Regulation of Receptor-Dependent Activation of the Innate Immune Response

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In the United States, >750,000 patients annually are thought to be at high risk for developing septic shock, with mortality rates reaching 60%. Thus, huge societal and financial costs are associated with this syndrome. Because of the high incidence and poor prognosis of septic shock, basic research has focused on the innate immune system for >2 decades. The pathophysiology of severe sepsis/shock is exceedingly complex, but there is little doubt that infection often progresses from systemic inflammatory response to severe sepsis and shock. Infection is the primary event in this sequence. There is evidence that the severity of the systemic reaction to infection (severe sepsis) is strongly influenced by the intensity of the inflammatory process at the infection sites. Efforts to understand the molecular mechanisms involved in recognition of bacterial products by members of the Toll-like receptor family are described, as well as some events that occur after receptor ligand binding that lead to new gene activation.

Because the underlying causes of shock in hospitalized patients may be quite different, outcomes vary widely. One common form is hypovolemic shock due to blood loss associated with trauma. Cardiogenic shock due to myocardial failure or obstructive events, such as pulmonary embolus, is also observed. Patients with no complications are easily identified and usually respond to supportive therapy. Unfortunately a significant number of hospitalized patients develop septic shock, the most lethal of the infection-triggered physiologic disturbances. Central in the development of this syndrome is the plethora of inflammatory mediators produced via the innate immune system response to infection.

Septic shock has diverse causes and occurs in complex patient populations. Unlike other forms of shock, onset is often insidious, and progression of the syndrome is often irreversible. Despite many years of intensive research, septic shock continues to be a major cause of morbidity and mortality in intensive care units worldwide. Numerous clinical trials have failed to improve survival, even though the underlying scientific rationale was, in many cases, quite strong. For example, past research efforts provided strong evidence for the role of proinflammatory mediators such as tumor necrosis factor (TNF)–α and interleukin (IL)–1 in the disease process, yet clinical trials that used agents to block the activities of these mediators did not result in improved outcomes. Thus, there remain major unanswered questions related to the pathogenesis and proper treatment of septic shock, although the knowledge base to ultimately solve this problem is increasing dramatically [1–3].

Remarkably, ~90% of cases of severe sepsis and septic shock now attributed to infection are triggered by microorganisms that make up the body’s normal microbial flora [4]. Although the ability of invasive procedures and mechanical devices to breach skin and mucosal barriers has been appreciated for many years, explanations for the inability of the body’s other frontline defenses to defeat these commensal invaders are incomplete. How the host response to infection can
produce toxic systemic reactions mediated in large part, if not exclusively, by inflammatory mediators (severe sepsis/shock) is also poorly understood.

In a major conceptual advance, Janeway, in 1989, pointed out that the body’s innate immune defenses are fixed in the genome, rapidly mobilized, and able to recognize microbes that express conserved molecular patterns [5–12]. Some of the elements of this innate immunity are preformed and ready to act without modification (e.g., natural antibodies and the alternative complement system). Others (phagocytes) require activation by non-self signals; host molecules (pattern receptors) recognize conserved microbial molecular patterns and trigger signal-transducing pathways within host cells. When stimulated in this way, many types of myeloid lineage cells activate their own antimicrobial killing mechanisms and release mediators that increase local blood flow, attract neutrophils to the site of infection, and provoke fluid extravasation. In other words, the sensory or recognition arm of innate immunity is tightly coupled to the antimicrobial effector functions provided by local inflammation. Here, I discuss some aspects of how signal transduction regulates the sensory arm of innate immunity after discussing what we know about the innate immune system in its broadest sense.

**INNATE IMMUNE RESPONSES DURING INFECTION**

Our understanding of essential features of the innate immune system has increased dramatically during the past dozen years, beginning with the discovery of the lipopolysaccharide (LPS)—binding protein/CD14 pathway and continuing to the recent findings of the importance of the Toll-like receptor (TLR) family of proteins [5–9, 13–17]. The accepted paradigm is that activation of innate immunity occurs when products of infectious organisms bind to specific plasma membrane receptors on host cells. This response is characterized by the synthesis and release of multiple pro- and anti-inflammatory mediators. These include lipid mediators, cell-surface proteins, and a myriad of cytokines and other bioactive proteins. Among the issues that must be considered are which mechanisms are used by the innate immune system to recognize microbial products, the signaling pathways that elicit production of these mediators, the temporal sequence of mediator production, and the specific actions of mediators throughout the body and the counter-regulatory responses that they set in motion.

