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Review Article

ROLE OF TOLL-LIKE RECEPTORS IN THE DEVELOPMENT OF SEPSIS

Hironori Tsujimoto,* Satoshi Ono,* Philip A. Efron,† Philip O. Scumpia,† Lyle L. Moldawer,† and Hidetaka Mochizuki*

**Department of Surgery, National Defense Medical College, 3-2 Namiki, Tokorozawa, Japan; and †Department of Surgery, University of Florida College of Medicine, Gainesville, Florida*

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ABSTRACT—The outcome of sepsis and septic shock has not significantly improved in recent decades despite the development of numerous drugs and supportive care therapies. To reduce sepsis-related mortality, a better understanding of molecular mechanism(s) associated with the development of sepsis and sepsis-related organ injury is essential. There is increasing evidence that Toll-like receptors (TLRs) play a key role in the mediation of systemic responses to invading pathogens during sepsis. However, the role of TLRs in the development of sepsis and in sepsis-related organ injury remains debatable. In this review, we focus on the biological significance of TLRs during sepsis. Medline was searched for pertinent publications relating to TLRs, with emphasis on their clinical and pathophysiological importance in sepsis. In addition, a summary of the authors' own experimental data from this field was set in the context of current knowledge regarding TLRs. In both animal models and human sepsis, TLRs are highly expressed on monocytes/macrophages, and this TLR expression may not simply be a ligand-specific response in such an environment. The fact that TLR signaling enables TLRs to recognize harmful mediators induced by invading pathogens may be associated with a positive feedback loop for the inflammatory response among different cell populations. This mechanism(s) may contribute to the organ dysfunction and mortality that occurs in sepsis. A better understanding of TLR biology may unveil novel therapeutic approaches for sepsis.

KEYWORDS—Pathogen-associated molecular pattern, systemic response, innate immunity, organ injury

INTRODUCTION

Sepsis affects more than 500,000 patients in the United States annually, and its incidence continues to increase (1). Despite continuous progress in the development of antibiotics and other supportive care therapies, sepsis remains a leading cause of morbidity and mortality in the intensive care unit (2). The outcome of sepsis and septic shock has not improved significantly in the past 50 years (1). It is apparent that the clinical trials of anti-inflammatory agents and anticytokine therapies have, in general, failed (3, 4). These disappointing results may be partially due to the lack of understanding of the molecular mechanisms associated with the development of sepsis and sepsis-related organ injury. This review focuses on the role of Toll-like receptors (TLRs) during sepsis and their association with sepsis and sepsis-related organ injury.

Medline was searched for publications relating to TLRs, with emphasis on their clinical and pathophysiological importance in sepsis. In addition, a summary of the authors' own experimental data from this field was set in the context of current knowledge regarding TLRs and their ligands.

SEPSIS AND SEPSIS SYNDROME

Historically, sepsis has been defined as a clinical syndrome consisting of a severe infection with fever, leukocytosis or leucopenia, elevated cardiac output, and reduced systemic vascular resistance (5). In 1991, the American College of Chest Physicians/Society Critical Care Medicine Consensus Conference altered the definition of sepsis to the systemic response to a microbial infection. Recently, the term "sepsis" has been supplanted by the term "sepsis syndrome" to include patients manifesting the physiological and metabolic responses associated with sepsis but without a documented severe infection (6). Sepsis/sepsis syndrome is a complex clinical syndrome that results in both the activation and dysfunction of the innate and adaptive branches of the immune system. The systemic administration of bacterial LPS is known to recapitulate many of the clinical features of septic shock (7), including the early release of a number of proinflammatory mediators. However, there are a number of critical differences between LPS- and bacteria-induced septic shocks, including the pattern of cytokine expression. Thus, the LPS and other classical models of sepsis may be limited in their applications because they do not properly reflect all forms and presentations of sepsis in a clinical setting. This supports the proposal that other components, including other bacterial molecular elements, may contribute to the development of sepsis (8).

