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Original Article

Activating transcription factor-3 protects against lipopolysaccharide-induced acute liver inflammation and reduces mortality in mice

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ABSTRACT

Objectives: Sepsis is a complex clinical problem that is caused by excessive secretion of proinflammatory and inflammatory mediators; these factors can result in pathophysiological hematological abnormalities and multiple organ failure. To date, long-term investigations into septic pathophysiology and treatment remain a major challenge. The aim of this study was to assess possible role of activating transcription factor 3 (ATF3) in lipopolysaccharide (LPS)-induced acute liver inflammation and mortality in mice.
Materials and Methods: ATF3-deficient (knock out, KO) mice and wild type (WT) mice were injected with LPS to induce endotoxemia and scored for their survival rate. Automatic multiparameter blood cell counting was used for white blood cell (WBC) analysis, and liver histology was evaluated for acute hepatic injury.
Results: At 3 to 6 hours after LPS (50 mg/kg, intraperitoneally) challenge, AFT3-KO mice were found to show a significant elevation in circulating WBCs. This was accompanied by persistent elevation of WBCs at 24 hours after LPS challenge compared to WT mice. The histopathological changes to the livers of the ATF3-KO mice after LPS challenge consisted of prominent inflammation with more severe structural disruption compared to WT mice. The above findings are in agreement with the decreased survival rate of AFT3-deficiency mice after LPS challenge compared to WT mice.
Conclusion: We demonstrated the beneficial role of ATF3 during LPS-induced endotoxemia in mice. ATF3 would seem to be a potential therapy regimen when treating LPS-induced endotoxemia.

1. Introduction

Sepsis is a serious clinical problem worldwide and its incidence and mortality rates remain high. In addition, severe sepsis is the most common cause of mortality in intensive care units [1]. The sepsis syndrome is a systemic inflammatory response caused by excessive secretion of proinflammatory mediators that results in hypotension, hematological abnormalities, and multiple organ failure [2]. Multiple organ dysfunction is the most critical determinant of prognosis in endotoxemia and sepsis [3]. Early organ dysfunction in sepsis is a result of cellular activation by bacterial products such as lipopolysaccharide (LPS), the complex interaction of inflammatory cytokines and the presence of hemodynamic abnormalities that causes reduced oxygen delivery. Early organ dysfunction due to sepsis is generally not related to cell death. Many studies have indicated that cells undergo a metabolic shutdown to protect against tissue injury in response to the early phases of infection and sepsis [4]. High levels of reactive oxygen species can occur during early sepsis and these damage mitochondria and other organelles [5]. Human hepatocytes have abundant mitochondria and as a result it is reasonable that, during sepsis, liver injury occurs before lung or heart injury. Successful management of sepsis needs therapies that disrupt the key pathways of sepsis. However, controversy remains concerning the relative roles of the various pathways involved in sepsis.

Toll-like receptors (TLRs) are membrane-bound pattern recognition receptors that detect a variety of microbial specific motifs in

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order to elicit an innate immune response [4]. TLR4 recognizes LPS, a major outer membrane component of Gram-negative bacteria and does this in cooperation with its co-receptor MD2 [5]. The binding of LPS to TLR4-MD2 leads to activation of various downstream signaling pathways [6]. In macrophages, activating transcription factor 3 (ATF3) has been shown to be induced by the TLR4 ligand, LPS, using human peripheral blood mononuclear cells [7]. ATF3 belongs to the ATF/cAMP responsive element-binding protein family of transcription factors. Previous studies have revealed a role for ATF3 in various negative feedback mechanisms that control immune cell activation including in macrophages [8]. In macrophages, ATF3 has been demonstrated to participate in the negative feedback loop that modulates TLR4-stimulated inflammatory responses as previously mentioned [9]. The proof that ATF3 negatively regulates the transcription of nuclear factor (NF)-κB-dependent genes was provided many years ago [10]. Recently it has been suggested that ATF3 contributes to the negative regulation of inflammatory cytokine gene expression by recruiting histone deacetylase-1 to ATF/NF-κB binding sites in order to curtail the production of inflammatory cytokine genes and that this prevents damaging inflammatory responses after acute kidney injury [11]. Promising findings have also suggested that the TLR and ATF3 pathways act as host defense mechanisms against invading pathogens. Indeed, TLRs are able to activate various complicated cellular signaling pathways that induce antiviral and antibacterial reactions [12]. ATF3 may suppress the later phases (>16 hours) of TLR-stimulated IL-6 production in response to LPS treatment [9]. ATF3-deficient (knockout, KO) mice have recently been reported to exhibit better protection against murine cytomegalovirus infection [13] and to show moderate hyper-responsiveness to asthma and airway inflammation. Thus, ATF3 would seem to mediate inflammatory responses by restricting the expression of different cytokines in a number of biologically relevant disease models. Given the evidence that LPS significantly elevates ATF3 protein expression in macrophages, and that ATF3 is a negative transcriptional regulator of TLR-mediated cytokine expression, it may be postulated that ATF3 acts as a crucial regulator of immunity against invading pathogens and inflammatory diseases.

