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Innate Immune Mechanisms in Ischemia/Reperfusion

David J. Kaczorowski[1], Allan Tsung[1], Timothy R. Billiar[1]

[1]*Department of Surgery, University of Pittsburgh Medical Center, Pittsburgh, PA*

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1. ABSTRACT

Ischemia/reperfusion (I/R) injury remains a major problem in solid organ transplantation, as it adversely impacts both short and long term outcomes. It has been well established that the innate immune system plays a significant role in the pathogenesis of I/R injury. In contrast, the proximal molecular signaling events that initiate activation of the innate immune system are less clear. Recent findings have demonstrated that Toll-like receptors (TLR) play a role in I/R injury. Specifically, TLR4 is central to early activation of the innate immune response in the setting of I/R. Furthermore, recent evidence has shown that endogenous molecules, such as high mobility group box-1 (HMGB1) and others that are released from ischemic, damaged, or dying cells and tissues in the setting I/R, can serve as triggers for activation of the innate immune system and exacerbate tissue injury. This evolving body of literature, which has provided insight into the early molecular events that activate the innate system after I/R, is reviewed here.

2. INTRODUCTION

Injury caused by ischemia followed by reperfusion occurs when the blood supply to an organ is interrupted and subsequently returned, resulting in damage to the organ and a robust inflammatory response. Warm I/R injury occurs in a number of clinical settings including percutaneous coronary interventions, revascularization procedures, hemorrhagic shock and resuscitation, while cold I/R injury is seen in cardiac surgery involving cardiopulmonary bypass and solid organ transplantation. I/R injury is particularly important in solid organ transplantation because it can influence both graft function and patient survival (1,2,3,4,5,6,7). Furthermore, I/R injury may influence the allo-immune response to the transplanted graft (8).

While the pathogenesis of I/R injury is multifactorial, it is has been established the innate immune system plays a significant role in the etiology of both warm and cold I/R injury. Pro-inflammatory cytokine release,

endothelial cell activation, and tissue infiltration by neutrophils are among the known factors that contribute to I/R injury (9,10,11,12,13,14). While these and other factors have been well characterized, the early molecular events that initiate I/R injury are poorly defined. More recent evidence suggests that Toll-like receptors (TLRs) and endogenous mediators of inflammation play important roles as triggers of inflammation and organ injury after I/R injury.

3. TOLL-LIKE RECEPTORS AND INNATE IMMUNITY

Lamaître and colleagues initially demonstrated in 1996 that the *Drosophila* protein Toll, which was known to play a role in development, was also central to the host response to fungal infection in adult flies (15). It was subsequently discovered by Medzhitov and Janeway that homologous receptors exists in higher organisms (16). These receptors, known as Toll-like receptors (TLRs), are a family of transmembrane receptors that play a critical role in the innate immune response to invading microbial pathogens (17,18). Through recognition of conserved microbial motifs, know as pathogen-associated molecular patterns (PAMPs) or microbial-associated molecular patterns (MAMPs), TLRs serve as sentinels that alert the host to the presence of microbial invaders by activating the innate system.

TLR4, one of the most studied TLRs, was originally identified as the molecular sensor for bacterial lipopolysaccharide (LPS) when Poltorak and co-investigators demonstrated that mutations in the tlr4 gene were responsible for defective LPS signaling in mutant mice (19). In conjunction with other molecules, including CD14 and MD-2, TLR4 senses the presence of bacterial LPS at the cell surface and transmits a signal into the cytoplasm through two distinct pathways. One pathway is dependent upon an adaptor known as myeloid differentiation factor 88 (MyD88) (20). The other pathway is MyD88-independent and relies on an adaptor known as TIR domain-containing adaptor-inducing IFNβ (TRIF) (21,22). Importantly, recent data has demonstrated that TLR4 also has the ability to recognize molecules released from stressed or damaged cells and tissues and initiate an inflammatory response. Heparan sulfate, hyaluronan, fibrinogen, and high mobility group box-1 (HMGB1) are among molecules that have been identified as potential endogenous activators of the TLR4 signaling complex (23,24,25,26,27,28,29,30).

4. TLR4 IN I/R INJURY

Experimental evidence from animal models of liver, renal, pulmonary, and myocardial injury has pointed to a key role for TLR4 in I/R injury. Furthermore, TLR4 appears to mediate I/R injury in both warm I/R and cold I/R injury. While further work will be required to more completely delineate the molecular mechanisms involved, it is becoming clear that TLR4 is central to activation of the innate immune system in the setting of I/R.

