P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(5):1038-1044
World Journal of Gastroenterology
 ISSN 1007-9327

• BASIC RESEARCH •

The mRNA expression patterns of tumor necrosis factor-a and TNFR-I in some vital organs after thermal injury

Wen-Hui Fang, Yong-Ming Yao, Zhi-Guo Shi, Yan Yu, Ye Wu, Lian-Rong Lu, Zhi-Yong Sheng

Wen-Hui Fang, Yong-Ming Yao, Zhi-Guo Shi, Yan Yu, Ye Wu, Lian-Rong Lu, Zhi-Yong Sheng, Department of Microbiology and Immunology, Burns Institute, 304th Hospital of PLA, Beijing 100037, China
Supported by the National Key Program for Fundamental Research and Development, No.G1999054203, the National Natural Science Outstanding Youth Foundation of China, No.30125020, and the National Natural Science Foundation of China, No.30200293
Correspondence to: Yong-Ming Yao, M.D., Department of Microbiology and Immunology, Burns Institute, 304th Hospital of PLA, 51 Fu-Cheng Road, Beijing 100037, China. c_fanf@sina.com
Telephone: +86-10- 66867394 **Fax:** +86-10-68429998
Received: 2002-10-30 **Accepted:** 2002-11-25

Abstract

AIM: To investigate changes of tumor necrosis factor-α (TNF-α) and TNFR-I expression in vital organs and their significance in the pathogenesis of multiple organ damage associated with endogenous endotoxin following major burns.

METHODS: Wistar rats subjected to a 35 % full-thickness scald injury were sacrificed at 12 h, 24 h, 48 h, and 72 h postburn, respectively. Meanwhile, eight rats were taken as normal controls. Tissue samples from liver, spleen, kidney, lung and intestine were collected to assay tissue endotoxin levels and measure TNF-α and TNFR-I expression. In addition, blood samples were obtained for the determination of organ function parameters.

RESULTS: Endotoxin levels in liver, spleen and lung increased markedly after thermal injury, with the highest level in liver. The gene expression of TNF-α in liver, lung and kidney was up-regulated after thermal injury, while the TNFR-I mRNA expression in liver, lung, kidney and intestine was shown decreased throughout the observation period. Thus, the mRNA expression ratio of TNF-α to TNFR-I was significantly increased postburn, particularly in pulmonary tissue (67-fold). In addition, the significant correlations between the expression of TNFR-I or the expression ratio of TNF-α/TNFR mRNA in liver tissue and serum aspartate aminotransferase levels were noted (P <0.05-0.01). Similar results were also obtained between pulmonary TNF-α mRNA expression and myeloperoxidase activities (P<0.01), whereas there was a highly negative correlation between levels of renal TNFR-I mRNA expression and serum creatinine.

CONCLUSION: Burn injury could result in the translocation of gut-derived endotoxin that was mainly distributed in the liver, spleen and lung. The translocated endotoxin then made the expression of TNF-α and TNFR-I mRNA up-regulated and down-regulated respectively in various organs, which might be involved in the pathogenesis of multiple organ damage following burns.

Fang WH, Yao MY, Shi ZG, Yu Y, Wu Y, Lu LR, Sheng ZY. The mRNA expression patterns of tumor necrosis factor-α and TNFR-I in some vital organs after thermal injury. *World J Gastroenterol* 2003; 9(5): 1038-1044
http://www.wjgnet.com/1007-9327/9/1038.htm

INTRODUCTION

Gram-negative bacterial sepsis with resulting multiple organ dysfunction syndrome (MODS) and death continues to be a major problem in critical surgical patients. Lipopolysaccharide (LPS), an integral component of the gram-negative bacterial cell membrane, is responsible for many, if not all, of the toxic effects that occur during gram-negative sepsis. Both experimental and clinical data recently implicated that gut-derived bacteria or endotoxin translocation might play a role in the development of sepsis and multiple organ damage in critically ill patients, especially after trauma, thermal injury, hemorrhagic shock and major elective surgical procedure[1-3]. It has also become clear that endotoxin initiates an inflammatory cytokine and mediator cascade, and these mediators, in turn, act on additional target cells to produce an array of pro-inflammatory cytokines leading to cardiovascular shock, MODS, or even death[4-7]. Among these inflammatory mediators, tumor necrosis factor-α (TNF-α) in particular occupies a pivotal role in the pathogenesis of inflammation, cachexia, septic shock and tissue injury.

