

Lipopolysaccharide signaling in endothelial cells

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Sepsis is the systemic immune response to severe bacterial infection. The innate immune recognition of bacterial and viral products is mediated by a family of transmembrane receptors known as Toll-like receptors (TLRs). In endothelial cells, exposure to lipopolysaccharide (LPS), a major cell wall constituent of Gram-negative bacteria, results in endothelial activation through a receptor complex consisting of TLR4, CD14 and MD2. Recruitment of the adaptor protein myeloid differentiation factor (MyD88) initiates an MyD88-dependent pathway that culminates in the early activation of nuclear factor- κ B (NF- κ B) and the mitogen-activated protein kinases. In parallel, a MyD88-independent pathway results in a late-phase activation of NF- κ B. The outcome is the production of various proinflammatory mediators and ultimately cellular injury, leading to the various vascular sequelae of sepsis. This review will focus on the signaling pathways initiated by LPS binding to the TLR4 receptor in endothelial cells and the coordinated regulation of this pathway.

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Sepsis is the leading cause of mortality in critically ill patients.¹ The development of sepsis occurs as a result of a systemic inflammatory response to a severe bacterial infection.² Under normal conditions, a controlled cellular response to bacterial products protects the host from infection. In sepsis, hyperactivation of the immune response leads to the excessive production of various proinflammatory cytokines and cellular injury.² In mammals, the innate immune system is the first line of host defense involved in detecting the wide variety of invading microbial pathogens.³ Receptors of the innate immune system are activated by microbial components such as lipopolysaccharide (LPS) (also known as endotoxin), which is a key molecule involved in the initiation of the sepsis syndrome.³

Endothelial dysfunction in sepsis

The endothelium plays a major role in the pathogenesis of sepsis. Endothelial cells line the inner wall of blood vessels, lying at the interface between circulating blood and the surrounding tissue.⁴ Although these cells are potentially highly susceptible to injury given that they are the first cells exposed to invading pathogens circulating in the bloodstream, endothelial cells have a remarkable capacity to protect themselves.⁴ The endothelium serves a multitude of functions that help to maintain organ homeostasis, including vasoregulation, selective vascular permeability and providing an anti-coagulant surface.⁵ However, during infection, the normal physiological functions of the endothelium are perturbed, contributing to the organ failure characteristic of sepsis.⁶ Although there is debate on the extent of vascular dysfunction that is due to the direct effects of LPS on the endothelium, relative to the effects that are secondary to the release of inflammatory mediators, such as tumor necrosis factor α (TNF α), interleukin- 1β (IL- 1β), interferons (IFNs) and others, from macrophages and immune cells,⁷ this review will concentrate on the direct cellular and molecular effects of LPS on the endothelium.

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Vasoregulation

The endothelium secretes a diversity of paracrine agents that mediate vascular tone.⁸ Vasodilating compounds such as nitric oxide (NO) and prostacyclin increase the diameter of the intravascular space by relaxing adjacent smooth muscle cells within the vessel wall.⁹ These mediators counteract vasoconstrictors such as angiotensin to maintain a balance in vascular resistance.¹⁰ Upon exposure to LPS, endothelial cells upregulate the expression of inducible NO synthase (NOS2), which leads to an increase in the production of NO.¹¹ The use of pharmacological NOS inhibitors in patients with septic shock increases blood pressure and restores vascular resistance.¹² Furthermore, NOS2-deficient mice do not experience the impaired vasoconstriction associated with endotoxemia.¹³ This excessive production of vasoactive mediators, such as NO, results in the impaired vasoconstriction and associated hypotension seen in patients with sepsis.¹⁴

Vascular Permeability

Endothelial cells serve as a selective barrier for the exchange of fluid and macromolecules from the vascular compartment to the tissue.⁵ An increase in vascular permeability is mediated by retraction of the endothelium and phosphorylation of the light chain of nonmuscle myosin.¹⁵ This phosphorylation event induces a conformational change in the myosin light chain that promotes the interaction of actin and myosin, thereby supporting the contractile state.¹⁵ A recombinant form of LPS derived from the horseshoe crab, *Limulus polyphemus*, that is designed to neutralize endotoxin action, has been shown to prevent LPS-induced actin depolymerization, thereby preventing an LPS-induced increase in endothelial permeability.¹⁶ LPS also directly contributes to endothelial barrier dysfunction through a caspase-mediated cleavage of junctional proteins involved in regulating transport of material between the vascular space and tissue.¹⁷

Leukocyte Recruitment and Adhesion

During the healing process, circulating leukocytes are recruited to the endothelium, where they adhere and traverse between the endothelial cells to enter the site of inflammation.¹⁸ The initial step in this process involves recruitment of the leukocyte to the endothelial surface through the association between selectin molecules, which are found on the surface of both the circulating leukocytes (L-selectin) and the endothelium (E- and P-selectin), and sialylated, carbohydrate moieties on the contacting cell.¹⁸ The initial selectin-mediated rolling followed by tighter adhesion to the endothelium is also mediated by the integrin family. Integrins are heterodimeric molecules expressed on the surface of leukocytes that

facilitate a strong association with cell-adhesion molecules (CAMs) on the surface of cytokine-stimulated endothelial cells (ICAM-1, VCAM-1).¹⁹ LPS directly increases expression of E-selectin and integrin counter receptors,²⁰ and the upregulation of this adhesion molecule expression requires the nuclear localization of nuclear factor-kappa B (NF- κ B).^{21,22} In septic patients, elevated levels of endothelial selectins correlate with poor prognosis.²³ Furthermore, mice deficient for endothelial selectins show increased survival in an animal model of sepsis.²⁴

Hemostasis

Under normal physiological conditions, the endothelium is maintained in an anticoagulatory state by the action of thrombin on the surface of the endothelial cell.²⁵ Thrombin, bound to thrombomodulin, cleaves protein C into its active form, activated protein C, which inhibits the activated coagulation factors, Va and VIIIa.²⁶ During sepsis, the endothelium shifts from an anticoagulant surface to a procoagulant surface by reduced expression of anticoagulatory molecules such as thrombomodulin, thereby shifting the action of thrombin towards the cleavage of fibrinogen and the generation of fibrin clots.^{27,28} Furthermore, LPS may directly induce the prothrombotic state by upregulating the endothelial expression of tissue factor through an NF- κ B-dependent mechanism.²⁹ These changes contribute to the disseminated intravascular coagulation characteristic of sepsis.