ATF3-KO mice show no developmental deformities and manifest phenotypes only when faced with stressors [14]. ATF3-KO mice, when challenged with intraperitoneal (ip) LPS, show a considerably raised morbidity and mortality due to their increased susceptibility to endotoxic shock as compared to wild type controls (data not show). In this study, we have demonstrated the beneficial effects of ATF3 on the LPS-induced endotoxemic mortality and acute liver inflammation in mice. ATF3 maintains the circulating WBC balance and protects against hepatic injury. We also explored how ATF3 affects the survival rate of mice after LPS challenge. It can be concluded that ATF3 exerts a beneficial effect in terms of mortality and its presence helps to limit liver dysfunction during sepsis.

2. Materials and methods

2.1. Animals

Eight-week-old male mice weighing 20 to 25 g were bred and housed under a 12-hour light/dark cycle at Tzu Chi University's Animal Center. The ATF3-KO mice were kindly provided by Dr. Tsonwin Hai. C57BL/6J (B6) mice (age 8 to 12 weeks) were purchased from Lasco Laboratories (Taipei, Taiwan) and provided by Professor Sung-Ho Chen (Tzu Chi University, Hualien, Taiwan). Food and water were provided *ad libitum*. All experimental procedures and protocols were approved by the Animal Care and Use Committee of the University of Tzu Chi and all animal care and experiments were performed according to the *Guide for the Care and Use of Laboratory Animals* (NRC, USA, 1996).

2.2. Mouse model for endotoxemia

In order to evaluate the beneficial effects of ATF3 in terms of sepsis-related liver injuries, animals were divided randomly into four groups: Groups 1 and 2 (Control): wild type (WT) mice and ATF3-KO mice, respectively, which received an ip injection of normal saline as solvent; and Groups 3 and 4 (LPS): WT mice and ATF3-KO mice, respectively, that received ip LPS (50 mg/kg) in normal saline. Each group was subdivided into four subgroups ($n = 4$) and these subgroups were observed and examined at 0, 3, 6, 12, and 24 hours after LPS challenge. Blood (0.5 mL) was sampled from the right atrium, transferred to 1.6 mL tubes, centrifuged at 8000*g* at 4 °C for 10 minutes and the plasma samples were frozen at −80 °C for further analysis. The livers of the mice were harvested from the animals sacrificed at 24 hours after LPS challenge and these were then studied further.

2.3. Whole blood WBC and LYM measurement

Immediately after the drawing of the whole blood samples (100 μL), these were analyzed on an automatic multi-parameter blood cell counter (KX-21; Sysmex, Kobe, Japan).

