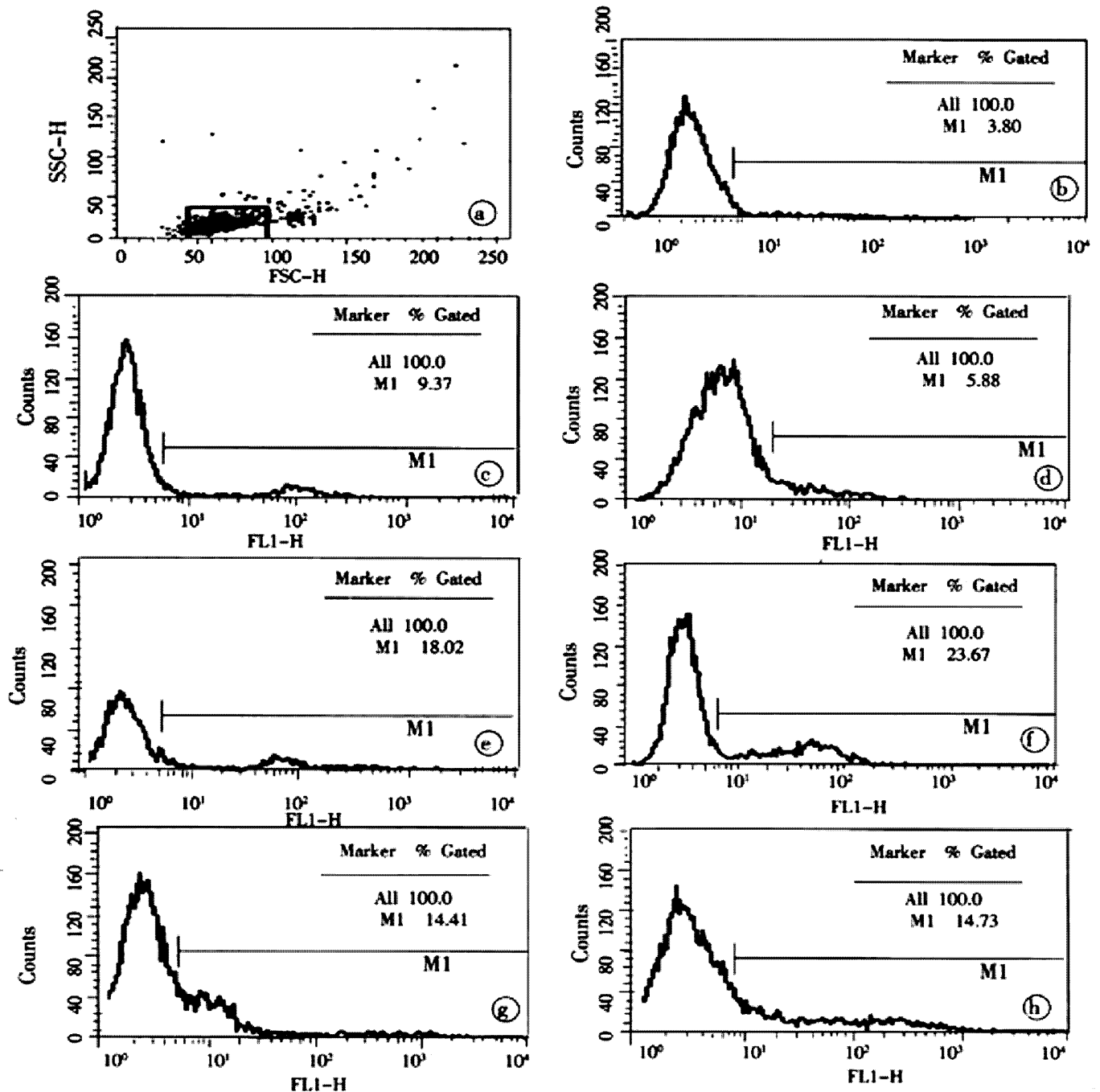


Figure 3 The results of typical flow cytometric analysis of postburn apoptotic lymphocytes. a, b, c, d, e, f, g and h were the relative proportion of apoptotic lymphocytes isolated from one rat in the control and group 3, 6, 12, 24, 48 and 72 PBH, respectively.