TNF-α exerts its pleiotropic effect by interacting with two high affinity receptors termed TNFR-I (55 kilodaltons) and TNFR-II (75 kilodaltons) on a variety of cells[8]. Both TNFRs also exit in soluble forms[9]. The soluble TNFRs (sTNFRs) are produced by proteolytic cleavage of the extracellular domain of TNFRs. The biological role of sTNFR appears to be dose-dependent. For instance, at low concentrations, sTNFRs stabilize the trimeric structure of TNF-α, thereby slowing its spontaneous decay of bioactivity, and augmenting the long-term effects of TNF-α by providing a reservoir of bioactive TNF-α that is slowly released. When more quantities of sTNFRs are present, however, they reduce the bioactivity of TNF-α by competing for TNF-α binding with cell-associated receptors. These above regulatory processes may modulate TNF-α activity in response to inflammation. In this regard, to obtain a complete view of the *in vivo* pathophysiologic roles of TNF-α, one should further study the regulation of TNF-α/TNFR and the balance between them. Since most of the known cellular TNF-α responses occur through TNFR-I, we investigated the expression changes of TNF-α and TNFR-I in vital organs, and their significance in the pathogenesis of multiple organ damage associated with endogenous endotoxin following major burns.

MATERIALS AND METHODS

Animals and thermal injury

Male Wistar rats (weight range 250-300 g), purchased from the Laboratory Animal Center, Beijing, China, were used for the study. The animals were housed in separate cages in a temperature-controlled room with alternating 12-h light-dark cycles, and were allowed to acclimatize for at least 7 days

before being used. All animals had free access to water, but were fasted overnight prior to the experiment. After the rats were anesthetized by the intraperitoneal (i.p.) injection of pentobarbital sodium (40 mg/kg), their dorsal hair was shaved and a 35 % of total body surface area with full-thickness burn was inflicted by immersion of the dorsal skin in a 100 ℃ water bath for 12 s. All animals received Ringer' s solution (50 ml/kg) administered i.p. after burn injury for resuscitation, and the burn wounds were treated with an antibacterial agent everyday as a prophylactic measure against wound infection. All experimental manipulations were undertaken in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and with the approval of the Scientific Investigation Board of the Burns Institute, Postgraduate Medical College, Beijing.

Experimental design

A total of 46 animals were sacrificed at each of following time points: 12 h, 24 h, 48 h and 72 h postburn. Meanwhile, eight rats were taken as normal controls. Under anesthesia, systemic blood samples were obtained. Then tissue specimens were taken from mesenteric lymph nodes (MLNs), liver, spleen, kidneys, lungs, and intestine.

Bacterial translocation

The MLNs, spleen, liver, kidney, lung and subeschar tissues were removed aseptically in this order. Each organ sample was weighed, homogenized in 2 ml of sterile saline under aseptic conditions, and 100 μl of the homogenate were plated on Chinese blue agar and 5 % sheep blood agar. After 48 h of aerobic incubation at 37 ℃, all agar plates were examined for growth. The number of viable microorganisms per gram of organ tissue was calculated, and the obtained organisms were identified by standard bacteriological techniques.

Tissue endotoxin measurement

Tissue specimens from liver, spleen, kidneys and lungs were aseptically removed, and were homogenized in 3-fold volume pyrogen-free saline on ice. The homogenate was stored at -20 ℃ until analyzed. The tissue endotoxin levels were measured by the chromogenic Limulus Amebocyte Lysate (LAL) assay with the procedure based on the LS-1 kit (Seikagaku Corp., Tokyo, Japan) protocol modified by perchloric acid (PCA) treatment of samples to remove nonspecific activators or inhibitors of the lysate[10]. Briefly, 150 μl of 0.32 M PCA were added to 75 μl of homogenate in an ice bath, and the mixture was incubated at 37 ℃ for 20 min. After centrifuged (Kubota Corp., Japan) at 3 000 rpm for 15 min, the supernatant was neutralized with an equal volume of 0.18 N NaOH. Then, 100 μl of the supernatant was incubated for 18 min at 37 ℃ with 50 μl of amebocyte lysate. Chromogenic substrate then was added, and after 3 min of further incubation, the reaction was stopped. The amount of *p*-nitroaniline (pNA) released from the substrate was determined, after diazo-coupling, by the absorbance of the solution at 545 nm (Beckman Corp. U.S). The endotoxin concentration expressed as endotoxin units per gram of organ tissue was calculated from a standard curve derived from the assay of s standard endotoxin (Lot EC-5, endotoxin unit (EU) =100 pg of U.S. standard endotoxin). The detection limit with this method was 0.01 EU/ml.

RNA extraction and reverse-transcription-polymerase chain reaction

Tissue samples from liver, lungs, kidneys and intestine were stored in liquid nitrogen until analysis. Extraction of total tissue RNA was performed with guanidine isothiocyanate according to the method by Chromczynski and Sacchi[11]. First-strand cDNA was synthesized using oligo-dT primer and the AMV reverse transcriptase (Promega Corp., Madison, WI). In brief, 2 μg of total RNA was reversely transcribed by adding 20 μl of a master mixed with 1 U/μl RNase inhibitor, 0.025 μg of random hexamers, 5 mmol/L $MgCl_2$, 1×reverse transcriptase buffer, 1 mmol/L of dNTP mixture, and 0.7 U/ml AMV reverse transcriptase (final concentrations indicated). Samples were incubated at 42 ℃ for 60 min.