TOLL-LIKE RECEPTORS AND THEIR LIGANDS

The innate immune system is phylogenetically conserved and present in almost all organisms (9). The mechanisms used by the innate immune system to recognize nonself have been elucidated only recently, and the discovery of TLRs has revolutionized the field of microbial pathogenesis and human immunology. The Toll-signaling pathway was initially described in *Drosophila* for its role in dorsal–ventral patterning

Address reprint requests to Hironori Tsujimoto, MD, PhD, Department of Surgery, National Defense Medical College, 3-2 Namiki, Tokorozawa 359-8513, Japan. E-mail: Tsujiflorida@aol.com.

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during embryogenesis. Medzhitov et al. (10) previously demonstrated that human TLR-4 was the principal receptor for LPS that mediates the activation of nuclear factor–κB and the synthesis of proinflammatory cytokines. In general, TLRs are a family of transmembrane receptors consisting of an extracellular leucine-rich repeat domain that interacts with relevant pathogen-associated molecular patterns and an intracellular Toll/IL-1 receptor domain, which is involved in signaling. To date, at least 11 human TLRs have been identified, and each is known to detect a specific pathogen-associated molecular pattern and have a specific intracellular signaling pathway. Toll-like receptors 1, 2, 4, 5, and 6 mainly recognize bacterial products, whereas TLR-3, TLR-7, and TLR-8 are specific for viral detection. Toll-like receptor 9 seems to be involved in both microbial and viral recognition (Table 1).

TOLL-LIKE RECEPTOR EXPRESSION DURING SEPSIS

Increasing experimental and clinical evidence demonstrates the importance of TLR expression on various cell types during sepsis. Our laboratory (11) and Armstrong et al. (12) reported that monocytic expression of TLR-2 and TLR-4 in septic patients was significantly up-regulated compared with the expression in healthy individuals. In addition, we have demonstrated that the expression of TLR-2 and TLR-4/MD-2 in hepatic and splenic macrophages is significantly up-regulated in mice with experimental peritonitis induced by cecum ligation and puncture (CLP) (Fig. 1A) (13). Williams et al. (14) also demonstrated that TLR-2 and TLR-4 mRNA expression in the lungs and liver of CLP mice were significantly up-regulated as compared with that in sham-operated mice, which occurred as early as 1 h after the onset of peritonitis. Andonegui et al. (15) reported that expression of TLR-4, particularly on alveolar endothelial cells, played an important role in neutrophil recruitment into the lungs after LPS administration, suggesting that TLRs on nonimmune cells and immune cells may be involved in tissue injury during sepsis. Thus, both experimental models of sepsis and septic human patients display significantly up-regulated TLR expression in various organs (Table 2).

Viemann et al. (16) demonstrated that TLR-4 showed no remarkable changes in neonates with sepsis as compared with healthy individuals, and Renshaw et al. (17) indicated that TLR expression declined with age. In addition, our laboratory reported differential regulation of TLRs during sepsis between

TABLE 1. **Exogenous and endogenous ligands for TLRs**

	Ligands	
TLR	Exogenous	Endogenous
1	Triacyl lipopeptide*	
2	Peptidoglycan	Necrotic cells
	Lipoprotein	HSPs (HSP-60, HSP-70, Gp-96)
		Biglycan
3	Double-stranded RNA	Self-messenger RNA
4	LPS	Extra domain A–containing fibronectin
	Taxol (mouse TLR-4 only)	Fibrinogen
		Polysaccharide fragments of heparan sulfate
		Oligosaccharides of hyaluronic acid
		β-Defensin 2
		Oxidized low-density lipoprotein
		HSPs
		Surfactant protein A in the lung epithelium 1
		Neutrophil elastase
		High mobility group box 1 protein
		Biglycan
5	Flagellin	
6	Diacyl lipopeptide*	
7	Single-stranded RNA	
8	Single-stranded RNA	
9	Unmethylated CpG DNA	Chromatin-IgG complex
10	Unknown	
11	Uropathogenic *Escherichia coli*	

Ig indicates immunoglobulin; TLR, toll-like receptor; HSP, heat shock protein; CpG, deoxy-cystidylate-phospate-deoxy-guanylate.