Toll-like receptor (TLR)4 signaling

LPS is a key component of the cell wall of Gram-negative bacteria.³⁰ It is composed of three structural elements: a core oligosaccharide, an O-specific chain made up of repeating sequences of polysaccharides and a lipid A component, which is responsible for the proinflammatory properties of LPS.³⁰ The binding of LPS to the surface of endothelial cells results in endothelial activation, as demonstrated by the expression of proinflammatory cytokines and adhesion molecules, and, in some cases, endothelial apoptosis.³¹ LPS also activates monocytes and macrophages to stimulate the production of proinflammatory mediators, which in turn modulate endothelial function. Collectively, this initiates a parallel cascade of events that contribute to the clinical manifestations of sepsis.

The TLRs are a family of pattern recognition receptors that are classified on the basis of homology of the cytoplasmic domain with that of the interleukin-1 receptor (IL-1R) family, which is known as the Toll/IL-1R (TIR) domain.³² To date, there have been 12 TLRs identified in mice³³ and 10 TLRs in humans.^{34–36} TLR4 was established as the LPS signaling receptor based on genetic evidence from

the LPS-insensitive mouse strain, C3H/HeJ, which has a single point mutation in the TIR domain of TLR4. In addition, the C57BL/10ScCr strain of mice has a null mutation in the *TLR4* gene that also confers resistance to LPS.³⁷ However, it should be noted that LPS from some bacterial species, such as *Porphyromonas gingivalis*, activate cells through TLR2.³⁸

The first host protein involved in the recognition of LPS is LPS-binding protein (LBP).³⁹ LBP is an acute-phase protein, the role of which is to bring LPS to the cell surface by binding to LPS and forming a ternary complex with the LPS receptor molecule, CD14.³⁹ In endothelial cells, LBP also serves to enhance LPS uptake.⁴⁰ Formation of the complex between LPS and CD14 facilitates the transfer of LPS to the LPS receptor complex composed of TLR4 and MD2.⁴¹ CD14 is found in two forms: a membrane-bound glycosylphosphatidylinositol (GPI)-anchored protein (mCD14) and as a soluble proteolytic fragment lacking the GPI anchor (sCD14).⁴² Endothelial cells lack mCD14 found on many cell types, such as macrophages and monocytes, and thus require sCD14 cleaved off cells bearing mCD14 and found circulating in the plasma.⁴³ In addition to the production of sCD14 by proteolysis of the membrane-bound receptor, monocytic cell lines also secrete sCD14 molecules that never acquire a GPI anchor.⁴⁴ Although it is widely accepted that endothelial cells do not express mCD14, early-passage human umbilical vein endothelial cells (HUVECs) have been reported to synthesize and express mCD14 at levels that are capable of supporting LPS-induced cell activation.⁴⁵ However, LPS activation of endothelial cells is primarily achieved using sCD14.⁴⁶ Nevertheless, *in vivo* expression of mCD14 on endothelial cells has not been examined. Since CD14 lacks a transmembrane domain, it does not have the capability to initiate intracellular signaling events.⁴² Concerted efforts over several years have led to the discovery of the TLR4/MD2 receptor complex as the signaling entity for LPS. MD2 is a secreted glycoprotein that functions as an indispensable extracellular adaptor molecule for LPS-initiated signaling events,⁴⁷ perhaps by aiding in ligand recognition.⁴⁸

In addition to CD14, TLR4 and MD2, Triantifilou *et al*^{49,50} have proposed that other molecules may be involved in CD14-independent signal initiation in macrophages. Studies using affinity chromatography, and later confirmed by fluorescence resonance energy transfer (FRET), revealed that LPS associates with the heat shock proteins, Hsp70 and Hsp90, chemokine receptor 4 (CXCR4) and growth differentiation factor 5 (GDF5).^{49,50}

In human coronary artery endothelial cells, TLR4 functions intracellularly,⁵¹ suggesting that LPS uptake may be necessary for optimal signal transduction. Similarly, TLR4 colocalizes with LPS within the Golgi of intestinal epithelial cells⁵² and functions within this intracellular compartment to

recognize internalized LPS.⁵³ Indeed, TLR4 is the primary receptor for transduction of the LPS signal, but it is not responsible for the internalization of LPS, pointing to the existence of another receptor for the uptake of LPS.⁴⁰ LPS internalization in endothelial cells via scavenger receptor-dependent pathways is suggested to be important in the clearance of LPS.⁵⁴ However, overexpression of a fluorescent TLR4 in human embryonic kidney (HEK293) cells showed that TLR4 cycled between the plasma membrane and the Golgi complex, but the initiation of LPS signaling occurred at the membrane, as evidenced by an inability to disrupt signaling using brefeldin A, a pharmacological agent that disrupts the Golgi.⁵⁵ Thus, further studies are required to definitively elucidate the subcellular location in which LPS activates endothelial cells. In contrast, in monocytes that express mCD14, TLR4 is found exclusively on the cell surface localized within discrete lipid microdomains following LPS stimulation.⁵⁶

The TLR4 signaling cascade initiated following LPS binding is enhanced by homodimerization of the receptor and subsequent recruitment of TIR-domain-containing adaptor molecules (TIRAP) to the cytoplasmic domain of the receptor.^{57,58} These adaptors include myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like protein (Mal), also called TIRAP, TIR-containing adaptor inducing IFN β (TRIF), also known as TIRAP-1 (TICAM-1), and TRIF-related adaptor molecule (TRAM), also called TIRAP-2 (TICAM-2).⁵⁹

Activation of TLR4 leads to stimulation of both a MyD88-dependent and a MyD88-independent pathway.⁶⁰ The major players involved in eliciting the functional effects of LPS within endothelial cells are activated through the NF- κ B, mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways. These pathways regulate the balance between cell viability and inflammation.