For the cDNA amplification, the PCR with hot start technique was used, in which *Taq* polymerase was added to each tube at 88 ℃ with Thermal Cycler (Perkin-Elmer Corp., U.S.). The PCR mixture contained a final concentration of 0.2 μmol/L specific primers for TNF-α and TNFR-I (from Beijing Medical University), 1×PCR buffer, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP and 0.5 U/25μl *Taq* polymerase (Promega Corp., Madison, WI). After a 5-min initial melting step at 97 ℃, the PCR with 28 to 32 cycles was carried out [94 ℃ 1 min for denaturation; 58 ℃ (TNF-α) or 63 ℃ (TNFR-I) 1 min for annealing; and 72 ℃ 1 min for extension]. The final cycle was followed by a 10-min soak at 72 ℃. The sequences of primer pairs, and predicted sizes of the amplified PCR fragments were shown in Table 1. The house keeping gene β-actin was used as internal controls for standardization of PCR product[14].

Table 1 Primer sequences used for polymerase chain reaction (PCR)

Target gene	Oligonucleotide primers	Size of PCR production, bp	Ref.
TNF-α	5'-AGA ACT CCA GGC GGT GTC TCT G-3'	415	8
	5'-GT GGC AAA TCG GCT GAC GGT GT-3'		
TNFR	5'-CC ATC TGC TGC ACC AAG TGC CA-3'	347	9
	5'-AA TCC TCG GTG GCA GTT ACA CA-3'		

PCR products and molecular weight markers were subjected to electrophoresis and visualized by means of ethidium bromide staining. The cycle number of each study was chosen in a linear range to avoid the plateau effect. The gel then was photographed, and the negative scanned with a densitometer (Pharmacia Corp., Sweden). Final data were expressed as a ratio of the band of interest to the unregulated control (β-actin).

Tumor necrosis factor-α protein analysis

Liver, lung, kidney and intestine tissues were respectively homogenized in 9-fold volume 0.01 mmol/L sodium phosphate buffer, pH 7.2 in an ice bath, and centrifuged (Haerus Corp., Germany) for 20 min at 1 400 rpm, 4 ℃. The supernatant was stored at -20 ℃ until analysis. TNF-α concentration was determined by a commercially available protocol (rat tumor necrosis factor ELISA test kit; Endogen Corp., U.S.). In brief, aliquots of freshly diluted standard concentrations of recombinant rat TNF-α or samples were incubated in duplicate on ELISA plates. ELISA wells were then sequentially exposed to biotinylated antibody, streptavidin-HRP, and finally TMB substrate. The reaction was terminated using stop solution. The sample absorbance was detected at 450 nm with a plate reader (Denley Corp., England) and the TNF-α concentrations were determined by the reading of the standard curve. The sample protein was measured by the method of Bradford[15], and the detected tissue TNF-α was finally expressed as pg/mg protein.

Myeloperoxidase assay

Lung tissue was homogenized in a 9-fold volume of 20 mmol/L potassium phosphate buffer, pH 7.4, and centrifuged (Hitachi Corp., Japan) for 30 min at 35 000 rpm, 4 ℃[16]. The pellet was

resuspended in 1 ml of 50 mmol/L potassium phosphate buffer, pH 6.0, containing 0.5 mg/dl hexadecyltrimethylammonium bromide and frozen overnight at -70 ℃. Then, the samples were thawed, sonicated for 90 s at full power, incubated in a 60 ℃ water bath for 2 h, and centrifuged for 10 min at 35 000 rpm, 4 ℃. 0.1 ml of supernatant, was added to 2.9 ml of 50 mmol/L potassium phosphate buffer, pH 6.0, containing 0.167 mg/ml o-dianisidine and 0.0005 hydrogen peroxide. The sample absorbance was measured at 460 nm visible light (A_{460}) for 2 min (Beckman Corp., U.S.). Myeloperoxidase (MPO) activity per gram wet lung (gwl) was calculated by the following formula: Myeloperoxidase activity (units/gwl)= (ΔA_{460})×(13.5)/ lung weight (g), where ΔA_{460} was the changes in absorbance at 460 nm from 30 to 90 s after the initiation of the reaction. The coefficient 13.5 was empirically determined such that 1 unit MPO activity was the amount of enzyme that would reduce 1mmole peroxide/min.

