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Role of Toll-like receptor responses for sepsis pathogenesis

Heike Weighardt[a,b,*], Bernhard Holzmann[a]

[a]Department of Surgery, Technische Universität München, Ismaninger Strasse 22, 81675 Munich, Germany
[b]Institut für Umweltmedizinische Forschung GmbH, University of Duesseldorf, Auf'm Hennekamp 50, 40225 Duesseldorf, Germany

Abstract

Sepsis remains a serious clinical problem because of high patient morbidity and mortality. Despite significant advances in critical care, there is still no efficient causal therapy applicable to patients indicating the need to further elucidate the molecular pathways leading to the immunopathology of sepsis. The importance of Toll-like receptors (TLR) for the induction of immune responses against sepsis was demonstrated in humans exhibiting polymorphisms in TLR genes and in animal models using genetically modified mouse strains. Because of the clinical heterogeneity in human sepsis and the complex pathomechanisms underlying sepsis, several different animal models might be used to cover the diverse features of sepsis. TLR receptors induce signaling through the adapter proteins MyD88 and TRIF. TLR signaling is tightly controlled at different steps of the signaling cascade by series of regulatory proteins. Using a model of severe polymicrobial septic peritonitis we could show that single TLRs are dispensable for the induction of innate immune responses under those conditions. However, genetic ablation of MyD88 or TRIF/type-I interferon signaling pathways prevented hyper-inflammation and attenuated the pathogenic consequences of sepsis indicating that dampening common signaling pathways may create a moderate signal strength which is associated with favorable immune responses. Therefore, broad knowledge about the regulation of TLR-induced signaling pathways may further elucidate the immune mechanisms during sepsis and targeting of TLR adapter molecules may provide a new therapeutic strategy against severe sepsis.
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Introduction

Sepsis is a complex pathophysiological response of the body to systemic infection and may result in severe disorders such as septic shock and multiple organ failure (Bone, 1995; van Deventer, 1992). The mortality rate of sepsis may range from 30% to 50% for severe cases (Bernard et al., 2001). Despite continued efforts and significant advances in critical care, there is still no efficient causal therapy applicable to patients (Imahara and O'Keefe, 2004). The immunopathogenesis of sepsis is characterized by an overwhelming inflammation and suppressed adaptive immunity (Hotchkiss and Karl, 2003; van der Poll and Deventer, 1999; van der Poll, 2001). Although activation of the innate immune system by microbial pathogens and their products was reported to contribute to hyper-inflammation and organ injury during systemic inflammatory responses, many aspects of sepsis immuno-pathogenesis need further elucidation. In the present review, we summarize current evidence for the contribution of Toll-like receptor (TLR)-mediated responses to sepsis pathogenesis.

Toll-like receptor signaling

Activation of TLR signaling by conserved molecular structures of microbial pathogens is crucial for the induction of innate immune responses to infection. TLRs induce inflammatory reactions by the activation of signaling pathways mediated by the adapter proteins Myeloid differentiation factor 88 (MyD88) and Toll/IL-1-receptor domain-containing adapter inducing IFN (TRIF) (Beutler, 2004; Beutler et al., 2005; Kawai and Akira, 2005; Takeda and Akira, 2004). MyD88 is the central adapter protein for signal transduction of all TLRs, except TLR3, and the interleukin (IL)-1 receptor family (Adachi et al., 1998; Kawai et al., 1999). MyD88 needs the bridging adapter MyD88-adapter-like /TIR-associated protein (Mal/TIRAP) for signaling induced by TLR2 and TLR4. MyD88 recruits IL-1 receptor-associated kinase (IRAK)-4 (Janssens et al., 2003) which phosphorylates IRAK-1. Activated IRAK-1 further recruits TNF-receptor-associated factor (TRAF)-6 (Li et al., 2002; Medzhitov et al., 1998; Muzio et al., 1997; Wesche et al., 1997), which activates the transforming growth factor -β-activated kinase (TAK)-1/TAK1-binding protein (TAB)-2 complex (Takaesu et al., 2000) as well as MAP-kinases. Finally, nuclear factor κB (NF-κB) and activator protein 1 (AP-1) transcription factors are activated resulting in the transcription of inflammatory genes.