MyD88-Dependent Signaling

NF- κ B activation

MyD88 was originally cloned as an adaptor molecule within the IL-1R complex and shown to possess a C-terminal TIR domain and an N-terminal death domain (DD) that recruits the serine/threonine kinase, IL-1 receptor-associated kinase (IRAK).⁶¹ During MyD88-dependent signaling, MyD88 is recruited to the TLR4 receptor through interaction with the TIR domain of TLR4.⁶² This complex in turn facilitates the recruitment of IRAK1 and IRAK4 via a DD present in both molecules.^{61,63} Upon LPS stimulation, TIRAP also associates with TLR4 via a TIR-TIR interaction, and is essential for MyD88-dependent signaling.⁶⁴ This is apparent in TIRAP-deficient mice, which exhibit delayed kinetics with respect to NF- κ B and MAPK activation.⁶⁵ In

endothelial cells, TIRAP has been shown to mediate LPS-induced NF- κ B activation and apoptosis.⁶⁶ However, the precise role of TIRAP has not been fully elucidated. The binding of IRAK4 to the receptor complex facilitates the transphosphorylation of IRAK1, inducing IRAK1 kinase activity.⁶⁷ Ectopic expression of a kinase-deficient IRAK4 dramatically reduced both IRAK1 phosphorylation and downstream NF- κ B activation.⁶³ Furthermore, IRAK4 knockout mice have severely impaired TLR signaling, indicating that IRAK4 kinase activity is essential for LPS signaling.⁶⁸ The autophosphorylation and activation of IRAK1 results in the ability to bind TNF receptor-associated factor-6 (TRAF6).⁶⁹ TRAF6 contains an N-terminal domain comprised of a RING finger and several zinc fingers, which mediate downstream signaling, and a C-terminal domain involved in self-association and heterologous protein interactions.⁷⁰

The complex of IRAK1 and TRAF6 dissociates from the receptor to form a complex at the membrane with transforming growth factor- β (TGF- β)-activated kinase 1 (TAK1) and the adaptor molecules, TAK1-binding protein 1 and 2 (TAB1 and TAB2).⁷¹ TAB1 functions as an activator of TAK1,⁷² while TAB2 links TAK1 to TRAF6 and facilitates the ubiquitination of TRAF6.^{73,74} More recently, a third adaptor molecule has been identified, TAB3, which also interacts with TAK1 and mediates activation of NF- κ B.⁷⁵ Although TAK1 phosphorylation occurs at the membrane and is dependent on the formation of the TRAF6-TAK1-TAB1-TAB2-TAB3 complex, it does not become active until the complex translocates to the cytoplasm.⁷¹ Here, it forms a complex with the ubiquitin conjugating enzyme Ubc13 and the Ubc-like protein, Uev1A, to catalyze the formation of a polyubiquitin chain linked through lysine 63 (K63) of ubiquitin.⁷⁶ When TAK1 is activated, IRAK1 is released from the complex and eventually degraded by the ubiquitin-proteasome system.⁷⁷ TRAF6 is recycled through the process of deubiquitination.⁷⁸ The formation of K63-linked polyubiquitin chains on TRAF6 results in TAK1-mediated phosphorylation of the I κ B kinase (IKK) complex, composed of two catalytic subunits, IKK α and IKK β , and a regulatory subunit, IKK γ , also known as NF- κ B essential modulator (NEMO).⁷⁹ Activation of IKK involves the TRAF-interacting protein with a forkhead-associated (FHA) domain (TIFA) protein.⁸⁰ TIFA promotes the oligomerization of TRAF6 and enhances the auto-ubiquitinating activity of TRAF6, thereby facilitating downstream activation of NF- κ B and JNK.⁸⁰ Activation of the IKKs leads to downstream phosphorylation of members of the inhibitor of NF- κ B (I κ B) family, resulting in ubiquitin-directed proteasome-mediated degradation of the I κ B members, thus permitting the release and nuclear translocation of the transcription factor, NF- κ B.⁸¹ In endothelial cells, degradation of the I κ B protein is dependent on its tyrosine phosphorylation and is

specific to the I κ B α isoform, while I κ B β and I κ B γ protein levels remain unchanged following LPS stimulation.⁸² As will be discussed below, several other parallel pathways have an impact on TLR4-mediated NF- κ B activation, including PI3K, receptor-interacting protein (RIP) and the MAPKs. In non-endothelial cells, various PKC isoforms have also been implicated in NF- κ B activation^{83,84} (Figure 1).

PI3K activation

PI3Ks are a family of kinases that catalyze the phosphorylation of phosphoinositides.⁸⁵ The phosphorylated lipid products, phosphatidylinositol (PtdIns) 3,4-bisphosphate and PtdIns 3,4,5-triphosphate, then act as second messengers to activate downstream events, including activation of Akt.⁸⁶ B cells isolated from PI3K-deficient mice fail to