Innate immunity protects the body from infection by a very broad range of microbes, including those found among the vast microbial flora that normally inhabit the surfaces of human skin and mucosae. Failure of innate immune mechanisms renders the body unable to contain commensal microbes when they invade, often through a break in an epithelial barrier, and allows them to multiply within tissues. The local inflammatory response intensifies, and, for poorly understood reasons, severe sepsis/shock sometimes ensues. Moreover it is beginning to be appreciated that this local inflammatory response initiates key processes at the interface between innate and adaptive immunity.

In the past, basic and clinical research focused on gram-negative sepsis, mainly through studies that used LPS. It is generally acknowledged that LPS plays an important role in septic shock, and a large body of important data has emerged from studies of the effects of LPS on the innate immune system. However, a broader approach is required for the study of inflammatory reactions to invading bacteria, as indicated by recent investigations that implicate gram-positive bacteria in severe sepsis and septic shock [18–21].

Until recently, little was known about the cellular mechanisms involved in recognition and responsiveness to gram-positive bacteria. Now it is clear that membrane constituents from gram-positive bacteria use a TLR distinct from that used by LPS (i.e., TLR2 rather than TLR4). The distinct and overlapping features of signaling via the individual TLRs have not yet been fully elucidated. Another key unanswered question concerns the role of multiple bacterial products in the propagation of septic shock. To progress from our current state of understanding to improved patient care, I believe that it is essential to bridge these gaps in our knowledge. Thus, I will discuss some recent advances from my laboratory that provide a new level of understanding of the structure-function relationships of TLRs and that expand our knowledge about specific signaling pathways used by TLRs.

It is now well established that activation of the innate immune system first requires recognition of one or more diverse products of invading pathogens. This is mediated by a family of cell membrane receptors known as TLRs [5–9]. The 10 known members of the TLR family have common structural features, including multiple leucine-rich repeats in the ectodomains and cytoplasmic tails, with sequence homology (Toll-IL receptor domains) to the IL-1 receptor (IL-1R) family [5]. To date, distinct ligands have been identified for 6 of the 10 TLRs: TLR2 is a receptor for a number of outer membrane constituents of gram-positive bacteria, TLR3 is a receptor for double-stranded viral RNA, TLR4 is a receptor for gram-negative LPS, TLR5 is a receptor for bacterial flagellin protein, TLR7 acts a receptor for heterocyclic antiviral/immunostimulatory compounds, and TLR9 is involved in recognition of unmethylated cytosine-phosphodiester-guanine–rich motifs in bacterial DNA. In some circumstances, TLR6 and/or TLR1 and TLR2 appear to interact synergistically to recognize some bacterial outer membrane lipopeptides [22].
TLR4 AS A RECEPTOR FOR GRAM-NEGATIVE ENDOTOXIN

The role of specific membrane proteins in recognizing the endotoxin (LPS) of gram-negative bacteria was established with the identification of CD14 as an LPS-binding protein. Since CD14 is present in the outer leaflet of the plasma membrane as a glycosylphosphatidyl inositol-linked protein, the presence of one of more additional transmembrane proteins was thought to be required for signaling [13–16]. The identity of the putative transducing molecule eluded many investigators until the identification of TLR4 as the key component of the LPS receptor complex (LPSR) through the analysis of genetically unresponsive mouse strains by Beutler et al. [23, 24]. Subsequently, other researchers provided information about a second protein, MD-2, as another essential protein of the membrane receptor for LPS. Thus, the physiologic LPSR comprises at least 3 proteins, CD14, TLR4, and MD-2. Hereafter, I will refer to this heteromeric complex as the LPSR.

My colleagues and I recently explored several aspects of the function of the LPSR. The structural features of some proteins of the innate immune system involved in mediating responses to microbial pathogens are highly conserved throughout evolution. Examples include members of the Drosophila Toll (dToll) and the mammalian TLR protein families. Activation of dToll is believed to occur via an endogenous peptide, rather than through direct binding of microbial products to the Toll protein. In mammals, LPS initiates its biologic activities through a heteromeric receptor complex containing CD14, TLR4, and MD-2. LPS binds directly to CD14, but whether LPS then binds to TLR4 and/or MD-2 is not known. Thus, we addressed the question as to whether LPS binds directly to each of the components of the LPSR and whether this binding is directly linked to signaling.