Diamine oxidase activity measurement

The small intestines were removed and stored in liquid nitrogen before analysis. For measurement of intestinal diamine oxidase (DAO) activity, the small intestine was homogenized in a threefold volume of ice-cold phosphate buffer (0.1M, pH 7.2) and centrifuged (Haerus Corp., Germany) for 30 min at 10 000 rpm, 4 ℃. After the resulting upper layer was discarded, the supernatant left was used as the source of the enzyme. DAO activity was assayed according to the modified method[17]. The assay mixture contained 3 ml of phosphate buffer (0.2 M, pH7.2); 0.1 ml (4 μg) of horseradish peroxidase solution (Shanghai Biochemistry Institute, China), 0.1 ml of o-dianisidine (Sigma Chemical, St. Louis, MO) methanol solution (500 μg), 0.5 ml of intestinal homogenate or freshly diluted DAO standard, and 0.1 ml of substrate (cadaverine dihydrochloride from Sigma Chemical, St. Louis, MO) solution (175 μg), which was incubated for 30 min at 37 ℃. Then, the absorbance was measured at 436 nm (Beckman Corp. Germany), and the DAO activity was read on the standard curve. Sample protein was measured as described previously[15]. The intestinal DAO activity was calculated by the following formula: DAO activity (units/mg protein) = DAO activity×(60)/protein concentration.

Organ function parameters measurement

Systemic blood samples were collected and serum was prepared by centrifugation for 10 min at 2 000 rpm. Then, the samples were stored at -20 ℃ until analysis. Serum aminoleucine transferase (ALT), aspartate aminotransferase (AST), MB isoenzyme of creatine kinase (CK-MB) and creatinine (Cr) levels were determined with a biochemical autoanalyzer (Model 7170; Hitachi Ltd., Japan).

Statistical analysis

Statistical analyses were done by using the statistical package SAS 6.04, and the data were expressed as the mean ± SEM. Statistical evaluation of the continuous data were performed by one-way analysis of variance (ANOVA), following by either Dunnett' s *t* test or Kruskal-Wallis test for inter-group comparisons. Correlations between variables were tested by Spearman' s correlation coefficients. The level of significance was considered to be $P<0.05$.

RESULTS

Bacterial translocation and endotoxin levels in tissues

Microbiologic cultures from subeschar tissues were all negative throughout the observation period, while the bacterial translocation was significantly increased at various time points after severe burns ($P<0.05$), which was predominantly detected in MLNs (the incidence was 30.0 %) with the higher cultured bacterial number than that in other organs (control: negative, postburn: 4.748±0.313 $\log_{10}$CFU/g). The translocating organisms were predominantly *E. coli*, but *Klebsiella spp.* and *Enterecoccus* species were also identified. Meanwhile, the endotoxin levels in liver, spleen and lungs were significantly elevated at 12 h postburn, with the liver highest (control: 1.388±0.312 EU/g, 12 h postburn: 17.337±3.687 EU/g, $P<0.01$). Endotoxin levels in liver and spleen then fell quickly, and elevated again at 48 h (2.805±0.306 EU/g in liver, 2.623±0.321 EU/g in spleen, $P<0.01$ compared with the controls). On the other hand, endotoxin levels in lungs maintained a high level till 72 h (control: 4.510±1.139 EU/g, 72 h postburn: 6.938±1.715 EU/g). There was no significant difference in endotoxin levels in kidneys at any time point postburn.

Expression of TNF-α and TNFR-I in Tissues

The expressions of TNF-α mRNA in tissues (liver, lungs, kidneys and intestine) were examined by RT-PCR as shown in Figure 1, which revealed a constitutively lower level in the control animals and a marked increase in burned subjects. The expression of TNF-α mRNA in liver was increased to a maximum at 12 h postburn (control: 0.020±0.013, 12 h postburn: 0.140±0.032, $P<0.05$) and decreased thereafter (Figure 1A), whereas that in the kidneys and lungs peaked at 48 h postburn with the increased expression by 10-fold and 8-fold respectively (Figure 1B), among which the pulmonary mRNA expression maintained at a higher level up to 72 h postburn ($P<0.05$ compared with the controls). However, there was a trend of lower expression of TNF-α mRNA in intestine (Figure 1B).

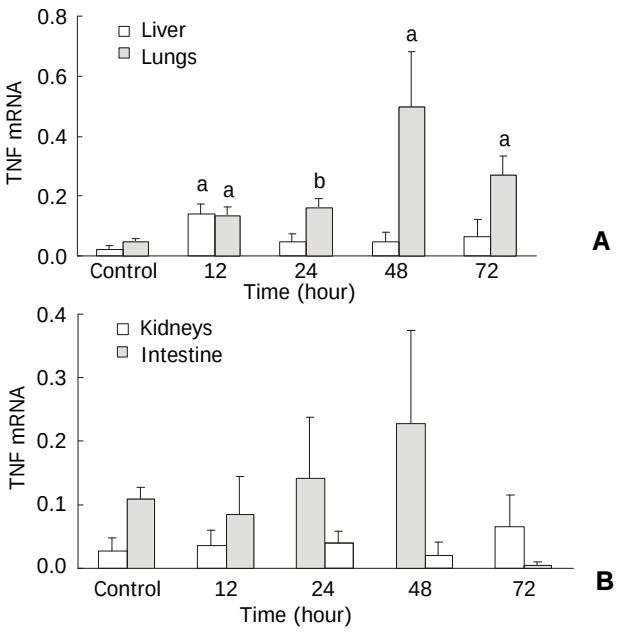

Figure 1 Semiquantitive analysis of tumor necrosis factor-α (TNF-α) mRNA in various organs after thermal injury. Values are reported as the ratio of TNF-α to β-actin signals. [a]$P<0.05$ and [b]$P<0.01$ as compared to the control values.