Apoptotic lymphocytes in spleen and MLN

It was shown by the results of TUNEL-POD staining and the counts of apoptotic lymphocytes that the apoptotic cells were few in normal MLN (Figure 4a), but increased postburn obviously ($P < 0.01$), especially at 3 PBH (Figure 4b, Table 1). Opposite to MLN, apoptotic lymphocytes were concentrated in spleen cortex before burn (Figure 4c), but decreased obviously during 3 to 72 PBHs ($P < 0.01$) though a slight rise was found in the medulla after 24 PBH (Figure 4d, Table 1). Since the principle of TUNEL-POD is to label the fragmented genomic DNA, the biochemical hallmark of apoptotic cell, the color density of nuclei staining could indirectly reflect the extent of DNA fragmentation. Optical density of TUNEL-POD staining in apoptotic lymphocytes was significantly reduced in spleen in the 24 PBHs, but raised in MLN during 48 PBHs than that before burn, respectively (Table 2).

Table 1 Apoptotic lymphocyte counts in 2000 total cells of spleen and MLN

Tissue	Healthy control	t(postburn)/h					
		3	6	12	24	48	72
Spleen	499±186	12±8 ^b	19±15 ^b	12±7 ^b	100±15 ^b	123±25 ^b	226±26 ^b
MLN	3±1	546±83 ^b	285±39 ^b	149±30 ^b	58±10 ^b	36±11 ^b	33±9 ^b

^b $P < 0.01$, vs control

Table 2 The optical density values(transmission light) of TUNEL-POD positive lymphocytes in spleen and MLN

Tissue	Healthy control	t(postburn)/h					
		3	6	12	24	48	72
Spleen	0.54±0.03	0.30±0.02 ^b	0.35±0.12 ^b	0.36±0.03 ^b	0.36±0.02 ^b	0.44±0.19	0.52±0.12
MLN	0.24±0.06	0.83±0.16 ^b	0.96±0.25 ^b	0.62±0.16 ^b	0.45±0.04 ^b	0.35±0.07 ^b	0.29±0.04

^b $P < 0.01$, vs control

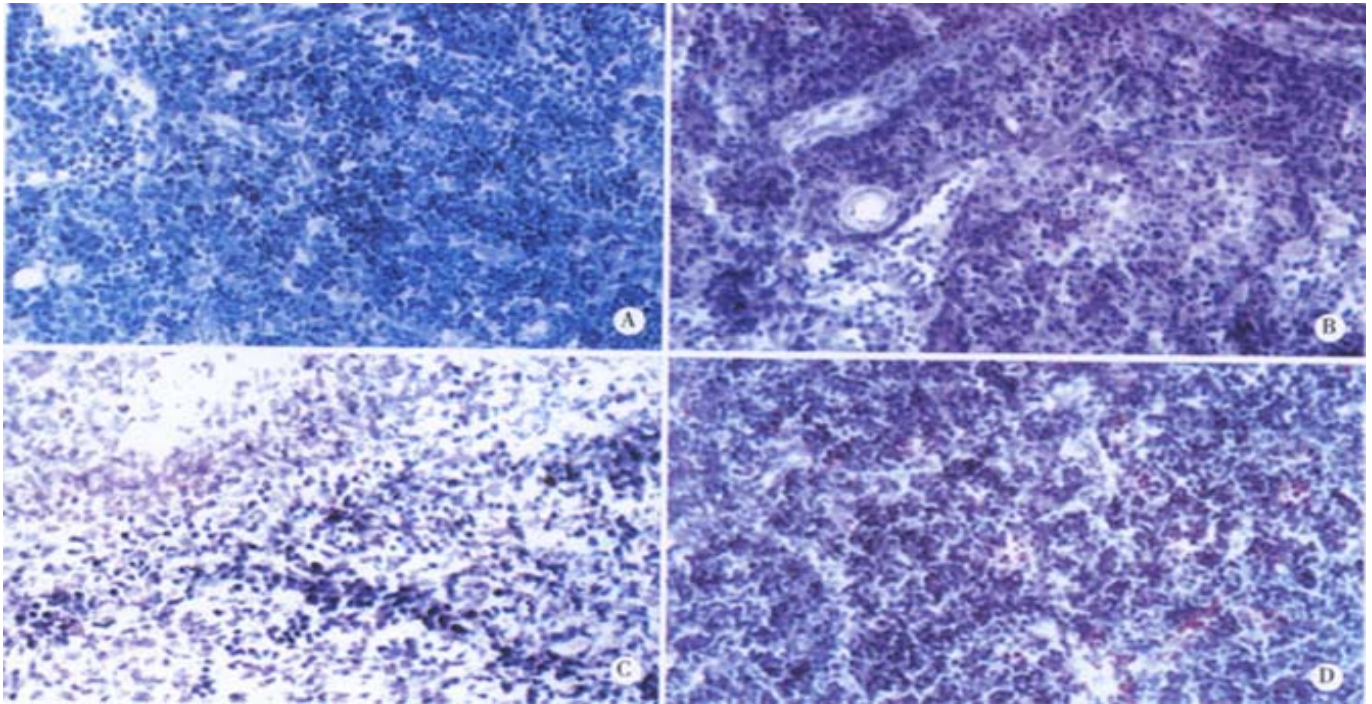


Figure 4 Apoptotic lymphocytes in spleen and MLN. (TUNEL-POD staining $\times 400$, the nuclei stained brown are positive apoptotic cells). a: MLN from the healthy rat; b: MLN from the rat at 3 PBH; c: Spleen from the healthy rat; d: Spleen from the rat 24 PBH.