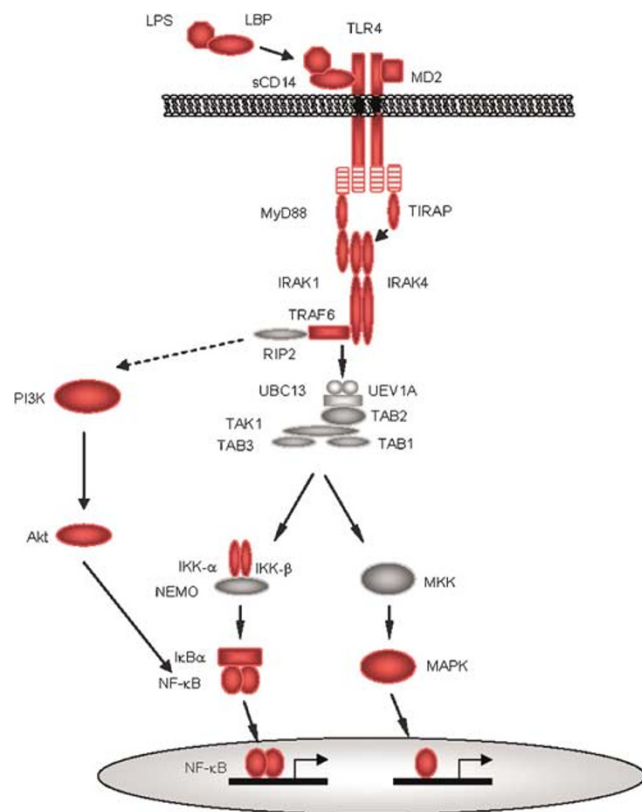


Figure 1 MyD88-dependent signaling pathway. In endothelial cells, LPS binds to the TLR4 receptor complex consisting of soluble CD14 (sCD14) and MD2. This results in the recruitment of the adaptor molecules MyD88 and TIRAP. Then, IRAK1 and 4 are recruited to the receptor complex via interactions between the DDs of MyD88 and IRAKs. IRAK1 recruits and activates TRAF6, leading to the downstream activation of the IKK complex and the MAPKs. Activation of the IKK complex results in phosphorylation and degradation of I κ B, permitting the nuclear translocation of the transcription factor, NF- κ B. This results in the expression of proinflammatory molecules. Concurrently, activation of the MAPKs, predominantly JNK, results in activation of the transcription factor AP-1, leading to a proapoptotic state. PI3K, through association with MyD88 or TRAF6, is also involved in NF- κ B activation through an Akt-dependent mechanism. Molecules which are known to be involved in endothelial TLR4-mediated signaling are highlighted in red.

proliferate in response to LPS, indicating that PI3K plays a role in LPS-induced signaling events.⁸⁷ Following LPS challenge, PI3K has been shown to exist in a complex with TLR4 and MyD88 in murine macrophages.⁸⁸ Consistent with this, enforced expression of various dominant negative mutants has shown that PI3K activation is downstream of MyD88/IRAK/TRAF6, but not TIRAP.^{89,90} Further, PI3K and its downstream target kinase, Akt, appear to be an important component of LPS-induced NF- κ B activity following its translocation to the nucleus.^{88,90}

RIP is a serine/threonine kinase that plays a critical role in LPS-induced activation of NF- κ B.⁹¹ RIP^{-/-} splenocytes exhibit decreased LPS-induced Akt phosphorylation, suggesting that RIP also connects TLR4 signaling to PI3K activation.⁹¹ RIP2 is a kinase related to RIP that contains a caspase recruitment domain (CARD) at its N-terminus.⁹² RIP2 knockout mice are resistant to the lethal effects of LPS-induced septic shock⁹³ and RIP2 participates in LPS signaling in a kinase independent manner, likely functioning as an adaptor molecule⁹⁴ (Figure 1).

Mitogen-activated protein kinases

In addition to the activation of NF- κ B and PI3K, activation of TAK1 also leads to activation of a family of MAPKs consisting of p38, extracellular signal-regulated kinase (ERK) and c-jun NH₂-terminal kinase (JNK)⁷⁹ (Figure 1). Activation of the MAPKs by TLR4 involves MyD88 and TIRAP.⁶² However, both MyD88 and TIRAP knockout mice are able to activate MAPKs albeit with delayed kinetics, pointing to the existence of a MyD88/TIRAP-independent pathway for the activation of MAPKs.⁶⁵ The mechanism of TLR4-mediated activation of MAPKs is not fully understood but it has been recognized that their activation occurs differentially during exposure to LPS.

Activation of p38 is critically dependent on MEKK3.⁹⁵ MEKK3 activates both NF- κ B and p38 kinase, but not ERK, by binding to TRAF6 in mouse embryonic fibroblasts (MEFs).⁹⁵ However, a dominant negative mutant of TRAF6 does not inhibit LPS activation of p38 in endothelial cells, suggesting that p38 does not lie downstream of TRAF6.⁹⁶ Similarly, TRAF6 is not required for LPS-induced activation of ERK in endothelial cells.⁹⁰ Nonetheless, in immune cells, the activation of p38 involves TRAF6 and apoptosis signal-regulating kinase 1 (ASK1).⁹⁷ LPS-induced intracellular reactive oxygen species (ROS) mediate the formation of a complex between TRAF6 and ASK1 to facilitate activation of p38 but not JNK.⁹⁷ Furthermore, ASK1 is required for LPS-induced cytokine production and ASK1-null mice are resistant to endotoxic shock.⁹⁷

The activation of ERK in response to LPS has not been well characterized. However, the MAP kinase kinase, Tpl2 (also known as Cot) has been implicated in LPS-induced ERK activation in macro-

phages.⁹⁸ Tpl2 has been shown to coordinate with the Ras pathway to induce activation of ERK in COS-1 monkey kidney cells⁹⁹ and the Ras pathway has been implicated in LPS-induced ERK activation.¹⁰⁰

In endothelial cells, JNK activation has been shown to require TRAF6 activation.⁹⁶ In macrophages, however, activation of JNK is dependent on interaction of the scaffolding protein JNK-interacting protein 3 (JIP3) with the cytoplasmic domain of TLR4.¹⁰¹ Inhibition of JIP3 function, either by expression of a dominant negative form of the protein or using RNA interference, inhibits JNK activation in macrophages.¹⁰¹ JIP3 is physically associated with MEKK1 in RAW264.7 cells.¹⁰¹ In neutrophils, LPS-induced JNK activation involves the participation of the protein tyrosine kinase, Syk, and PI3K.¹⁰² Taken together, these findings suggest that the receptor complex may include many more proteins than that shown in Figures 1 and 2.

Functional effects of MyD88/TIRAP-dependent signaling

LPS activates both NF- κ B and JNK through TRAF6, leading to proinflammatory and apoptotic signaling pathways, respectively.⁹⁶ TRAF6 is also the bifurcation point, downstream of which both death and survival signals are induced.⁹⁰ We have shown that LPS induces a proapoptotic signal through TRAF6-mediated activation of JNK, which in turn leads to caspase activation following mitochondrial depolarization.⁹⁶ Additionally, PI3K-dependent survival signals are mediated downstream of TRAF6 in endothelial cells.⁹⁰ The TRAF6-interacting protein, RIP, is essential for cell survival following LPS stimulation in splenocytes and provides a link between TLR4 and the PI3K-Akt survival pathway.⁹¹ LPS has also been shown to directly stimulate endothelial sprouting *in vitro* and angiogenesis *in vivo*.¹⁰³ The initiation of angiogenesis mediated by LPS is dependent on TRAF6 and the downstream effector molecules, NF- κ B and JNK.¹⁰³ The balance between endothelial survival and death pathways is crucial in the pathogenesis of sepsis as endothelial cell apoptosis could potentially lead to severe vascular collapse, as demonstrated by the disseminated endothelial apoptosis seen in C57BL/6 mice challenged with LPS¹⁰⁴ (Figure 2).