Tissue TNF-α protein assayed by ELISA exhibited higher levels in both liver and intestine (Figure 2, A and B), with the former that was increased by 25 % at 72 h postburn compared with control values, and the latter that peaked at 48 h postburn (control: 45.723±11.333 pg/mg protein, 48 h postburn: 80.448±14.018 pg/mg protein, $P<0.05$) and maintained at a higher levels until 72 h postburn (61.357±4.902 pg/mg protein).

However, there was no significant difference in TNF-α levels in lungs at each time point postburn compared with the controls. So was TNF-α levels in kidneys.

TNFR-I mRNA expression in vital organs by RT-PCR was shown in Figure 3, in which abundant expression was observed in the control animals, with the highest level in the liver (1.029±0.215). After burn injury, however, TNFR mRNA expression in liver, kidneys and lungs was shown to reduce dramatically, with the lowest levels at 24-48 h postburn (26.1-46.7 % of the control values). Though increased gradually thereafter, TNFR mRNA levels in these organs were shown lower than the control values at 72 h after thermal injury. A remarkable decrease in intestinal TNFR mRNA expression was also noted (17.3 % of the control values, $P<0.05$) and maintained throughout the observation period.

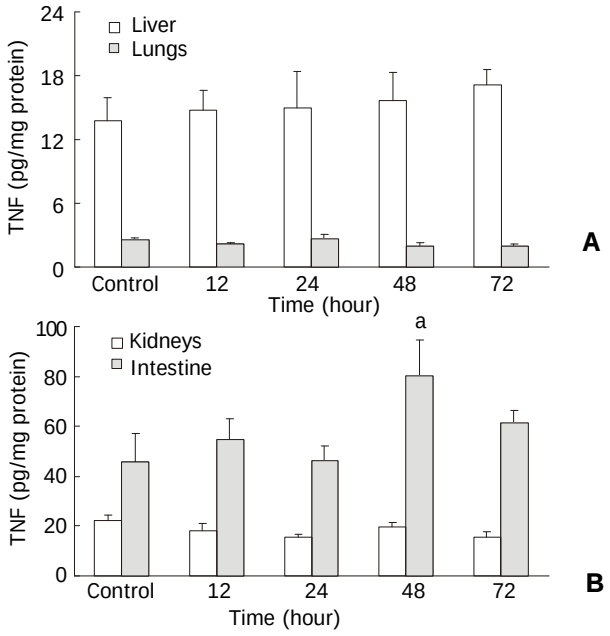

Figure 2 TNF-α protein levels in various organs after thermal injury. Values are reported as pg / mg protein. [a]$P<0.05$ as compared to the control values.

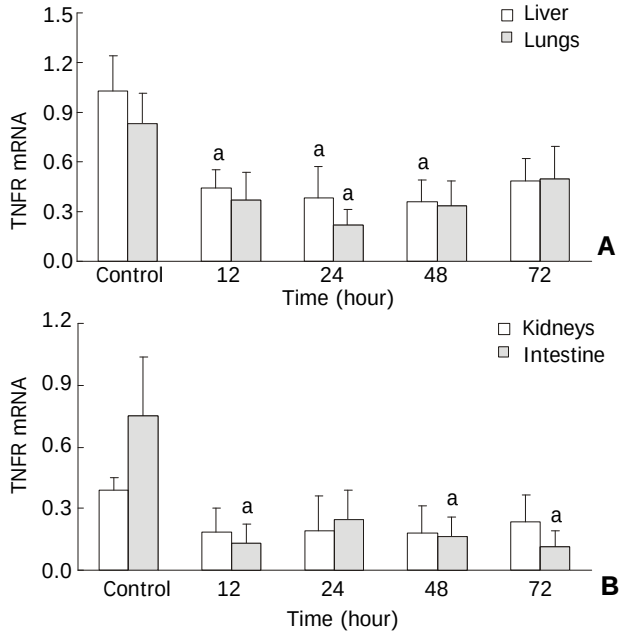

Figure 3 Semiquantitive analysis of tumor necrosis factor-α receptor-I (TNFR-I) mRNA in observed organs after thermal injury. Values are reported as the ratio of TNFR-I to β-actin signals. [a]$P<0.05$ as compared to the control values.