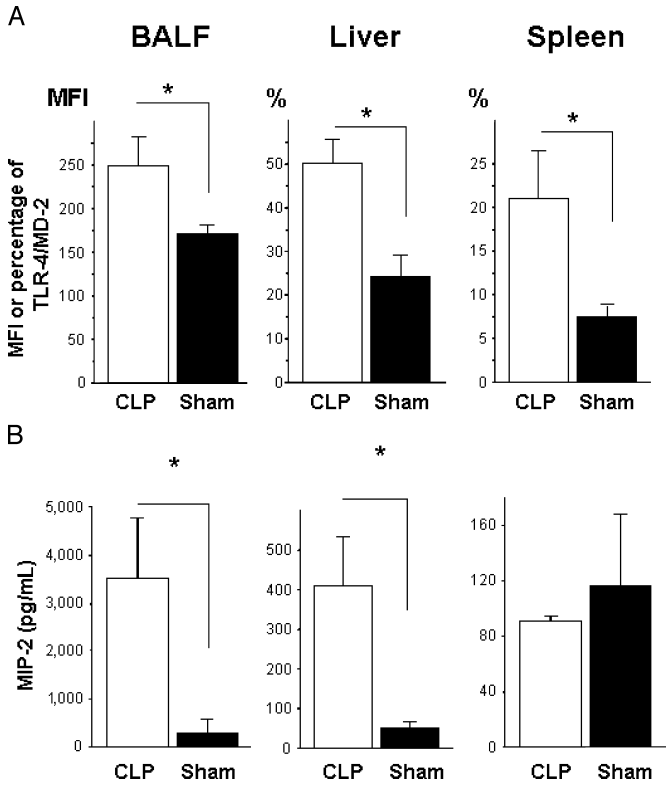

FIG. 1. **Toll-like receptor 4/MD-2 expression on liver and splenic macrophages and BALF cells, and MIP-2 production by LPS-induced MNC and BALF cells**. The expression of TLR-4/MD-2 on liver and splenic macrophages was significantly increased in CLP mice compared with sham-operated mice. Similarly, the MFI of TLR-4/MD-2 on BALF cells was significantly enhanced in CLP mice (A). LPS-induced MIP-2 production by BALF cells and liver MNC from CLP mice were significantly increased, whereas there was no difference in LPS-induced MIP-2 production by splenic MNC between CLP and sham-operated mice (B). All data are mean ± SEM. *$P < 0.05$ versus sham-operated mice. MFI, mean fluorescence intensity; MNC, mononuclear cells. n = 7 per group. Adapted from *Shock*. 2005;23:39–44.

men and women (18). Thus, it should be noted that there is a critical difference in TLR regulation and baseline TLR expression depending on age and sex in patients with sepsis.

EXPRESSION OF TOLL-LIKE RECEPTORS AND THEIR RESPONSE TO SPECIFIC LIGANDS UNDER NONSEPTIC CONDITIONS

In vitro studies have demonstrated that preexposure to LPS reduces responsiveness to subsequent LPS challenges. This phenomenon has been designated as LPS tolerance. LPS tolerance has also been observed in *in vivo* animal models, with a decreased response and protection from lethality in response to a secondary stimulation with a sublethal dose of LPS. Nomura et al. (19) concluded that one of the mechanisms responsible for hyporesponsiveness to LPS might be the down-regulation of TLR-4/MD-2 expression in purified murine peritoneal macrophages. Indeed, we obtained similar results using murine bone marrow–derived dendritic cells (20). On the other hand, Bihl et al. (21) reported that transgenic mice having several copies of TLR-4 showed an enhanced immune response to LPS, suggesting a good correlation between the level of TLR-4 mRNA expression and sensitivity to LPS both *in vitro* and *in vivo*. Paterson et al. (22) reported that thermal injury augments TLR-2 and TLR-4 expression and primes the innate immune system for enhanced TLR reactivity, resulting in LPS-induced mortality. Motegi et al. (23) have demonstrated that human peripheral blood mononuclear cells (PBMCs), which show up-regulated TLR-4 expression in monocytes after IL-12 stimulation, show augmented TNF-α production after subsequent LPS stimulation, and they conclude that this phenomena may be responsible for the generalized Shwartzman reaction. Thus, TLR-4 regulation may be associated with the extent of the biological response to subsequent TLR ligand stimulation under such conditions. One possible mechanism for regulation of TLR-4 expression in monocytes/macrophages involves proinflammatory cytokines such as interferon-γ and TNF-α (24), and an interferon-γ–responsive element was found in the promoter region of the gene encoding TLR-4 (25).