DISCUSSION

Translocation of gut bacteria and endotoxin is a common situation after severe trauma, probably as a consequence of loss of physical integrity of the mucosal barrier, increments in permeability, or impaired local immune function resulted from ischemia-reperfusion injury of intestine. The gut-origin LPS translocated to the portal circulation or gut lymphatics has been thought to subsequently initiate a septic process leading to the development of SIRS^[22]. In this study, the LPS levels in portal vein and vena cava increased sharply after the severe thermal injury (Figure 1) indicating that endotoxin could enter the blood circulation in the early period of trauma and might be a main cause of endotoxemia.

The gastrointestinal tract contains numerous immune effector and regulatory cells of lymphoid and myeloid origin that are thought to play a critical role in host defense against enteric infections^[23]. And at a systemic level, the lymphocytes, through the production of regulatory cytokines, are important in mediating and controlling the host immune response. This was well illustrated by the spontaneous appearance of gastrointestinal inflammatory disease in a number of animals deficient in different cytokines, including IL-2 and IL-10, etc^[24-26]. Thus, the increased lymphocyte apoptosis in circulatory blood and gut-associated lymphoid tissues must impair the balance between the functions of host defense and immune tolerance in the gut immune system^[27,28]. The observation presented here provided some evidence that local and systemic pathologic lymphocyte apoptosis following an initial event of injury could impair the immune system. In the scalded rats, the counts of apoptotic lymphocytes in peripheral blood and MLN increased dramatically during the whole 72 PBHs, and reached a peak level at 12-24 PBHs and 3 PBH, respectively (Figure 2,3, 4, Table 1, 2). The results of lymphocyte apoptosis in MLN were consistent with that reported by Mongini *et al*^[22]. Moreover, It was surprised to observe the apoptotic lymphocytes in the spleen decreased obviously after thermal injury in this study (Figure 4, Table 1, 2) though splenic lymphocyte seemed to be less sensitive to apoptosis triggered by LPS

and oxidative stress^[11,12,29]. Specifically, we also found that splenic apoptotic lymphocytes were located mainly in the cortex in healthy rats, but being present in the medulla after thermal injury. The implications of these results are unclear. During normal lymphocyte development, immature lymphocytes are located in the cortex and migrate to the medulla in the course of maturation. The depletion of cortical lymphocytes by apoptosis may be one mechanism of eliminating potentially autoreactive immune cells^[22]. As for the decreased splenic apoptotic lymphocytes in the scalded rats, whether it is a reaction induced by the lymphocyte apoptosis in peripheral blood remains to be clarified.

Taking that both change tendency of lymphocyte apoptosis and serum LPS level after thermal injury into consideration, it was found that the peak level of lymphocyte apoptosis appeared earlier in MLN and much later in circulatory blood than that of LPS in the blood. The results indicated that MLN was an advanced station in the routing of the LPS translocation and suggested that the translocation of gut-origin LPS was a major factor of lymphocyte apoptosis in rats with severe burn injury. Furthermore, LPS translocated into the blood could lead to a further breakdown of gut immune barrier and thus accelerate the entrance of enteric bacteria and their toxins into the circulation^[30] and result in an immunosuppressive state due to the induction of lymphocyte apoptosis^[14,22,31]. This might explain why the peak level of apoptotic lymphocytes came forth much later in circulatory blood than that in MLN.

In addition to our observations in the present study, thymocyte and mucosal lymphocyte apoptosis after thermal injury had been recently reported^[32,33]. Although in these two studies the increased lymphocyte apoptosis in thymus and gut-associated lymphoid tissue seemed mainly due to the increased corticosterone concentration in plasma and in the lymphoid tissue, the presence of lymphocyte apoptosis in the peripheral blood, thymus and gut-associated lymphoid tissue following injury could explain the immunosuppressive state following such injury. In fact, decreased lymphocyte apoptosis in

sepsis can improve survival by overexpression of Bcl-2 in transgenic mice^[34]. In summary, we can reach the conclusion that an immunosuppression state could follow the initial injury and favor a septic state based on apoptotic mechanism, and that the immunosuppressive state after the thermal injury can be the event induced by translocation of gut-origin LPS and bacteria spreading to other tissues, and which in turn causes a recurrence of sepsis.

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