2.4. Histopathology

Liver specimens were fixed in 4% paraformaldehyde (pH 7.6; Sigma Chemicals, St Louis, MO, USA) overnight at room temperature. After washing with tap water for 10 minutes, specimens were dehydrated through a graded ethanol series (75% for 30 minutes, 80% for 60 minutes, 95% for 3 hour, and 100% for 30 minutes, purchased from Sigma Chemicals) and then embedded in paraffin. Sections of 1-μm thickness were placed on glass slides and dried at 37 °C for overnight. The slides were then rinsed in nonxylene solution (three times for 5 minutes each time) to remove the paraffin. After rehydration (ethanol at 100% twice for 3 minutes, at 95% for 1 minute, and at 75% for 1 minute), the specimens were processed in hematoxylin for 3 minutes, and then stained with eosin Y for 45 seconds. All sections were then incubated in nonxylene solution (Sigma Chemicals) for 6 minutes. After mounting with a cover slip, each of the specimens was examined under a light microscope (Leica Microsystems, Wetzlar, Germany).

2.5. Measurement of mice survival rate

WT and ATF3-deficiency mice were used to evaluate survival rate. Using an *in vivo* endotoxemic model, the mice received doses of LPS ip that should be lethal to approximately 80% of the animals tested (LD_{80} dose). The mice were randomly divided into four groups as described above. After solvent ($n = 8$) or LPS ($n = 20$) challenge, the survival rate of each group was calculated at 0, 3, 6, 12, and 24 hours.

2.6. Drugs

Sodium chloride, *E coli* LPS (serotype 0127:B8), and Tween 80 were purchased from Sigma Chemicals. *E coli* LPS was diluted in normal saline to a concentration of 10 mg/ml before use.

2.7. Statistical analysis

Experimental data are presented as means ± SEM. One-way ANOVA was used to determine any significant differences.

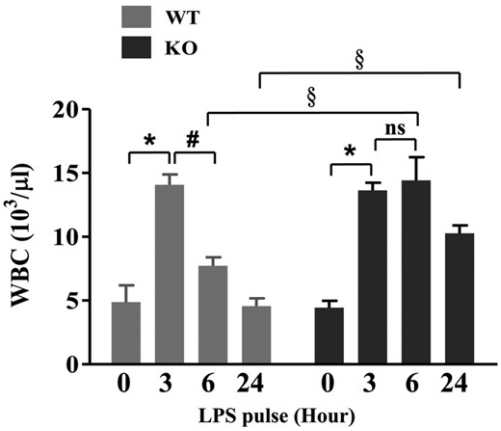

Fig. 1. Effects of ATF3 deficiency in mice on LPS-induced changes in physiological parameters after LPS challenge. Change in the physiological parameters of white blood cells (WBCs) in whole blood at 0, 3, 6, and 24 h for various different groups of wild-type or ATF3-deficient mice that received an injection of control saline or LPS saline (50 mg/kg, ip). The data represent means. $^{*}p < 0.05$ indicates a significant difference from the respective control, and $^{\#\&\S}p < 0.05$ indicates a significantly different from the respective LPS-treated group.

Measurements at a single time point were compared by Student unpaired t test. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Changes in physiological parameter upon LPS challenge in mice

Any changes in circulatory parameter WBC number during the mice model of LPS-induced endotoxemia were assessed at 0, 3, 6, and 24 hours. In the ATF3-KO mice, circulating WBC numbers were enhanced at 3 hours after LPS challenge and reached a maximum 6 hours after LPS challenge (Fig. 1). WBC numbers remained substantially increased until 24 hours (Fig. 1). However, when WT mice were investigated, although there as a significant increase in WBC numbers at 3 hours after LPS challenge, it was found that this initial increase in wild-type circulating WBC levels had already begun to decrease at 6 hours after LPS challenge (Fig. 1). These results indicate that during the early and later stages of inflammation, ATF3 helps to reduce the LPS-induced increase in WBC numbers (Fig. 1).

3.2. ATF3 prevents LPS-induced endotoxemic liver injury

To evaluate the therapeutic relevance of these findings, we examined the pathological changes to the liver caused by LPS administration and whether ATF3 prevents LPS-induced endotoxemic liver injury. Generally, near the central vein, the duct became filled with polymorphonuclear neutrophils (PMNs), and was found to have an abnormal structure at 24 hour after LPS challenge (Fig. 2). However, in the ATF3-KO mice, it was found that there was an accumulation of macrophages such as Kupffer cells and that there was PMN sequestration within the liver. In general, the severity of the liver damage among the ATF3-deficient mice after LPS challenge was much greater than that found in wild type mice after LPS challenge (Fig. 2).