TABLE 2. **TLRs expression during septic condition**

Organs	TLR	Change of expression	Reference/s
Lung	TLR-2 mRNA	Up-regulated	14, 80
	TLR-4 mRNA/protein	Up-regulated	14, 80
Liver	TLR-2 mRNA/protein	Up-regulated	13, 14
	TLR-4 mRNA/protein	Up-regulated	13, 14
	TLR-9 protein	Up-regulated	81
Spleen	TLR-2 protein	Up-regulated	13
	TLR-4 protein	Up-regulated	13
Kidney	TLR-4 protein	Up-regulated	29
Intestine	TLR-2 mRNA	Up-regulated	82
	TLR-4 mRNA	Up-regulated	82
Peripheral blood	TLR-2 protein	Up-regulated	16, 33
	TLR-4 protein	Up-regulated	30, 33
	TLR-9 protein	Up-regulated	83

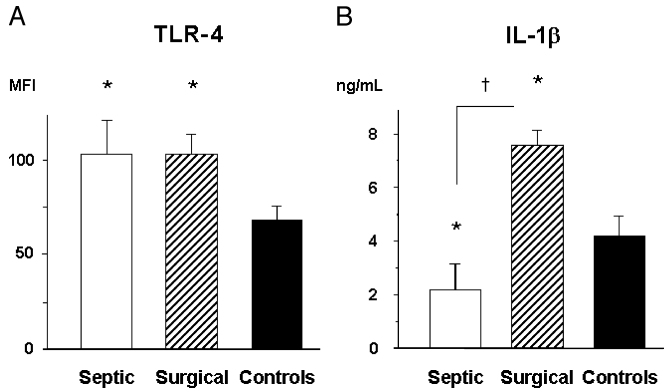

FIG. 2. **Toll-like receptors 2, 4, and CD14 expression on peripheral blood monocytes, and LPS-induced IL-1β production**. A, $CD14^+$ monocytes from both septic (n = 15) and surgical patients (n = 34) showed significantly increased expression of TLR-4 compared with healthy controls (n = 13), although no significant difference in TLR-4 expression was observed between $CD14^+$ monocytes from septic and surgical patients. B, Peripheral blood mononuclear cells from septic, surgical, and control patients were isolated, and 1.0×10^6 PBMCs were incubated in the presence of 1 μg/mL of LPS for 24 h. The supernatants were collected, and IL-1β concentrations were measured by enzyme-linked immunosorbent assays. All data are mean ± SEM. $^*P < 0.05$ compared with the controls; $^\dagger P < 0.05$ compared with the surgical patients. Septic patients were diagnosed with sepsis due to an intra-abdominal infection, and surgical patients had gastrointestinal cancer and underwent nonseptic elective surgery. MFI indicates mean fluorescence intensity. Adapted from *Clin Immunol.* 2006;119:180–187.