To begin to address this question, we used a derivative of LPS previously described by our laboratory [25]. This derivative is prepared from Re595 LPS isolated from Salmonella minnesota Re595 and contains a covalently bound chemical group providing a radioactive tag, together with a chemical substituent that permits UV-activated cross-linking [26]. This derivative is referred to hereafter as “azido-salicylamido-ethyldithiopropionate (ASD)–LPS.” We have used transient transfection to express human TLRs, MD-2, or CD14 alone or in different combinations in human embryonic kidney (HEK) 293 cells. With this approach we determined that LPS is cross-linked specifically to TLR4 and MD-2 only when coexpressed with CD14. These data support the contention that LPS is in close proximity to the 3 known proteins of its membrane receptor complex. These data further support the contention that CD14 acts to concentrate LPS at the cell surface and to present it to the TLR4/MD-2 complex. Thus, LPS binds directly to each member of the tripartite LPSR complex, and these interactions depend on coordinated binding events among each LPSR member [27].

To further address the relationship of LPS binding to the LPSR and cell activation, my colleagues and I performed the following series of studies. We noticed that human TLR4 and MD-2 contain 7 and 2 N-linked glycosylation sites, respectively. The other TLRs also contain numerous putative N-linked glycosylation sites, which suggests that a specific function exists for this posttranslational modification in the TLR family. Thus, we asked whether glycosylation of TLR4 and MD-2 play any role in determining the function of the LPSR. MD-2 contains 2 N-glycosylated sites at positions Asn560 and Asn564, whereas the amino-terminal ectodomain of human TLR4 contains 7 N-linked glycosylation sites spaced across the entire protein sequence. Site-directed mutagenesis studies showed that cell-surface expression of MD-2 did not depend on the presence of either N-linked site; in contrast, TLR4 mutants carrying substitutions in Asn560 or Asn564 failed to be transported to the cell surface.

By using ASD-Re595 LPS in cross-linking assays [27], we demonstrated a critical role of MD-2 and TLR4 carbohydrates in LPS cross-linking to the LPSR [28]. The ability of the various glycosylation mutants to support cell activation also was evaluated in transiently transfected HeLa cells. The double mutant of MD-2 failed to support LPS-induced activation of an IL-8 promoter-driven luciferase reported to induce IL-8 secretion or to activate amino-terminal c-Jun kinase (JNK). Similar results were observed with TLR4 mutants lacking ≥3 N-linked glycosylation sites. The reduction in activation resulting from expression of the Asn mutants of MD-2 and TLR4 can be partially reversed by coexpression with CD14. This suggests that the functional integrity of the LPSR depends both on the surface expression of at least 3 proteins, CD14, MD-2, and TLR4, and that certain N-linked sites of both MD-2 and TLR4 are essential in maintaining the functional integrity of the LPSR complex.

SIGNAL TRANSDUCTION MECHANISMS USED BY TLR FAMILY MEMBERS

Given the similarities in the cytoplasmic domains of members of this receptor family, it is not surprising that signaling through TLRs uses intracellular pathways that overlap in part with those used by the IL-1R family. However, it appears that there may be distinct signaling pathways that lead to common events associated with activation of innate immunity. For example, my colleagues and I previously showed differences in the protein subunit composition of the NF-κB complex formed in response to LPS (TLR4 dependent) or heat-killed Staphylococcus aureus [29]. Others have reported differences in gene expression patterns when activation of TLR2 and TLR4 is enabled in the
same cell type. More recent evidence by the use of pharmacologic inhibitors also supports the idea of receptor-specific signaling pathways. For example, Rabehi et al. [30] reported differential effects of the p38 inhibitor, SB203580, on expression of cytokines in monocytes induced by LPS or by a gram-positive bacterial product. Until now, there has been no information to explain molecular mechanisms that might account for the observed differences.

When cells of the innate immune system are activated by diverse ligands from microbial pathogens, various signaling pathways are activated including those of the NF-κB pathway and the mitogen-activated protein (MAP) kinase family extracellular signal-regulated kinase (ERK) 1/ERK2, JNK, and p38. A large body of literature suggests that each MAP kinase pathway regulates downstream events linked to activation of the innate immune response to infection. Although my laboratory has studied the ERK1/2, JNK, and p38 pathways, our main research emphasis has been on the p38 pathway. We discovered p38α, and then we and others showed that the p38 family comprises 4 isoforms: p38α, β, γ, and δ. The α and β isoforms are thought to play a major role in mediating p38-dependent events associated with activation of innate immunity.