4.1. Liver I/R

Evidence from several laboratories implicates TLR4 as an early mediator of inflammation and organ injury after hepatic I/R. Wu and co-investigators first demonstrated a role for TLR4 in hepatic warm I/R (31). Using a model of partial hepatic warm ischemia, they observed significantly lower levels of serum aspartate aminotransferase (AST) and tumor necrosis factor alpha (TNFα) in mutant mice defective in TLR4 signaling (C3H/HeJ) compared to wild-type controls (C3H/HeOuJ) at early time points (1 and 3 hours after reperfusion). Further, they found lower levels of TNFα mRNA at 1 hour after reperfusion and lower levels of myeloperoxidase (MPO) activity, a marker of neutrophil accumulation, at 3 hours after reperfusion in mice deficient in TLR4 signaling compared to control mice (31). These results not only provided evidence that TLR4 is an important mediator of I/R injury, but highlighted its early involvement in the time course of I/R injury.

Zhai and colleagues, as well as our group, confirmed these observations and extended upon them in a number of ways (32,33). These groups found that while TLR4 deficient mice exhibited lower serum alanine aminotransferase (ALT) levels, less hepatocellular injury by histology, and less induction of pro-inflammatory gene expression after I/R compared to control animals, TLR2 deficient animals did not. Further, Zhai *et al.* observed that interferon regulatory factor 3 (IRF3) deficient animals, but not MyD88 deficient animals, were protected from hepatocellular injury, suggesting that the TRIF dependent pathway may be the predominant pathway involved in this TLR4-dependent process (32).

While these studies established a role for TLR4 signaling in hepatic I/R injury, they did not address the whether TLR4 signaling on liver immune cells such as Kupffer cells and dendritic cells is required for inflammation and organ injury after I/R. In order to address this question, we generated chimeric mice with reciprocal combinations of TLR4 competent or TLR4 mutant bone marrow and subjected these mice to partial hepatic warm I/R (34). Either wild-type or mutant mice that received TLR4 mutant bone marrow were protected from organ injury and inflammation after I/R, while mice with TLR4 wild-type marrow were not. In addition, depleting liver phagocytic cells with gadolinium chloride resulted in reduced hepatocellular injury in wild-type mice, but further protection was not provided in TLR4 mutant mice (34). We also found that expanding the numbers of immature dendritic cells in the livers of TLR4 wild-type, but not TLR4 mutant animals significantly increased I/R induced liver damage (35). These results demonstrate that functional TLR4 signaling on bone marrow derived phagocytic cells is important in hepatic warm I/R injury.

Taken together, the results of these studies convincingly argue for a key role for TLR4 in hepatic warm I/R. In each of these studies, models utilizing warm ischemia were utilized. These models are representative of clinical settings, such as elective surgery, where hepatic warm ischemia is encountered. To address whether TLR4

is also involved in cold I/R, as seen in organ transplantation, Shen and co-investigators utilized an isogenic, orthotopic liver transplant model. The results of these experiments demonstrated that TLR4 is also a mediator of inflammation and organ injury in the setting of cold I/R (36). Specifically, the authors found that absence of TLR4 signaling in the donor organ ameliorated the consequences of I/R injury (36).

4.2. Renal I/R

Other studies have demonstrated a role for TLR4 in renal I/R. Kim and colleagues first noted that TLR4 mRNA and protein were upregulated after renal I/R injury in rat model involving bilateral warm I/R (37). A recent study by Wu and colleagues more definitively demonstrated a role for TLR4 as mediator of renal I/R injury (38). Using a murine model of warm renal I/R, they found that renal dysfunction, tubular damage, pro-inflammatory cytokine expression, and leukocyte infiltration were dependent upon functional TLR4 signaling. In contrast to the observations of Zhai and colleagues, the authors of this study observed that MyD88 deficient mice exhibited reduced renal injury and inflammation compared to wild-type animals in this model. The authors also generated chimeric mice and observed that while wild-type mice with TLR4 mutant bone marrow were afforded some protection from renal injury after I/R, TLR4 mutant mice with wild type bone marrow were protected to a greater degree. Contrary to what was previously observed in a hepatic I/R model, this observation argues that TLR4 signaling in cells that are not derived from bone marrow play a greater role in injury in renal warm I/R than do bone marrow derived cells (38).

4.3. Pulmonary I/R

I/R injury is a major problem in lung transplantation and the role of TLR4 has also been investigated in pulmonary I/R. As it does in hepatic and renal I/R, TLR4 also plays a prominent role in pulmonary I/R injury. Using a lung warm I/R model, Shimamoto and co-investigators found that TLR4 knock-out mice exhibited significantly reduced pulmonary vascular permeability, leukocyte infiltration, and cytokine production compared to wild-type mice after I/R (39). While a definitive role has not been established for TLR4 in the setting of human lung transplant I/R, TLR4 mRNA expression has been shown to correlate with IL-8 levels, which may serve as a potential prognostic marker in human lung transplantation (40).