EXPRESSION OF TOLL-LIKE RECEPTORS AND THEIR RESPONSE TO SPECIFIC LIGANDS DURING SEPSIS

In contrast to the resting state and nonseptic condition, it remains unclear whether TLR expression may be associated with the response after exposure to TLR-specific ligands during sepsis. It is well known that peripheral blood monocytes isolated from septic patients synthesize and/or secrete reduced quantities of proinflammatory cytokines after *ex vivo* LPS stimulation (26–28) regardless of their up-regulated TLR expression in both experimental and human sepsis (29–32). What determines such differential responses during sepsis? To elucidate this, we investigated whether LPS-induced chemokine (macrophage inflammatory protein 2 [MIP-2]) is produced by bronchoalveolar lavage fluid (BALF), liver, and spleen cells from CLP and sham-operated mice. Cecal ligation and puncture mice showed significantly increased TLR-4 expression on BALF cells and on macrophages of the liver and spleen as compared with sham-operated mice (13) (Fig. 1A). We demonstrated that LPS-induced MIP-2 production by BALF and liver mononuclear cells from CLP mice was significantly increased, although there was no difference in splenic MIP-2 production between CLP and sham-operated mice (Fig. 1B). In the human study, we demonstrated that PBMCs from the septic patients, having up-regulated TLR-4 expression, showed significantly reduced IL-1β production after LPS exposure as compared with healthy individuals. In contrast, PBMCs from patients after a nonseptic elective surgical operation who had up-regulated TLR-4

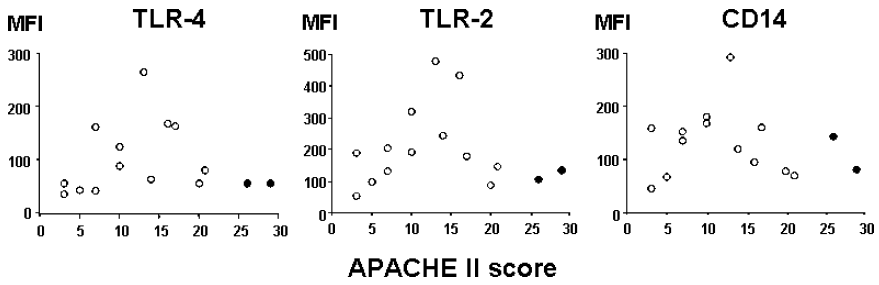

FIG. 3. **Relationship between APACHE II score and TLR-2, TLR-4, and CD14 expression in septic patients**. Acute physiology and chronic health evaluation II score showed no correlation with TLR-2, TLR-4, and CD14 expression on peripheral blood monocytes in septic patients. ○ indicates patients with favorable outcome; •, patients with unfavorable outcome. MFI indicates mean fluorescence intensity. Adapted from *Shock*. 2005;23:39–44.

expression showed significantly greater IL-1β production after LPS stimulation as compared with cells obtained from both the septic group and healthy controls (Fig. 2) (33). Taking these results together, the responses to specific ligands against TLR during sepsis seem to depend not only on the degree of TLR expression but also on organ specificity or the expression of intracellular inhibitory molecules (34).

TOLL-LIKE RECEPTOR EXPRESSION AND SEVERITY OF SEPSIS

A limited number of studies have investigated how highly expressed TLRs may contribute to the severity of illness or mortality during sepsis. We have previously investigated the relationship between the severity of illness and the expression of TLR-2, TLR-4, and CD14 on monocytes. There was no significant correlation between the acute physiology and chronic health evaluation II (APACHE II) scores (35) and the expression of these molecules (Fig. 3) (13). The reason may be that severely septic patients, especially patients with APACHE II scores greater than 20 and an unfavorable clinical outcome, did not have increased expression of TLRs relative to less severely injured patients. The association between TLR expression and the severity of illness in septic patients, however, remains elusive, and further investigations will be necessary.