Although our understanding of the downstream effects of this pathway is only beginning to emerge, it is becoming increasingly clear that p38 regulates expression of key proinflammatory cytokines such as TNF-α and IL-1. In part, our knowledge of the physiologic function of this pathway during infection stems from data obtained with selective pharmacologic inhibitors of the pyridinylimidazole series (i.e., SB203580) [31–34]. Further information has come from limited gene deletion studies involving genes encoding p38 substrates. In totality, numerous experimental observations suggest that p38 activation is linked to multiple events in the evolution of an innate immune response.

The paradigm for MAP kinase activation involves at least 2 coordinately activated upstream kinases known respectively as MAP kinase kinase kinase (MAP3K) and MAP kinase kinase (MAP2K). Nonetheless, many studies of intracellular signaling have revealed both cross talk among various pathways and alternative activation pathways. Until now there has been no evidence to suggest the latter for the p38 pathway in humans. Of interest are data showing at least 2 distinct pathways for p38 activation. The α and β isoforms are thought to play a major role in mediating p38-dependent events associated with activation of innate immunity.

Signal transduction is controlled by enzymes and by nonenzymatic adapters, scaffolds, and other “inert” proteins. Here, I refer to scaffold proteins as those capable of binding to multiple proteins in a given signaling pathway without directly activating the bound proteins. TAB1 is a protein that was originally identified by a 2-hybrid screen to identify TAK1-interacting proteins; TAB1 regulates TAK1 activity and also may act as a scaffold protein, since it may also interact with other proteins such as XIAP (X-linked inhibitor of apoptosis) [37]. A number of scaffolds or adapters for ERK and JNK MAP kinase pathways have been reported. Much like these adapters, TAB1 can bind different kinases such as TAK1, and now, as we have shown, p38α. The most significant difference between TAB1 and the other prototypic scaffold proteins is that, in addition to binding function, TAB1 interactions also result in enhanced kinase activity. Thus, TAB1 is not a classical scaffold protein because in some cases binding of TAB1 to a kinase activates the kinase. When expressed together with TAK1, TAB1 augments the kinase activity of TAK1.

TAK1 is a member of the MAP3K family. Its activation has been observed in TGF-β, bone morphologic protein, IL-1, and TNF-treated cells. TAK1 has been suggested to act as a MAP3K in the JNK and p38 MAP kinase cascades. TAK1 was also reported to be involved in NF-κB pathway by activating NF-κB–inducing kinase (NIK). Both TAK1-dependent and TAB1-independent TAK1 activation has been described. Hematopoietic progenitor kinase-1 was reported to be upstream of TAK1, which suggests that TAK1 may also be activated by an upstream kinase cascade. Because of the potential of having multiple downstream signaling pathways, TAK1 was proposed to have a central role in the coordinated activation of NF-κB, JNK, and p38. However, conflicting data challenge these possibilities. For example, an NIK knockout showed no defect in NF-κB activation, raising the question of whether a TAK1-NIK-IKK (1κB kinase) pathway is important in a physiologic setting.

My colleagues and I have now determined that binding of TAB1 to p38α induces an autoactivation as a result of phosphorylation of the adjacent tyrosine and threonine residues in the activation loop. The autoactivation requires complexation with TAB1 and a fully functional adenosine triphosphate (ATP) binding site. The presence of a p38 inhibitor (i.e., the SB203850 or a mutation in the ATP binding site) prevents TAB1-induced autoactivation. Remarkably, this pathway is restricted to the regulation of only p38α and none of the other p38 isoforms or other MAP kinase families. Of note, with respect to innate immunity, this pathway appears to predominate when p38α activation is induced by a TLR9 ligand but not by a TLR2 ligand. Activation of TLR4 by LPS appears to be regulated by both the TAB1 and the classical pathway. Thus, continued studies of this novel pathway may provide a new basis for understanding fundamental differences between events downstream
of the activation of TLR2 and TLR4. Finally, these efforts may point to novel means to interfere with signaling induced by a variety of bacterial products.

References