MyD88-Independent Signaling

The MyD88-independent signaling pathway was identified in studies using MyD88 deficient mice.¹⁰⁵ Although MyD88^{-/-} mice were resistant to LPS-induced death, delayed activation of both NF- κ B and MAPK pathways was observed.¹⁰⁵ Furthermore, MyD88-deficient cells failed to release proinflammatory cytokines in response to LPS suggesting direct activation of NF- κ B and the MAPKs by LPS.¹⁰⁶ Collectively, these results suggested the existence of a MyD88-independent pathway downstream of

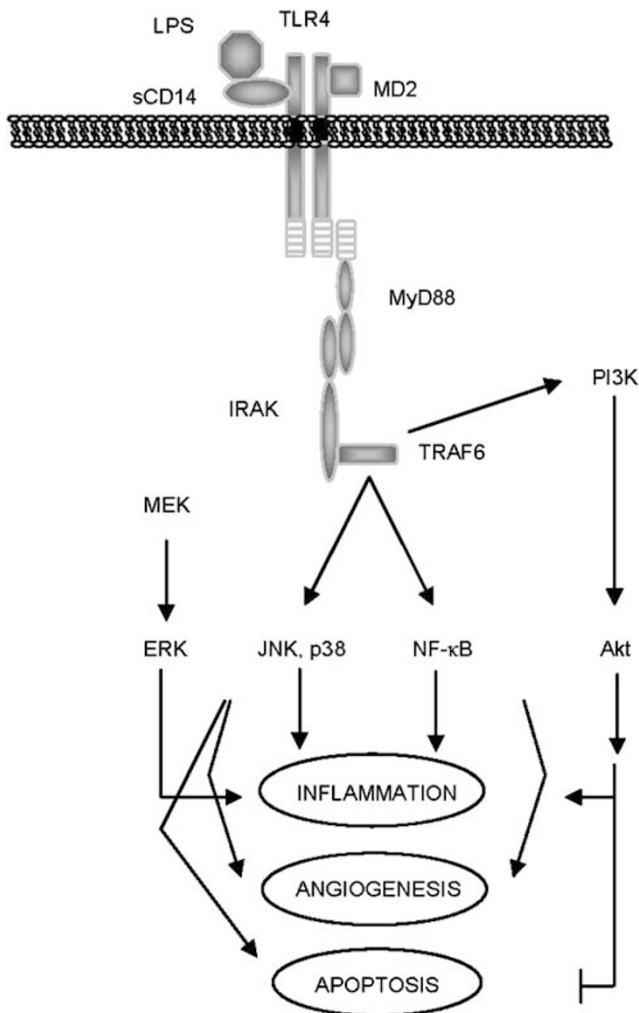


Figure 2 LPS-induced signaling events in endothelial cells. Stimulation of endothelial cells with LPS results in the activation of TRAF6, leading to activation of several downstream signaling pathways, including activation of JNK and NF- κ B, leading to the production of proinflammatory signaling molecules. Activation of TRAF6 also results in endothelial sprouting through JNK and NF- κ B-dependent signaling pathways. Furthermore, activation of TRAF6 also results in the activation of PI3K and Akt, which results in stimulation of inflammation, but inhibition of apoptosis. The activation of ERK in endothelial cells is TRAF6-independent.

ligand engagement of the TLR4 receptor. The MyD88-dependent pathway is largely responsible for controlling the expression of inflammatory cytokines, such as TNF α , IL-6 and IL-12, through activation of NF- κ B, whereas the MyD88-independent pathway induces expression of IFN-inducible genes, such as IP10 and glucocorticoid-attenuated response gene 16 (GARG16), through activation of the transcription factor, IFN regulatory factor 3 (IRF3).¹⁰⁷ MyD88-independent signaling begins with recruitment of the adaptor molecule, TRAM, to the cytoplasmic domain of TLR4.¹⁰⁸ TRAM is specific to the TLR4 signaling pathway and was cloned as a TLR4-binding protein in yeast that facilitates the binding of TRIF to the receptor complex by forming

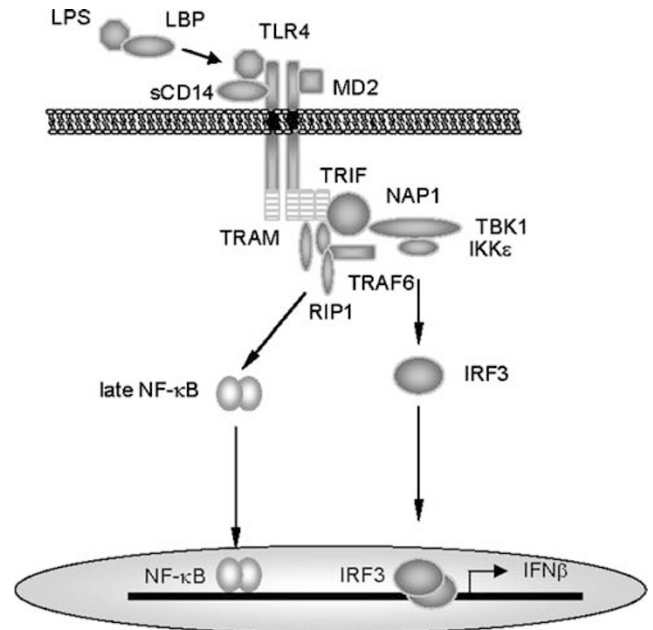


Figure 3 MyD88-independent signaling pathway. The late-phase activation of NF- κ B occurs through the MyD88-independent pathway involving the adaptor molecules, TRIF and TRAM. TRIF associates with TRAF6 and RIP1, as well as NAP1 and TBK1, to lead to the activation of the transcription factors, NF- κ B and IRF3, respectively. MyD88-independent signaling has not been extensively studied in endothelial cells.