GENETIC DETERMINATION OF THE INFLAMMATORY RESPONSE

Epidemiological studies suggest a strong genetic influence on the outcome of sepsis, and genetics may explain the variation in the individual response to infection that has long puzzled clinicians (36–39). The *TLR-4* gene is mutated or deleted in the LPS-resistant mouse strains—C3H/HeJ and C57BL10/ScCr—exhibiting a greatly diminished LPS response (40, 41). Hagberg et al. (42) demonstrated that C3H/HeJ mice had significantly increased susceptibility to gram-negative bacteria in experimental urinary tract infection. On the other hand, it has been reported that the presence of mutant TLR-4 does not correlate with either cytokine response or the development of organ injury in polymicrobial sepsis (43, 44). There has been extensive research on whether genetic variations can be used to identify patients at high risk for the development of sepsis and organ dysfunction during severe infection (37, 45, 46). Single-base variations, known as single-nucleotide polymorphisms, are the most commonly used variants. Several mutations within the extracellular region of TLR-4 were identified, including the Asp(299)Gly and Thr(399)Ile mutations (47). Some groups have shown that individuals with polymorphisms in TLR-4 are hyporesponsive to endotoxin (47, 48), whereas other investigators have not (49, 50). Although septic patients with TLR-4 polymorphism have been shown to have reduced levels of circulating inflammatory cytokines (51) and an increased risk of bacterial infection (52, 53), the association of mortality with polymorphism in TLRs during sepsis is still controversial (Table 3) (44). It has been reported that genetic polymorphisms vary according to race and certain other factors (54, 55); in particular, Asian people seem to have a very rare TLR-4 Asp(299)Gly mutation and/or Thr(399)Ile polymorphisms (54–56). Although positive or negative association between a polymorphism and clinical outcome has been identified in septic patients, the confidence is often tenuous because of small sample sizes. Thus, further research is required to determine whether genetic variation in

TABLE 3. **TLR-4 polymorphism and its correlation with clinical outcome**

Diagnosis	Association with clinical outcome	Odds ratio	Reference
SIRS patients	Yes	4.3	84
Severe RSV bronchitis	Yes	ND	85
Chronic periodontitis	Yes	5.6	86
Brucellosis	Yes	2.9	87
Candida infection	Yes	3.0	88
Acute pancreatitis	Yes	ND	89
Aggressive periodontitis	Yes	ND	90
Thermal injury	Yes	ND	91
Acute pancreatitis	No	ND	92
Necrotizing enterocolitis in very low birth weight infant	No	ND	93
Sepsis	No	ND	44
Cardiac surgery	No	ND	94
Esophagectomy	No	ND	95
Pneumococcal infection	No	ND	96
RSV infection	No	ND	97

ND indicates not defined; RSV, respiratory syncytial virus; SIRS, systemic inflammatory response syndrome.

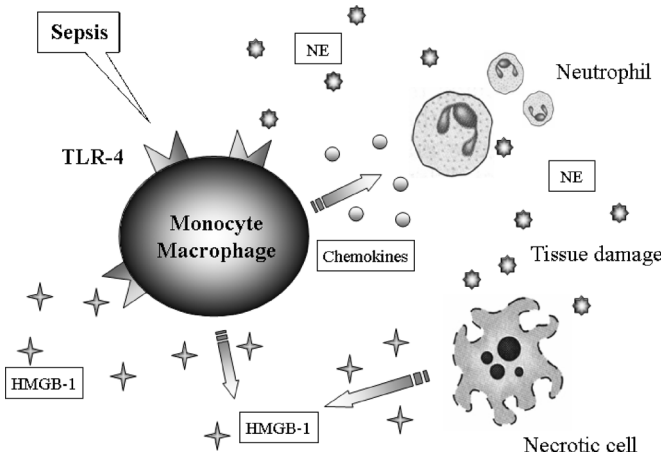

FIG. 4. **Schema of possible mechanisms of sepsis-related organ injury by the endogenous mediators through the binding with TLRs**. This mechanism may contribute to the organ dysfunction and high mortality that occurs in sepsis.

TLRs affects the organ injury and/or mortality in polymicrobial sepsis.