4.4. Myocardial I/R

Understanding the molecular basis of myocardial I/R injury is also of intense interest, as it is relevant to multiple clinical settings including cardiac ischemia and infarction, percutaneous coronary interventions and cardiac surgery with cardiopulmonary bypass, and cardiac transplantation. Multiple TLRs are expressed on cardiomyocytes, including TLR4 (41). Further, TLR4 signaling can affect myocardial function. TLR4 stimulation by LPS appears to mediate myocardial dysfunction in the setting of sepsis (42). Recent evidence demonstrates that TLR4 signaling plays a prominent role in myocardial I/R injury.

The role of TLR4 in warm myocardial I/R injury has been evaluated by several laboratories. Chong and co-investigators found that TLR4 deficient mice exhibited significantly smaller infarcts following reperfusion in a coronary occlusion model (43). They also noted reduced cytokine production and mitogen-activated protein (MAP) kinase activation in hearts of TLR4 deficient animals. Oyama and colleagues observed similar results in a temporary coronary occlusion model. There was also less neutrophil infiltration, lipid peroxidation, and complement deposition in hearts of TLR4 deficient animals (44). Others have confirmed these findings and extended upon them, demonstrating that phosphoinositide 3-kinase-dependent (PI3K) may be at least partially responsible for myocardial protection in TLR4 deficient mice (45). At least one study, which utilized an adenoviral delivery system to introduce a dominant-negative MyD88 construct in to the myocardium, has suggested that the MyD88-dependent pathway plays a role in this response (46). In addition, we have demonstrated that TLR4 plays a key role in myocardial injury after cold I/R, as a central mediator of the early inflammatory response (47). Furthermore, both donor and recipient TLR4 signaling participate in this response (47).

To date, most of the studies designed to evaluate the role of TLR4 in I/R injury have relied on the use of either TLR4 mutant or knock-out animals. However, pretreatment of animals with eritoran, a soluble structural analog of the lipid A portion of LPS that functions as a specific inhibitor of TLR4, has also been shown to reduce infarct size in a transient coronary occlusion model (48). Pretreatment with eritoran also resulted decreased Jun N-terminal kinase (JNK) phosphorylation, nuclear factor-κB (NF-κB) nuclear translocation, and cytokine expression (48). These results not only provide a separate line of evidence distinct from data from mutant or knock-out animal experiments, but also highlight the possibility of targeting TLR4 from a therapeutic standpoint.

4.5. Future Directions

The results of these studies strongly argue for a role for TLR4 signaling in the in the activation of inflammatory pathways that lead to organ injury following I/R in several organs. Further investigation will be required to more definitively determine the mode of TLR4 activation and the intracellular pathways that are involved in mediating the molecular events downstream of TLR4 in a range of cell types. Modulating TLR signaling for therapeutic purposes in the setting of I/R injury is an exciting possibility that also merits further study.

5. ENDOGENOUS MEDIATORS OF INFLAMMATION IN I/R INJURY

Although the role of the adaptive immune system in transplant rejection has been well studied, it has only recently become apparent that the innate immune system contributes not only to I/R-mediated damage but also to the adaptive immune response. Innate immunity typically refers to the initial pro-inflammatory response that occurs in response to an invading microorganism. This response

serves as the front-line defense mechanism against infection. However, clinicians have long realized that tissue injury activates many of the same inflammatory pathways. This observation, among others, led to the hypothesis proposed by Polly Matzinger (49,50) that the innate immune system is designed to recognize events that are potentially dangerous to the host. In this scenario, both pathogens and tissue damage represent a threat that leads to disruption of homeostasis. Recent observations show that both microbial products (pathogen-associated molecular patterns (PAMPs)) or endogenous molecules (damage-associated molecular patterns (DAMPs)) can be recognized through the TLRs (51,52,25,28,33,53). These endogenous molecules or DAMPs can be classified as normal cell constituents released by damaged or dying cells or components of the extracellular matrix, released by the action of proteases at the site of tissue damage. They can then activate the innate immune response by interacting with TLRs. There is accumulating evidence that these endogenous molecules can mediate early organ injury from ischemia/reperfusion (I/R) as well as contribute to adaptive allograft rejection by signaling through the TLRs.