Engagement of TLR4 and TLR3 induces the TRIF-dependent signaling pathway, which is essential for the production of interferon (IFN)-β (Hoebe et al., 2003a; Yamamoto et al., 2002) and contributes to the expression of cytokines and costimulatory molecules through both IFN-β-dependent and -independent mechanisms (Hoebe et al., 2003b; Weighardt et al., 2004). TLR4-induced TRIF signaling involves the adapter Toll-receptor-associated molecule (TRAM), whereas the interaction of TLR3 with TRIF is TRAM-independent (Fitzgerald et al., 2003; Oshiumi et al., 2003; Yamamoto et al., 2003b). TRIF-induced production of IFN-β is critically regulated by the noncanonical inhibitory factor κB (IκB) kinases TRAF family member associated NF-κB activator (TANK)-binding kinase 1 (TBK1) and IκB kinase (IKK)ε followed by phosphorylation and nuclear translocation of interferon regulatory factor (IRF)3 (Sato et al., 2003; Han et al., 2004). Type-I interferon production can also be triggered by TLR7, TLR8 and TLR9 in an MyD88-dependent fashion (Hoshino et al., 2002; Sirén et al., 2005; Kawai et al., 2004).

Type-I interferons are involved in the regulation of both innate and adaptive immune responses and induce signaling through the common type-I IFN receptor (Decker et al., 2002; Bogdan et al., 2004; Theofilopoulus et al., 2005). Type-I interferons are not only essential to establish anti-viral immunity (Matsumoto et al., 2004; Biron, 1998; van den Broek et al., 1995), but they also influence immune responses against various non-viral pathogens including *Leishmania major* (Diefenbach et al., 1998), *Listeria monocytogenes* (Carrero et al., 2004; O'Connell et al., 2004; Auerbuch et al., 2004), or *Streptococcus pneumoniae* (Weigent et al., 1986). IFN-β was found to contribute to endotoxic shock (Karaghiosoff et al., 2003).

Termination of TLR signaling

There is a growing number of molecules reported to be involved in the negative regulation of TLR-induced signaling responses, acting on different steps of the TLR-induced signaling cascade through different mechanisms. Radioprotective 105 (RP105) is a member of the TLR family and interferes with TLR4 signaling by inhibition of ligand binding (Divanovic et al., 2005). Single immunoglobulin IL-1-receptor-related molecule (SIGIRR) is a member of the TIR family consisting of a single extracellular immunoglobulin domain and a cytoplasmic TLR/IL-1-receptor (TIR) domain (Thomassen et al., 1999). SIGIRR is not able to induce NF-κB signaling by itself, but inhibits LPS-induced signaling probably by interference with the TIR domain of TLR4 and TLR9 (Qin et al., 2005). The E3-ubiqiutin ligase TLR-ubiquitinating enzyme 3A (Triad3A), however, inhibits TLR signaling by the promotion of proteolytic degradation of TLR4 and TLR9 (Chuang and Ulevitch, 2004). ST2-Ligand (ST2L) is another transmembrane TIR family member, lacking NF-κB signal capacity. ST2L inhibits signaling induced by TLR4 and IL1-R but not by TLR3, probably by

sequestering MyD88 and Mal (Brint et al., 2004). MyD88-signaling is inhibited by a splice variant of MyD88, MyD88s, which lacks the intermediate domain of MyD88 and acts as dominant negative inhibitor of the MyD88/IRAK complex by preventing the phosphorylation of IRAK-1 (Burns et al., 2003). In addition, IRF-4 interacts with the IRAK-1/MyD88/TRAF-6 complex and selectively inhibits IRF-5-dependent TLR signaling (Honma et al., 2005; Negishi et al., 2005). TRIF-induced signaling, however, is negatively regulated by the TIR adapter sterile α HEAT-Armadillo motif protein (SARM) in humans (Carty et al., 2006). Furthermore, the TRIF pathway is negatively regulated by Src homology 2(SH2)-domain-containing inositol-5-phosphatase (SHIP)-2 through interference with TANK-binding kinase 1 (TBK)1-induced signaling (An et al., 2006). The function of the IRAK-family members is also controlled by several mechanisms. While IRAK-M inhibits the dissociation of IRAK-1 and IRAK-4 from the TLR/MyD88/IRAK signaling complex by either inhibiting the phosphorylation of IRAK-1 and IRAK-4 or stabilizing the complex (Kobayashi et al., 2002), IRAK-M is also involved in endotoxin tolerance, a mechanism to protect against endotoxin toxicity (Kobayashi et al. 2002). Toll-interacting protein (Tollip) was shown to inhibit phosphorylation and kinase activity of IRAK-1 (Zhang and Ghosh, 2002). Recently, Smad6 was identified to abrogate TLR signaling by complex formation with Pelle-interacting protein (Pellino), IRAK-1 and TRAF6 (Choi et al., 2006). IRAK-induced signaling is further regulated by splice variants of murine IRAK-2, IRAK-c and IRAK-d. Both splice isoforms lack the death domain of full length IRAK-2, thereby acting as dominant negative inhibitors (Hardy and O'Neill, 2004). Moreover, A20 removes K63-linked ubiquitin residues from TRAF6 and therefore inhibits TRAF6 dependent NF-κB activation, while β-arrestin complexes with TRAF6 and averts autoubiqitination and NF-κB activation by TRAF6.