heterodimers with TRIF.^{108,109} TRIF has been shown to play an essential role in IRF3 activation and production of IFN β .¹¹⁰ The recruitment of these adaptor molecules to the receptor complex results in binding of TRAF6 and RIP1 to TRIF, leading to the late-phase activation of NF- κ B, which contributes to the induction of IFN β .¹¹¹ In a parallel pathway, TRAF family member-associated NF- κ B activator (TANK) binding kinase 1 (TBK1) and IKK ϵ (also known as IKKi) interact with TRIF and mediate activation of IRF3, leading to the induction of IFN β .¹¹² IRF3 is a member of a family of transcription factors that are involved in the induction of type I IFNs that translocate to the nucleus upon phosphorylation and dimerization.¹¹³ Once inside the nucleus, IRF3 controls IFN β transcription by recruiting the coactivators, p300 and CBP.¹¹³ NF- κ B-activating kinase associating protein (NAP1) also interacts with TRIF and TBK1 to stimulate IFN β production through activation of IRF3.¹¹⁴ Although MyD88-independent pathways have not been directly studied in the endothelium, recent studies have demonstrated LPS-dependent induction of MyD88-independent genes (eg, IFN β , IP10, IL-6 and iNOS) in endothelial cells¹¹⁵ (Figure 3).

Negative regulation of TLR4 signaling

Modulation of signaling cascades involved in immune regulation is imperative for the prevention

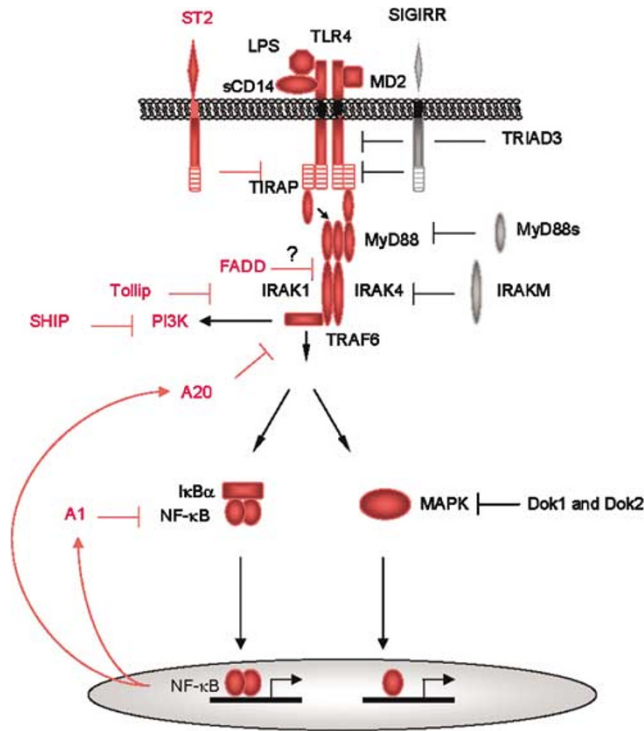


Figure 4 Modulation of TLR4-mediated signaling pathway. TLR4 signaling is regulated by several molecules. In endothelial cells, A20 functions as a deubiquitinase enzyme to inhibit activation of TRAF6 upstream of NF- κ B. A1 also inhibits NF- κ B activation through an unknown mechanism. Both A1 and A20 are upregulated by LPS through NF- κ B, suggesting a negative feedback loop to reduce the signaling by LPS. SHIP inhibits PI3K activity. PI3K itself plays a dual role in regulating LPS-induced signaling events, acting as both a positive and a negative regulator. FADD attenuates the activation of NF- κ B, perhaps by sequestering MyD88 or IRAK to prevent downstream signaling. ST2, an orphan receptor expressed in endothelial cells, sequesters the adaptor molecules MyD88 and TIRAP, preventing downstream signaling. In nonendothelial cells, a splice variant of MyD88, MyD88s, inhibits the association of MyD88 and IRAK. IRAKM prevents the association of IRAK and TRAF6. The orphan receptor, SIGIRR, negatively regulates TLR signaling, possibly by attenuating the recruitment of downstream signaling molecules. Dok1 and Dok2 inhibit LPS-induced ERK activation. Molecules that are involved in endothelial TLR4-mediated signaling, or implicated in the regulation of this pathway, are highlighted in red.

of an aberrant inflammatory response. Several molecules have been identified that regulate the TLR4 signaling pathway (Figure 4).

Downstream of Tyrosine Kinases (Dok)

Dok proteins are a family of adaptor proteins, consisting of Dok-1–5.^{116–119} Dok1 and Dok2 have been reported to be involved in the negative regulation of LPS signaling.¹²⁰ Mice that are deficient in Dok1 and Dok2 show an increase in TNF α and NO production in peritoneal and bone-marrow derived macrophages, as compared to wild-type cells.¹²⁰ Furthermore, these mice exhibit an in-

creased sensitivity to LPS, as demonstrated by an increase in TNF α production and increased lethality compared to wild-type mice.¹²⁰ Overexpression of Dok1 or Dok2 inhibits LPS-induced ERK activation.¹²⁰ Dok4 is highly expressed in endothelial cells¹²¹ and has recently been implicated as a positive regulator of TNF α -mediated NF- κ B activation in endothelial cells.¹²² However, the Dok family has not been studied in the LPS response of endothelial cells.

Phosphatidylinositol 3-Kinase

PI3K plays a dual role in LPS signaling events as it is both positively and negatively involved in TLR signal transduction. PI3K has been shown to negatively regulate the production of inflammatory mediators important in the pathogenesis of sepsis, such as proinflammatory cytokines and NO.^{123,124} In endothelial cells and monocytes, inhibition of the PI3K–Akt pathway has been shown to enhance LPS-induced cytokine production, suggesting a negative regulatory role for PI3K.¹²⁵ Activation of PI3K also reduces MyD88-dependent production of the proinflammatory molecules IL-12 in dendritic cells and TNF α in monocytes.^{126,127} In C6 glial cells stimulated with LPS, inhibition of PI3K results in the induction of NOS.¹²³ Moreover, the enforced expression of a constitutively active form of PI3K has been shown to inhibit the expression of NOS2 in human astrocytes.¹²⁸ Collectively, these results demonstrate that PI3K is a negative regulator of NO production in several cell types.