3.3. ATF3 improves the survival rate of LPS-induced endotoxemic mice

Overall, 10% of ATF3-deficient mice that were injected with ip 50 mg/kg LPS survived until 24 hours (Fig. 3). However, the wild-type mice showed a statistically significant improvement in the

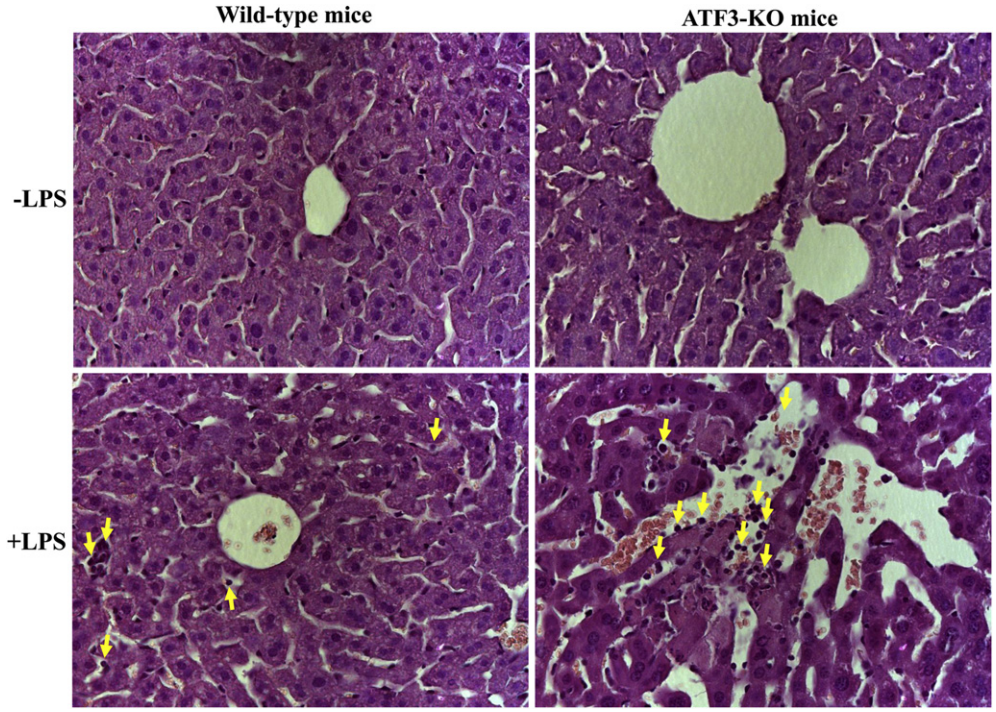

Fig. 2. ATF3 prevented LPS-induced severe liver injury in mice. Representative sections of liver tissues stained with hematoxylin for cell nucleus, and eosin Y dye for the cytoplasm obtained using the mice model of endotoxemia. The control section shows a normal central vein, normal liver structure, and few neutrophils. However, in ATF3-deficient mice, when the liver was examined at 24 h after LPS challenge (ip, 50 mg/kg), structural disruption and filling of the ducts with numerous neutrophils can be seen. Numerous activated macrophages (arrows) with PMNs can be seen to have accumulated near the central vein.

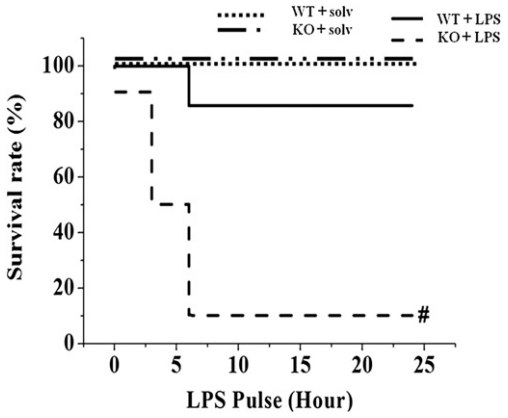

Fig. 3. ATF3 increases mice survival rate after LPS-induced endotoxemia. After intraperitoneal (ip) treatment with 50 mg/kg, it was found that wild-type mice showed a significant and time-depend increase in survival rate compared to ATF3-deficient mice. This suggests that ATF3 has a beneficial effect on survival during endotoxemia. $^{\#}p < 0.05$, significantly different from the LPS-treated group. *n* indicates the number of experiments.

survival compared to the wild type mice at 3, 6, 12, and 24 hours after LPS challenge (Fig. 3). These results support the hypothesis that ATF3 provides a survival advantage in the presence of LPS-induced endotoxemia.