Relevance of polymorphisms in TLR and TLR adapters for sepsis

The role of TLR signaling during sepsis can be analyzed in humans showing polymorphisms in TLR genes. In the TLR4 gene several polymorphisms have been identified with the extracellular ligand recognition domain being more variable than the cytoplasmic signaling domain (Smirnova et al., 2000). Thus far, the most extensively studied polymorphism is the D229G mutation. The incidence of heterozygosity of the mutant allele in the Caucasian population is 9.4% (Feterowski et al., 2003). This polymorphism is associated with an attenuated LPS response in vitro (Arbour et al., 2000),

even in heterozygotes, as well as with decreased susceptibility to LPS-induced airway inflammation and bronchitis induced by respiratory syncytial virus, which is a well-known TLR4 ligand (Kurt-Jones et al., 2000). Its association with the LPS response and with sepsis was analyzed in several studies. While it was shown that the D229G polymorphism increased the susceptibility to Gram-negative infections (Agnese et al., 2002; Lorenz et al., 2002), no correlation of this polymorphism with pre- or post-operative LPS-induced cytokine release could be demonstrated (Kumpf et al., 2006). Furthermore, no correlation of sepsis incidence and mortality was demonstrated during post-operative sepsis caused by mixed-bacterial infection (Feterowski et al., 2003). Finally, there are other rare TLR4 polymorphisms, which lead to missense mutations and influence meningococcal infections (Smirnova et al., 2003).

Polymorphisms in the TLR2 gene include the R753E and the R677W mutations. The R753E mutation affects susceptibility to Staphylococcus aureus infections (Lorenz et al., 2000) and to tuberculosis (Ogus et al., 2006) and may protect from late-stage Lyme disease (Schröder et al., 2005). The mutation R677W was reported to correlate with lepromatous leprosy in a Korean population (Kang et al., 2002), but recent data describing a pseudogene for TLR2 in different populations may have led to false positive signals (Malhotra et al., 2005).

Bacterial flagellin is recognized by TLR5. A mutation which leads to a premature stop (392stop) in TLR5 was found with an allelic frequency of 10%. The truncated protein acts as dominant negative inhibitor of wildtype TLR5 and is associated with enhanced susceptibility to infections with Legionella pneumophila (Hawn et al., 2003).

Null mutations in the TLR signaling protein IRAK-4 are associated with enhanced susceptibility to bacterial infections (Medvedev et al., 2003; Picard et al., 2003). Children with homozygous IRAK-4 mutations suffer from recurrent bacterial infections induced by pyogenic bacteria such as Staphylococcus aureus and Streptococcus pneumoniae which became less frequent with age, indicating the development of compensatory defense mechanisms over time.

These data indicate that polymorphisms in TLRs may influence the outcome of infections dependent on individual TLRs, while more complex infections appear not to be affected. Particularly during polymicrobial infections many TLRs might be activated so that the loss of a single TLR is likely to be compensated. Even in the case of IRAK-4, which is involved in several TLR-induced signal pathways only a limited immunosuppression is observed, pointing to a marked redundancy of anti-microbial signaling pathways. Notably, although polymorphisms in TLRs or TLR signaling proteins may increase the susceptibility to certain infections, they do not appear to have a major influence on the development or outcome of sepsis.