ENDOGENOUS LIGANDS FOR TOLL-LIKE RECEPTORS

Another recent important observation is that TLRs are also involved in the recognition of endogenous ligands, some of which have been recently named "alarmins" (57) (Table 1). Toll-like receptor 4 was shown to be involved in the recognition of extra domain A–containing fibronectin (58), fibrinogen (59), polysaccharide fragments of heparan sulfate (60), oligosaccharides of hyaluronic acid (61), β-defensin 2 (62), oxidized low-density lipoprotein (63), several heat shock proteins (HSPs) (64–67), surfactant protein A in the lung epithelium 1 (68), neutrophil elastase (13, 69), and high-mobility group box 1 protein (HMGB-1) (70, 71). Toll-like receptor 2 has also been suggested to play a role in the recognition of HSP-60 (72) and unidentified factors from necrotic cells (73). Recently, TLR-3 was also shown to recognize self-messenger RNA (74), and TLR-9 was shown to recognize self-DNA and chromatin–immunoglobulin G complexes (75). In addition, one of the small leucine-rich proteoglycans (biglycan) was demonstrated to be recognized by both TLR-2 and TLR-4 (76). Thus, the discovery that TLRs also have the capacity to recognize endogenous or harmful self-antigens suggests that their function may not be restricted to the recognition of extrinsic pathogens. Taking these findings together, we consider that TLRs play a key role in the development of sepsis and sepsis-related organ injury through both exogenous pathogens and endogenous ligands.

ENDOGENOUS LIGANDS CONTRIBUTE TO ORGAN INJURY LIKE A CYTOKINE THROUGH TOLL-LIKE RECEPTOR BINDING

Although several endogenous ligands have been implicated for TLRs, it is unclear how they contribute to sepsis-related organ injury through interaction with TLRs. High-mobility group box 1 is a nuclear protein that is released extracellularly as a late mediator of lethality in sepsis and after necrotic, but not apoptotic, death (77, 78). Recent *in vitro* studies suggest that some of the effects of HMGB-1 result from its interaction with TLR-2 or TLR-4, and with the receptor for advanced glycation end products (70, 71). Tsung et al. (71) clearly demonstrated that HMGB-1 mediates inflammation and organ damage in hepatic I/R injury depending upon the activation of TLR-4 signaling. Their findings suggest that a harmful mediator, HMGB-1, secreted from activated immunocompetent cells during sepsis, can in turn activate TLRs, resulting in further inflammation and organ injury. Johnson et al. (79) demonstrated that TLR-4 mutant mice were defective against the administration of heparan sulfate, which was degraded by proteases in inflammatory, traumatic, and septic conditions, whereas TLR-4 wild-type mice were killed. In addition, we investigated the roles of neutrophil elastase, MIP-2, and TLR-4 in organ injury in septic mice, showing that chemokine-induced recruitment of neutrophils into the lungs and liver in sepsis likely results in the augmented release of neutrophil elastase, which in turn may be associated with the production of higher levels of chemokines through binding with highly expressed TLR-4 (13). Together with these findings, the fact that endogenous ligands released through TLR signaling especially during sepsis engage with TLRs supports the idea of the perpetuation of a cycle of progressive organ injury during sepsis (Fig. 4). This mechanism may contribute to the organ dysfunction and high mortality that occurs in sepsis.

CONCLUDING REMARKS

It is likely that the expression and function of TLRs greatly influence the quality and control of innate immune response in patients with infectious disease. Modulation of TLR-4 expression may be a double-edged sword because TLRs play an important role in the host's defense against invading microbes. Indeed, mice with genetically mutated TLR-4 were reported to be highly susceptible to gram-negative bacterial infection compared with wild-type mice (42), although such mutant mice have defective responses against the endogenous danger signals that are subsequently produced in severe infection. Taking these findings together, we can conclude that TLRs are essential for triggering the host's immune response, acting as a sensor against invading pathogens. They may also serve as receptors for endogenous toxic signals, leading to tissue damage, especially in organs away from the site of infection or after successful elimination of microbes by drainage, antibiotics, or surgery. We believe that TLR antagonism should be useful in the latter case. Thus, new knowledge regarding TLRs suggests that the manipulation of TLR signaling pathways has great therapeutic potential especially in the treatment of organ injury accompanying sepsis. Further understanding of the biology of TLRs will open avenues for novel therapeutic approaches for sepsis.

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