The ratio of TNF-α to TNFR-I mRNA (T/R ratio) in various organs was investigated in the present study as shown in Figure 4, which revealed that T/R ratio in these organs was significantly increased postburn, particularly in pulmonary tissue that was 67-fold of control values at 48 h after burn injury. T/R ratios in liver and kidneys reached at their maximums between 24 and 48 h ($P<0.05$-0.01), whereas the elevated ratio in lungs and intestine existed till 72 h postburn.

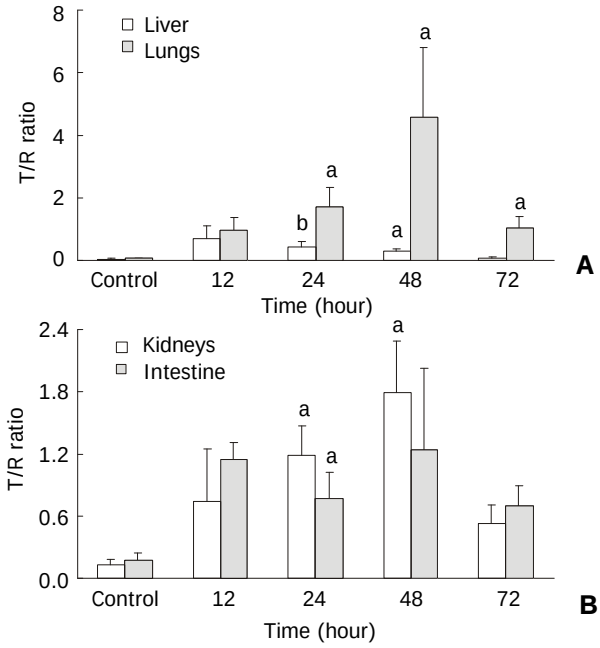

Figure 4 mRNA expression ratio of tumor necrosis factor-α to TNFR-I (T / R ratio) in observed organs after thermal injury. [a]$P<0.05$ and [b]$P<0.01$ as compared to the control values.

Organ function parameters

Serum ALT and AST levels markedly increased after scald injury, reaching a maximum between 12 and 24 h (4-5 folds of control values, $P<0.01$ compared with the controls), and remaining higher than control levels at 72 h postburn ($P<0.05$-0.01). Similarly, levels of serum CK-MB increased rapidly postburn, peaking at 12 h (control: 1 193.875±195.427 U/L, 12 h postburn: 3 626.100±410.073 U/L, $P<0.01$), and then decreasing gradually to baseline. However, serum Cr levels were not significantly different between normal and burned animals. There was a negative correlation between hepatic TNFR mRNA and serum AST levels (r=-0.3930, P=0.0121). Similar results were also obtained between renal TNFR mRNA and serum Cr levels (r=-0.9295, P=0.0001). In addition, it was noted significant correlation between T/R ratio in liver tissues and serum AST levels (r=0.4409, P=0.0056).

MPO and DAO activities

The pulmonary MPO activity that stood for the neutrophil sequestration was markedly increased after burns with the peaking at 12 h and elevating till 72 h postburn. The mucosal DAO activity, which might serve as a useful marker of intestinal injury, tended to be decreased at each time point postburn. It was noted that there was a significant correlation between pulmonary TNF-α mRNA levels and MPO activities (r=0.4289, P=0.0091), but there wasn' t between intestinal TNF-α mRNA levels and DAO activities, as well as between MPO or DAO activities and TNFR mRNA in lungs and intestine ($P>0.05$).

DISCUSSION

The role of endotoxin in the pathogenesis of septic shock and

organ failure has been well established. It is almost an opinion in unison that LPS initiates a cytokine cascade, and among these cytokines TNF-α is recognized as the pivotal one to evoke systemic responses to sepsis and injury[18]. Most of our current knowledge on the *in vivo* induction and regulation of TNF-α has been attained by measurement of serum cytokine levels. To date, only a few studies have addressed the gene regulation of the tissue-specific cytokines *in vivo*. In the current study, we investigated mRNA of TNF-α as well as its receptor and TNF-α protein expression in vital organs following severe burns. It was found that thermal injury induced a marked increase in mRNA of TNF-α. Hepatic TNF-α mRNA expression increased early and transiently with a peak at 12 h, whereas sustained elevation of TNF-α gene expression was observed in kidneys and lungs. Especially in the latter, the highest induced levels of TNF-α mRNA were found, suggesting a possible role for TNF-α in mediating pulmonary damage, which is commonly encountered in sepsis.