4. Discussion

Sepsis is frequently fatal and has been found to kill 20% to 50% of severely affected patients in the United States [15]. Moreover, sepsis significantly lowers the quality of life of those who survive [16]. Gram-negative bacteria are the group of major pathogens causing sepsis [17]. For this reason, we chose LPS to induce endotoxemia in the mice. The chance of organ failure increases over time and is an additive contributor to mortality; this remains consistent across patients of different races and sexes [17]. The pathophysiology of sepsis is supposed to be associated with a devastating or excessive host response involving dysregulated inflammation. Septic shock induced organ hypoperfusion, which progresses to multiple organ dysfunction; this is clinically characterized by liver, pulmonary, cardiovascular, renal, and gastrointestinal dysfunction [18].

According to Consensus Definition of SIRS/Sepsis by the American College of Chest Physicians and the Society of Critical Care Medicine [19], systemic inflammatory response syndrome can be seen following a wide variety of insults and occurs when more than one of the following clinical signs are present: (1) a body temperature $>38\,^{\circ}C$ or $<36\,^{\circ}C$; (2) a heart rate >90 beats/minute; (3) tachypnea, manifested by a respiratory rate >20 breaths/minute, or hyperventilation, as indicated by a $PaCO_2$ of >32 mmHg; and (4) a change in the WBC count, such as a count $>12 \times 10^9$/L, a count less than 4×10^9/L, or the existence of more than 10% immature neutrophils ("bands"). A change in WBC count is likely to be an early identification of an inflammatory response to an infection. In our study, after LPS challenge, ATF3 deficient mice showed a prolonged elevation of their WBC count compared to the WT mice. This implies that ATF3 is able to suppress SIRS during the early phase of endotoxemia. The result corresponded with research last year showing that, during sepsis, high ATF3 is found in patients who are admitted to an intensive care unit [20].

Sepsis represents a systemic inflammatory response accompanied by an infection. Severe sepsis is defined as sepsis combined with organ dysfunction. Retrospective clinical studies have noted that the main risk to survival is not the underlying disease, or even a particular complication, but is rather the development of progressive failure across several interdependent organ systems [21]. Acute or chronic liver failure and severe sepsis show a parallel degree of cellular immune depression [22]. However, there remains an inadequate pathophysiological basis for this conclusion and inclusion of sepsis as a precipitating episode to acute liver failure remains questionable [23]. Having examined the liver's response to sepsis, the present study found that ATF3-deficiency worsens LPS-induced liver injury (Fig. 2). Among the nonsurvivors with liver failure, High mobility group box 1 (HMGB-1) levels have been found to be much higher than among survivors [18] and HMGB-1 level increased with the number of organ failures. HMGB-1 has recently been identified as a cytokine mediator of lethal systemic inflammation [24]. Heme oxygenase-1 (HO-1) is important in the liver and other organs and is required to restore cellular homeostasis in response to infection and sepsis [25]. Thus, additional studies are required to address whether there is a crosstalk between ATF3 and HMGB-1 and/or HO-1, and whether this is a factor in the pathogenic progression of the inflammatory disorders that lead to liver failure.

In addition to protecting against liver injury in a septic animal model, our findings also demonstrated that ATF3 is capable of improving the survival of endotoxemic mice (Fig. 3). Therefore, it seems likely that ATF3 participates in a protective molecular pathway during sepsis. Understanding this role has important implications in relation to the pathophysiology of sepsis and further advances in this area may help to diminish morbidity and mortality due to sepsis.

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