Animal models of sepsis

To fully understand the pathophysiology of sepsis it is necessary to develop suitable and standardized animal models. Animal models should provide the opportunity to elucidate pathological processes and may allow for testing of novel therapeutic approaches in a preclinical setting. Accordingly, many models mimicking sepsis and septic shock have been developed (Buras et al., 2005). Some of them are based on the injection of toxins, or components of bacteria that activate TLRs, while others rely on the administration of viable bacteria. Another type of model involves surgical procedures to generate a septic focus leading to the immediate onset of peritonitis. Surgical models allow the influx of enteric bacteria into the peritoneal cavity thereby mimicking human postoperative sepsis. Currently, the surgical sepsis models used are cecal ligation and puncture (CLP) (Hubbard et al., 2005; Wichterman et al., 1980) and colon ascendens stent peritonitis (CASP) (Buras et al., 2005; Zantl et al., 1998). Both models show an acute inflammatory reaction caused by a continuous influx of different enteric bacteria into the peritoneal cavity. Although sepsis models may be designed to reproduce certain aspects of the human disease, it should be considered that the progression of human sepsis is highly complex and that the clinical outcome may vary due to age, pre-existing diseases, as well as oncologic and immune status of the patients. Because of the clinical heterogeneity in human sepsis and the complex pathomechanisms underlying sepsis it appears impossible to combine all these aspects in one single animal model. Instead it may be more appropriate to analyze different aspects of human sepsis in models modified according to specific clinical needs.

Role of TLRs in septic shock models

The role of TLRs in the recognition of conserved bacterial patterns was discovered by the identification of TLR4 as signaling receptor for LPS (Poltorak et al., 1998). C3H/HeJ and C57BL/10ScCr mice, which carry a missense mutation in the TIR domain of TLR4 or a null mutation for TLR4, respectively, are resistant to endotoxin challenge but are highly susceptible to Gram-negative infection (Poltorak et al., 1998). These observations were confirmed with TLR4-deficient mice, generated by homologous recombination (Hoshino et al., 1999). Mice deficient for TIRAP, MyD88, or TRIF also showed unresponsiveness to LPS and resistance to septic shock, indicating that receptor–proximal adapter proteins are essential for LPS-induced inflammatory responses (Hoebe et al., 2003a; Kawai et al. 1999; Yamamoto et al. 2002,2003a). Mice deficient for IRAK-1 or IRAK-4 also exhibit increased resistance to endotoxin

challenge (Suzuki et al., 2002; Swantek et al., 2000), confirming the important role of these kinases *in vivo*. Moreover, septic shock induced by bacterial lipopeptides was found to be dependent on TLR2 (Meng et al., 2004), while the toxic effects of CpG-DNA are mediated by TLR9 (Hemmi et al., 2000). It should be noted, however, that both TLR2- and MyD88-null mice are highly susceptible to infection with *Staphylococcus aureus* (Takeuchi et al., 2000). These findings indicate that TLR signaling may mediate the toxic effects of high doses of microbial components, but that TLRs may be required for generation of protective immune responses upon infection with individual bacterial pathogens.

Role of TLRs in polymicrobial infection models

In further studies, the contribution of TLRs to immune responses in sepsis caused by mixed-bacterial infections was examined. Using the CASP model of septic peritonitis, it was shown that the survival rates of mice with single or combined deficiency for TLR2 and TLR4 was comparable to those of wildtype mice (Weighardt et al., 2002), indicating that even deficiency of TLR2 and TLR4 does not have a major influence on the pathology of severe polymicrobial infections. In line with findings, LPS-nonresponder BALB/c mice which carry the mutated TLR4-gene of C3H/HeJ mice (Takakuwa et al., 1996) also showed no difference in survival compared with wildtype mice when analyzed in the CLP model (Echtenacher et al., 2001). These data are consistent with observations in surgical sepsis patients revealing that loss-of-function mutations in TLR4 did not correlate with incidence and mortality of mixed-bacterial sepsis (Feterowski et al., 2003). It therefore appears that during polymicrobial sepsis multiple TLRs are triggered and thereby strongly increase the complexity and intensity of the inflammatory response. As a consequence, individual TLRs may be dispensable for both protective and detrimental innate immune responses under these conditions.