Conversely, PI3K has been shown to positively regulate MyD88-dependent signaling through the TLR2 receptor, although whether this is true in endothelial cells has not been studied. Inhibition of PI3K leads to a reduction in the activation of NF- κ B and the MAPKs, p38 and p44/42.¹²⁹ PI3K also plays a role in MyD88-independent signaling. In the TRIF-dependent signaling cascade, PI3K physically associates with TRIF in response to LPS and inhibits TRIF-dependent NF- κ B transcriptional activity, but has no effect on IRF3 activation in dendritic cells.¹³⁰

A20

A20 is a zinc-finger protein that was initially identified as an antiapoptotic protein, but has subsequently been shown to have both ubiquitinase and deubiquitinase activities.^{131–133} In endothelial cells, expression of A20 is induced by LPS in a sCD14- and NF- κ B-dependent manner.¹³⁴ Mice that are deficient for the expression of A20 exhibit increased responsiveness to LPS and develop severe inflammation.¹³⁵ Expression of A20 inhibits TLR4-mediated NF- κ B activation by regulating MEKK-1 kinase activity.¹³⁶ The regulatory action imparted by A20 is dependent on its ability to act as a TRAF6 deubiquitinase, thereby reversing TRAF6

activation.¹³⁷ A20 also acts as a negative regulator of TNF α -induced NF- κ B activation through ubiquitination of RIP, targeting RIP for degradation.¹³² Whether A20 also regulates LPS signaling by ubiquitinating RIP remains to be shown. The upregulation of A20 by LPS suggests that A20 functions as part of a negative feedback mechanism to regulate the innate immune response to LPS.

A1

A1 is a homolog of the antiapoptotic molecule, Bcl-2, that also functions as a cytoprotective protein in response to TNF stimulation.^{134,138} Similar to A20, LPS upregulates mRNA expression of A1 in endothelial cells through an NF- κ B-dependent mechanism,¹³⁴ and protects cells from LPS-induced apoptosis. In addition to the antiapoptotic effects of A1, it has also been shown that the Bcl homology 4 (BH4) domain of A1 inhibits endothelial activation by inhibiting NF- κ B activity, and thus one of the major proinflammatory pathways activated by LPS.^{139,140} Thus, the NF- κ B-dependent upregulation of A1 by LPS acts as a negative regulator in a feedback loop to dampen the LPS signal.

Fas-Associated Death Domain (FADD)

FADD is a proapoptotic adaptor molecule that couples the cytoplasmic domain of death receptor molecules to effector caspases.¹⁴¹ Prior to identification of mammalian TLRs, we had shown that LPS regulates apoptosis through a FADD-dependent pathway that was independent of death receptor signaling.¹⁴² More recently, FADD has been shown to play an inhibitory role in LPS-induced NF- κ B-dependent gene expression. FADD^{-/-} MEFs stimulated with LPS demonstrate increased I κ B- β degradation compared to FADD^{+/+} cells, indicating that the role of FADD is upstream of I κ B degradation.¹⁴³ Although a definitive role for FADD in LPS signaling has been established, the mechanism through which FADD exerts its effects remains unclear. MyD88 is a mediator of TLR2-induced apoptosis through a FADD-dependent pathway through the binding of FADD to the DD of MyD88 and subsequent recruitment of caspase 8.¹⁴⁴ As such, it has been suggested that, during LPS signaling, FADD may bind to the DD of MyD88 and thereby prevent the obligatory interaction between MyD88 and IRAK.¹⁴³ Similarly, it is also possible that FADD binds to IRAK to sequester it in the cytosol, thus preventing the interaction with MyD88 and subsequently abrogating downstream signaling events.

Toll-Interacting Protein (Tollip)

Tollip was identified as a protein partner of the IL-1R complex following IL-1 stimulation. In unstimu-

lated cells, Tollip forms a complex with IRAK and is recruited to the receptor following stimulation.¹⁴⁵ Tollip has also been shown to associate with TLR4 and is phosphorylated by IRAK following stimulation with LPS.¹⁴⁶ Tollip plays a negative role in LPS signaling in macrophages by suppressing the activity of IRAK before receptor activation.¹⁴⁶ Furthermore, overexpression of Tollip results in the inhibition of NF- κ B activity downstream of IRAK.¹⁴⁵ Tollip is expressed in human dermal endothelial cells and ectopic expression of Tollip in these cells inhibits LPS-induced NF- κ B activation.¹⁴⁷

Src Homology 2 Domain-Containing Inositol-5-Phosphate (SHIP)

SHIP is a cellular phosphatase that catalyzes the removal of a phosphate group from PtdIns 3,4,5-triphosphate, a product of PI3K activity.¹⁴⁸ Upon initial exposure to LPS, intracellular levels of SHIP are increased, which promotes endotoxin tolerance.¹⁴⁹ SHIP1 negatively regulates LPS-induced activation of Akt downstream of PI3K. In addition, SHIP1 also inhibits LPS-induced MAPK activation and I κ B- α degradation, although this is independent of PI3K involvement. However, this function is thought to occur by SHIP inhibiting formation of a complex between TLR4 and MyD88.¹⁵⁰ SHIP1 is expressed in endothelial cells,¹⁵¹ and thus may play a role in the regulation of LPS signaling in these cells.