The investigations of Furse *et al*. have shown that TNF-α mRNA levels were increased in monocytes of trauma patients, and an increased amount of TNF-α protein was produced[19]. In the present study, we attempt to determine the association between TNF-α mRNA expression and TNF-α protein production by comparing their respective time course of exhibition. Recent studies document that cytokines exert their primary effects in a paracrine fashion at the local tissue level[20]. Andrejko *et al*. reported that the changes in TNF-α-dependent hepatic gene expression accompanied an animal model of the systemic inflammatory response syndrome correlated with intrahepatic, and not plasma concentrations of TNF-α[21]. Since circulating levels of cytokines may not appreciably reflect their true biological activity in the affected tissues[22], we determined tissue TNF-α protein expression in various organs instead of plasma levels of TNF-α. To our surprise, the results revealed that the changes in TNF-α protein did not correlate temporally with increased expression of TNF-α mRNA in vital organs. To explain the discrepancy, we offer the following possibilities. Firstly, TNF-α is short-lived in plasma[23]. There was a transient rise in TNF-α after the injection of *E. coli* or endotoxin, and TNF-α became undetectable by 4-6 h[24]. Therefore it is likely that the TNF-α response is too transient to be detected. Secondly, shed soluble TNF-α receptors may interfere with TNF-α activity in immunoassay[25]. Finally, despite of transcriptional control, TNF-α production is also regulated posttranscriptionally[26]. Thus, TNF-α protein concentration is a cesspool of complex interactions regarding binding and release from cell membranes, magnitude and kinetics of the soluble receptors and natural cytokine antagonists, and rates of catabolisms. Account of the aforementioned interfering factors, we suggest that determination of TNF-α mRNA expression may be more reliable to reflect the kinetics of cytokine at the local tissue level.

A large body of clinical and experimental evidence suggests that gut-derived endotoxemia following trauma, burns and circulatory shock could result in TNF-α production, which may play a key role in mediating subsequent septic response and systemic tissue injury[27-29]. In burn injury, however, the causative effect of locally accumulated endotoxin on TNF-α mRNA expression in various organs is still not completely understood. In the present study, it was found that endotoxin levels in the liver, spleen and lung rose markedly after thermal injury, peaking at 12 h. Endotoxin levels in liver and spleen then lowered gradually, while pulmonary endotoxin levels maintained a high level till 72 h. The temporal relationship between the kinetic changes of TNF-α mRNA and that of endotoxin levels in the visceral organs, as observed in the present study, suggests that endotoxin originated from the gastrointestinal tract appears to be one of the most potential triggers for TNF-α gene expression. Hadjimines *et al*. demonstrated that TNF-α mRNA in peritoneal exudated macrophages of mice rose fourfold after intraperitoneal administration of endotoxin[30]. Similarly, cecal ligation and puncture was shown to induce a rapid increase in hepatic and pulmonary TNF-α mRNA by 3 h, remaining high for 18 h[31]. These findings are consistent with our data suggesting that endotoxin can induce TNF-α gene expression in various tissues.

Monocytes have been demonstrated to be major producers of the elevated TNF-α seen post-trauma. In the model used here, hepatic macrophages appear to be the principal cellular source of TNF-α, as the Kupffer cell population within the liver is the largest fixed tissue macrophage population in the body. Recent study had shown that Kupffer cells were the major source of TNF-α in culture supernatants of hepatic perfusate mononuclear cells from ethanol-consuming rats injected LPS[32,33]. Additionally, blood monocytes, pulmonary macrophages and peritoneal macrophages were also possibly involved in TNF-α formation[30, 34-36], since endotoxin distributing to lung and spleen was evident after burns in the present study. Unexpectedly, correlation analysis between tissue endotoxin concentrations and TNF-α mRNA levels did not reveal any significant correlation. From that, endotoxin translocation originated from the gut may serve purely as an initial trigger. Moreover, although endotoxin translocation occur following severe burns, endotoxin may not be the sole agent responsible for the subsequent induction of cytokine, whereas a variety of factors, such as hypoxemia, shock, and necrosis tissues, may be also involved in cytokine production[37].

The majority of human cells express high-affinity receptors that mediated the biological activities of TNF-α. The potent regulatory abilities of TNF-α are transduced by two distinct cell surface receptors, namely 55 kd (TNFR-I) and 75 kd (TNFR-II) relative molecule mass[38]. In inflammation, the receptors activate both unique and synergistic responses. Studies of TNFR knockout mice have established that TNFR-I plays a predominant role in LPS-induced inflammatory diseases and mediate the lethal effects of endotoxin[39-41]. Furthermore, mice lacking TNFR-I showed resistance to endotoxin challenge[42]. Therefore, we attempted to observe the mRNA expression of this receptor in vital organs. It was found that although normal tissue expressed TNFR mRNA abundantly, with the highest level in liver, TNFR mRNA expression in various organs plummeted after burn injury. Previous study has shown that loss of monocyte surface TNFRs is an early event in the response to endotoxiemia[43]. The nadir of this response occurred 2 h after endotoxin administration, and the monocyte surface TNFR levels then returned to baseline within 6 h after endotoxin challenge. These results indicate that monocyte surface TNFR levels rapidly decrease in response to an inflammatory stimulus *in vivo* and also rapidly normalize when the stimulus is no longer present. Thus, the significant and sustained decrease in TNFR mRNA levels observed in present study may represent an ongoing condition of systemic inflammation. Our findings are consistent with the recent observations of Calvano *et al*., who reported a significant and sustained (up to 4 days) decrease in monocyte surface TNFR levels in non-surviving patients with sepsis[44]. On the other hand, the extracellular domains of the TNFR apparently are shed from the cell surface in response to many of the same inflammatory stimuli that are known to induce TNF-α production[25]. Our data may thus imply that a dual deregulation in TNFR reflects decreased expression of TNFR on the cell surface concomitant to decreased cell shedding of TNFR.