Endogenous molecules that have been shown to act as a DAMP, playing a role in the innate and adaptive immune response in transplantation, include high mobility group box-1 (HMGB1), hyaluronan, and heparan sulfate. High mobility group box-1 (HMGB1) is a fascinating nuclear protein with multiple functions. It was initially identified in 1973, and the early studies focused on its role as a DNA-binding, nuclear protein that co-purified with chromosomal DNA (54,55). HMGB1 is present in almost all eukaryotic cells, functions to stabilize nucleosomes, and acts as a transcription factor that regulates the expression of several genes (56,57). During the course of experiments to identify late-acting mediators of endotoxemia and sepsis, HMGB1 was discovered to be secreted by activated macrophages (58). Whereas HMGB1 is a late mediator of systemic inflammation in sepsis, HMGB1 can also acts as an endogenous DAMP, serving as a key link between the initial damage to cells and the activation of inflammatory signaling. In a model of warm, partial hepatic I/R in mice, HMGB1 protein expression was found to be rapidly upregulated in hepatocytes as early as 1 hour after reperfusion and then increased in a time-dependent manner up to 24 hours. Nuclear HMGB1 appears to be mobilized and released from ischemic hepatocytes in the absence of cell death through a process involving reactive oxygen species, calcium signaling and calcium-dependent kinases (59). Further support for the role of HMGB1 in the inflammation and injury seen after hepatic I/R was provided by studies demonstrating that inhibition of HMGB1 activity with neutralizing antibody significantly decreased liver damage after I/R while administration of recombinant HMGB1 worsened I/R injury (33).

In addition to its role of inflammation and early organ injury in I/R, HMGB1 also appears to contribute to the initiation of adaptive immune responses. In a murine cardiac transplantation model, HMGB1 was shown to be passively released by damaged cells in allografts after transplantation. HMGB1 was also found to be actively secreted by immune cells infiltrating the allograft, suggesting the role of HMGB1 in further enhancing allograft rejection (60). Interestingly, blockade of endogenous HMGB1 significantly prolonged cardiac allograft survival compared to controls. These findings suggest that HMGB1 is involved as an early mediator in the pathogenesis of I/R injury as well as acute allograft rejection after transplantation.

Hyaluronan and heparan sulfate are two other DAMPs that have been shown to play a role in initiating the innate immune response during organ harvest and I/R insults. Hyaluronan and heparan sulfate are glycosaminoglycan and proteoglycan molecules found in the extracellular matrix of many tissues. Recent studies suggest that these molecules, components of the extracellular matrix, are released during tissue injury associated with organ harvest and I/R. The release of these DAMPs may activate the innate immune response, leading to the initiation of the adaptive immune responses causing allograft rejection. Both hyaluronan and heparan sulfate have been shown to interact with TLRs (51,25,26). Their roles in triggering inflammation in response to ischemic settings such that occurs in transplantation have also been demonstrated. Hyaluronan has been shown to be upregulated in the kidney after I/R injury and to initiate the innate inflammatory response through activation of TLR4 or CD44 receptors (61,38). Similarly, exposure of heparan sulfate in the basement membrane after damage to the endothelial layer encountered during kidney I/R is important of the influx of inflammatory cells important for the immune response (62). Thus, endogenous activators of innate immune responses may serve as adjuvants for the initiation or propagation of alloimmune responses.

6. SUMMARY AND PERSPECTIVE

Taken together, the results of the studies summarized in this review provide convincing evidence demonstrating that TLR4 signaling is involved in the early activation of the innate immune system in the setting of I/R injury. Further, evidence is accumulating that endogenous molecules that are released from stressed or damaged cells or tissues during the course of I/R, including heparan sulfate, hyaluronan, and particularly HMGB1, are important triggers of innate immunity in the setting of I/R. Studies dating back to the observations Land and colleagues have implicated early ischemic tissue damage to rejection rates (63). The identification of specific pathways for injury induced activation of innate immunity are likely to provide a molecular basis linking tissue injury with activators of the innate immune response. A better understanding of the molecular interactions involved in these processes, as well as greater knowledge of the intracellular pathways that mediate these signaling cascades, may ultimately allow for the development of therapeutics aimed at ameliorating I/R injury and its adverse consequences.

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Drs David J. Kaczorowski, Allan Tsung contributed equally to this manuscript

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Send correspondence to: Timothy R. Billiar, M.D., Department of Surgery, University of Pittsburgh School of Medicine, 200 Lothrop Street, Presbyterian Hospital F1200, Pittsburgh, PA 15213, Tel: 412-647-1749, Fax: 412-647-3247, E-mail: billiartr@upmc.edu

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