To analyze the role of common TLR signaling pathways, MyD88-deficient mice were subjected to the CASP model. MyD88$^{-/-}$ mice exhibited improved survival, while bacterial clearance and recruitment of effector neutrophils to the infected peritoneal cavity were normal (Weighardt et al., 2002). The systemic hyper-inflammatory reaction was strongly attenuated, but not absent, in MyD88-deficient animals. In the absence of MyD88, TRIF-regulated inflammatory genes including Monocyte chemoattractant protein-1 (MCP-1) and Monocyte chemoattractant protein-5 (MCP-5) were found to be induced during sepsis (Feterowski et al., 2004; Weighardt et al., 2006b). Antibody blockade of CCR2, which functions as a

receptor for MCP-1 and MCP-5, demonstrated that this pathway is essential for neutrophil recruitment, bacterial clearance and prevention of kidney injury in septic peritonitis (Feterowski et al., 2004). Thus, genetic ablation of MyD88 may protect mice from the deleterious effects of polymicrobial sepsis by preventing inflammatory injury. Notably, even in the absence of a major TLR signaling pathway there appears to be sufficient residual gene expression to ensure protective neutrophil responses and anti-bacterial defense.

To further test this hypothesis, mice deficient for the type-I interferon receptor (IFNARI) were analyzed in the CASP model (Weighardt et al., 2006a). Type-I interferons are considered important effector cytokines of the TRIF-dependent TLR signaling pathway (Hoebe et al., 2003a; Yamamoto et al., 2003a). Polymicrobial sepsis was found to cause production of IFN-β, but not IFN-α subtypes, by macrophage-like cells. Similar to the findings with MyD88$^{-/-}$ mice, IFNARI$^{-/-}$ mice showed an increased early influx of neutrophils and enhanced bacterial clearance in the infected peritoneal cavity. The late, but not early, systemic levels of inflammation were reduced in IFNARI-null mice indicating that type-I interferons act as late mediators of septic hyper-inflammation.

Conclusions

Sepsis remains a major clinical problem due to high morbidity and mortality. Many aspects of the immuno-pathology of sepsis are still unclear and suitable animal models are necessary to further elucidate molecular mechanisms. TLR signaling through the MyD88 and TRIF pathways is crucial for induction of hyper-inflammatory responses and tissue injury during sepsis (Fig. 1). Experimental evidence derived from animal models indicates that, by dampening of TLR-induced inflammatory pathways, it is possible to interfere with the progression of sepsis. We propose that different thresholds of mediator production exist, either leading to detrimental processes or inducing protective immune reactions during sepsis. Full activation of multiple TLR signaling pathways during sepsis may lead to hyper-inflammation, thereby enhancing organ failure and death. Attenuation of common signaling pathways may create a moderate signal strength which might exert protective functions. Thus, the limitation of the immune signal, but not the complete absence of inflammatory mediator production, may give rise to protective immune functions. Available data suggest that there is a considerable degree of redundancy between MyD88- and TRIF-dependent signaling pathways in mediating these responses. Thus, it is tempting to speculate that targeting individual TLR signaling pathways during sepsis by affecting the function of
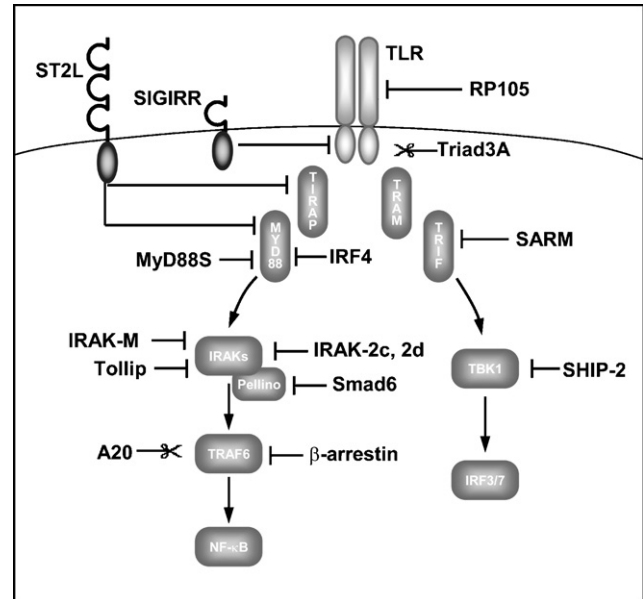

Fig. 1. Regulation of TLR signaling. TLR signaling procedes via the adapters TIRAP/MyD88 or TRAM/TRIF to activate NF-κB or IRF3 and IRF7 transcription factors. Several negative regulators influence TLR signaling at different steps of the cascade through competitive inhibition, sequestration (—|), or degradation (✂) of signaling proteins.

adapter molecules may provide a new therapeutic strategy against severe sepsis.

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