The shedding of TNFR and resultant acute decrease in the number of TNFR on the cell surface may serve to transiently desensitize cells, thereby providing a mechanism for inhibition of TNF-α activity. This process may have additional

significance, as released soluble receptors (sTNFR) may inhibit TNF-α bioactivity by binding to the molecule and preventing ligand binding to the cellular TNFR. *In vitro* studies demonstrated that sTNFR in critical ill patients and in experimental endotoxemia are sufficient to neutralize the bioactivity associated with TNF-α concentrations observed in mild inflammation, whereas such levels are inadequate to neutralize the cytotoxicity associated with TNF-α levels seen in overwhelming or lethal sepsis[45]. Thus, imbalance of TNF-α to TNFR may reflect more than one dysfunction in monocyte TNF-α production and regulation, which may contribute to overwhelming activation of the inflammation cascade.

Pellegrini *et al*. reported that monocyte membrane associated TNF-α (mTNF-α) elevated and TNFR shedding decreased in trauma patients[46]. There was a correlation between increased mTNF-α and decreased TNFR shedding to increased MODS score, but this lacked specificity. However, when mTNF-α and TNFR are assessed as the mTNF-α/TNFR ratio, an increased ratio correlated with higher specificity to development of organ failure. In this paper, we investigated for the first time the ratio of TNF-α to TNFR mRNA (T/R ratio) in various tissues. Our results revealed that tissue T/R ratio was significantly increased postburn. It is impressive that the magnitude of T/R ratio elevation was much higher than TNF-α (10-60 folds in T/R ratios *vs.* 8-10 folds in TNF-α). Combined with the persistently elevated levels found in tissues, this suggests that T/R ratio would better reflect the inflammatory status than TNF-α or TNFR alone. Moreover, it was noted that T/R ratio in liver tissue and serum AST level were significantly correlated, suggesting that this kind of cytokine regulation appears to be in sufficient in counteracting overwhelming sepsis, and the imbalance of TNF-α to TNFR might be involved in liver dysfunction. Hence, assessment of T/R ratio might provide a more sensitive indicator of organ damage or even outcome.

In this study, it was found that significant correlation between T/R ratio in liver tissues and serum AST levels. Similar results were also obtained between pulmonary TNF-α mRNA and MPO activities, whereas there were highly negative correlations between hepatic TNFR mRNA and serum AST levels, and between renal TNFR mRNA and serum Cr levels, suggesting imbalance of TNF-α to TNFR might be involved in the development of multiple organ damage following burns. Since TNF-α has been shown to be a pivotal cytokine of sepsis and MODS induced by endotoxin translocation post-trauma, we have evaluated the protective effect of anti- TNF-α monoclonal antibody (MoAb) for vital organs following intestinal ischemia-reperfusion or thermal injury. In rat intestinal ischemia-reperfusion model, prophylactic treatment with anti-TNF-α MoAb resulted in striking decreases in systemic TNF-α levels, and associated with such a neutralization effect were amelioration of hypotension and distant organ injury, including liver, kidneys, and lungs, as well as significant improvement in survival[47-49]. Similarly, early treatment with anti- TNF-α MoAb could attenuate endotoxemia-mediated multiple organ damage resulting from severe burns, which might be associated with the down-regulation effects of tissue lipopolysaccharide binding protein (LBP) gene expression by use of MoAb[50]. Moreover, a recent series of investigations have shown that recombinant sTNFR protected animals from death caused by endotoxin and gram-negative bacteria, and these promising experimental results prompted large scale randomized clinical trials[51]. Additionally, TNFR-deficient mice were resistant to endotoxin shock and sepsis induced by cecal ligation and puncture[42, 52]. Thus, appropriate use of agents to interrupt the chains of cytokine cascade might provide more balanced cytokine response, which may be beneficial to the host.

In summary, the present study demonstrated that burn injury *per se* could result in gut-derived endotoxin translocation that was mainly to the liver, spleen and lungs. The translocated endotoxin might be responsible at least in part, for the up-regulating of TNF-α and down-regulating of TNFR-I mRNA expression in these organs. The expression imbalance between TNF-α and TNFR might play a part in the development of multiple organ damage following burns.

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Edited by Zhu L