ST2

ST2 (also known as T1) is a TIR-domain-containing orphan receptor expressed on TH2 cells and mast cells.^{152,153} Mice that are deficient for ST2 exhibit an increase in LPS-induced cytokine production.¹⁵⁴ Although ST2 contains a TIR domain and is able to activate JNK, ERK and p38, it is unable to activate NF- κ B.¹⁵⁴ Forced overexpression of ST2 in HEK293 cells results in a decrease in LPS-induced NF- κ B activation.¹⁵⁵ The inhibitory effect of ST2 is the result of the interaction between its TIR domain and the TIR domains of MyD88 and TIRAP, thus sequestering these critical adaptors during LPS signaling.¹⁵⁴ A soluble ST2 receptor was immunoprecipitated from endothelial cells, indicating that ST2 is expressed in these cells.¹⁵⁶ The presence of ST2 in endothelial cells suggests that this molecule may also be involved in the regulation of TLR4-mediated signaling in these cells.

Additional Inhibitors of TLR4 Signaling

The suppressors of cytokine signaling are a family of intracellular proteins that regulate cytokine signaling in a classical feedback mechanism.¹⁵⁷ LPS-induced upregulation of the SOCS1 protein has

been observed in a macrophage cell line and SOCS1^{-/-} mice are hyperresponsive to LPS, showing increased serum TNF α levels and increased lethality.¹⁵⁸ Furthermore, ectopic expression of SOCS1 in a macrophage cell line inhibits LPS-induced NF- κ B activation.^{158,159} SOCS1 is expressed in endothelial cells,¹⁶⁰ but its involvement in regulating LPS signaling in these cells has not been examined.

RP105 is a TLR homolog that lacks an intracellular TIR domain.¹⁶¹ Expression of RP105 is induced in the presence of the MD2 homolog, MD1.¹⁶² The complex of RP105 and MD1 interacts with TLR4/MD2, impairing its ability to bind LPS.¹⁶¹ Moreover, dendritic cells derived from RP105-deficient mice produce higher levels of proinflammatory cytokines following LPS stimulation, compared to wild-type controls.¹⁶¹ Enforced expression of TLR4, MD1 and MD2 in HEK293 cells showed that RP105-mediated inhibition of IL-8 production was the result of inhibition of NF- κ B activity.¹⁶¹ RP105 expression in endothelial cells is low, suggesting that RP105 may play only a minor role in TLR4-mediated signaling in the endothelium.¹⁶³

The chemokine receptor CXCR4 plays a putative role in LPS signaling as part of a proposed LPS activation cluster.⁴⁹ Inhibition of CXCR4 in HEK293 cells expressing TLR4, MD2 and CD14, using anti-CXCR4 antibodies, resulted in an increase in NF- κ B luciferase activity in response to LPS, suggesting that CXCR4 inhibits signaling through TLR4.¹⁶⁴ CXCR4 is expressed in endothelial cells¹⁶⁵ and thus may be involved in regulating endothelial responses to LPS.

Single immunoglobulin IL-1 receptor-related molecule (SIGIRR) (also known as TIR8) is another member of the TIR-domain-containing receptor family that is unable to induce inflammatory responses. SIGIRR is thought to play a role in regulation of inflammatory events as its expression is downregulated in tissues of mice injected with LPS.¹⁶⁶ Furthermore, the role of SIGIRR in LPS signaling is thought to be negative, since it interacts with both TLR4 and TRAF6.¹⁶⁶ SIGIRR has recently been shown to interact with TLR4 through its TIR domain to attenuate recruitment of downstream signaling molecules.¹⁶⁷ Although SIGIRR functions as a modulator of TLR signaling, expression of SIGIRR is relatively low in endothelial compared with epithelial cell lines, suggesting that it may play only a minimal role in signaling in the endothelium.¹⁶⁶

Using a yeast two-hybrid approach, TRIAD3 was identified as a RING-finger protein that interacts with the cytoplasmic tail of several TLRs, including TLR4.¹⁶⁸ The most abundant isoform identified in a range of cell lines is TRIAD3A, which contains structural features reminiscent of an E3 ubiquitin-protein ligase and promotes degradation of the receptor by targeting the receptor for ubiquitination and proteolytic degradation.¹⁶⁸ Furthermore, deple-

tion of intracellular TRIAD3A results in an increase in TLR4-stimulated NF- κ B luciferase activity.¹⁶⁸ TRIAD3A expression has not been examined in endothelial cells.

MyD88s is a splice variant of MyD88 that lacks the intermediate domain required for recruitment of IRAK4.¹⁶⁹ MyD88s inhibits TLR4 signaling by preventing the recruitment of IRAK4 to MyD88 in the receptor complex, thus abolishing phosphorylation and activation of IRAK1.⁶⁷ As a result, MyD88s functions as a dominant-negative inhibitor and is unable to activate NF- κ B. However, activation of JNK is unaffected by MyD88s expression, suggesting that pathways leading to NF- κ B and MAPK activation may diverge at the level of MyD88.¹⁷⁰ MyD88s expression has not been determined in endothelial cells.

IRAK-M is a functional member of the IRAK/Pelle family that is expressed exclusively in monocytic cell lines. IRAK-M can interact with both MyD88 and TRAF6 and possesses intrinsic kinase activity, although it is incapable of autophosphorylation.¹⁷¹ IRAK-M plays a negative role in TLR4 signaling by preventing the dissociation of IRAK4 and MyD88 and thus preventing the formation of the IRAK-TRAF6 complex. Furthermore, IRAK-M knockout mice show an increased responsiveness to bacterial LPS.¹⁷²

Conclusion

The mechanism of LPS-induced signaling events mediated by TLR4 has been extensively studied over recent years. Much work has been carried out to elucidate the role of specific molecules comprising the signaling pathways and to identify the negative regulators of the LPS signaling cascades such that therapeutic approaches may be derived from these discoveries. Novel players involved in TLR4 signal transduction, such as Nod1 and Nod2,¹⁷³ as well as the demonstration of crosstalk interactions with other signaling pathways, such as those seen between TGF β ¹⁷⁴ and TLR4 signaling cascades, will provide further insight into the molecular mechanisms of innate immunity.

Although research in the field of TLR signaling has predominantly focused on the pathways initiated in macrophages and monocytes, it is evident that the endothelium plays a critical role during sepsis. A deeper understanding of the regulatory elements of TLR4-mediated survival and death pathways in endothelial cells may facilitate the development of novel therapies to avoid the pathological events associated with endothelial injury during sepsis.

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