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Lipopolysaccharide, Mediator of Sepsis Enigma: Recognition and Signaling

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Review Article

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ABSTRACT

The outer leaflet of Gram-negative bacterial membrane contains a great amount of lipopolysaccharides, also known as endotoxins, which play a central role in the pathogenesis of sepsis and ultimately septic shock. Lipopolysaccharide (LPS) is potent inducer of acute sepsis or chronic inflammation. Sepsis can strike anyone, but is most likely to develop from infection associated with events such as pneumonia, trauma, surgery, and burns, or serious illnesses such as cancer and AIDS. In fact, people whose deaths are ascribed to complications of cancer, AIDS, or pneumonia, often actually die as a direct result of sepsis. Sepsis involves a complex interaction between bacterial toxins and the host immune system. LPS stimulates Toll-like receptor (TLR)-4 which leads to the formation and release of range of proinflammatory mediators which are essential for the potent immune response. The massive host response to this single bacterial pattern recognition molecule is sufficient to generate diffuse endothelial injury, tissue hypoperfusion, disseminated intravascular coagulation and refractory shock. LPS recognition involves LPS binding protein (LBP), CD14 ending up in TLR4/MD-2/LPS complex. The complex leads to activation of TLR4 and subsequent signaling cascade via two pathways i. e., myeloid differentiation protein 88 (MyD88)-dependent and TRIF-dependent. Here is a brief review of TLR4 signaling and LPS recognition biology with its impact if any on downstream pathways.

Keywords: LPS, Sepsis, NF-κB, endotoxin, Toll-like receptor (TLR), MyD88;

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1. INTRODUCTION

Nearly quarter a century ago, late Charles A. Janeway, Jr. proposed the existence of an innate immune recognition mechanism that would identify conserved molecular structures expressed by microbes but not by eukaryotic hosts (Janeway, 1989; Medzhitov et al., 1997). Such a recognition mechanism (termed pattern recognition) was hypothesized to explain ability of eukaryotic host to reliably detect a microbial infection. Today we know that pathogen associated molecular patterns (PAMPs) are structural motifs characteristically expressed by bacteria, viruses and fungi, that stimulate Toll like receptors (TLRs) receptors which are expressed by cells of the innate immune system (Akira et al., 2006; Jin and Lee, 2008). TLRs, named so because of their homology with Toll receptor of Drosophila, have a vital role in innate immunity in mammals (Medzhitov et al., 1997; Rock et al., 1998). To date 10 members of TLRs have been identified in human and 13 in mice and a series of genetic studies have revealed their respective ligands (Takeda and Akira, 2005).

Lipopolysaccharide (LPS) or endotoxin is one of the best studied among PAMPs of bacteria and is an important structural component of the outer membrane of Gram-negative bacteria. LPS activates TLR4 which leads to a signaling cascade thereby activates transcription factor NF-κB, mitogen-activated protein kinases (MAPKs) and interferon response factors (IRFs) and then culminates with the release of pro-inflammatory mediators including interleukin (IL)-1, IL-6, IL-12, tumor necrosis factor (TNF)-α, interferon (IFN)- γ, β and nitric oxide. Also LPS activates production of anti-inflammatory mediators such as IL-10, IL-14. Balance of two controls LPS response and dysregulation can lead to acute inflammation and a fatal septic syndrome if the inflammatory response is amplified and uncontrolled. Septic shock is still an unresolved problem as far as treatment is concerned, and much of disease state (initial stage) is mimicked by LPS administration in animal models. So researchers are exploring the LPS signaling pathway to evaluate novel targets to counteract the condition. In mammalian cells LPS stimulates the TLR4 by binding to co-receptor MD-2, but the initial recognition needs accessory proteins like LBP and CD14.

2. LPS STRUCTURE

LPS consists of three parts: lipid A, core oligosaccharide, and O side chain (Rietschel et al., 1985). Lipid A moiety of LPS is the minimal fragment that triggers the cellular response so is sometimes referred as 'endotoxin principle' (Galanos et al., 1985). Lipid A is composed of phosphorylated (1,6 linked) D-glucosamine disaccharide that carries upto six acyl residues.

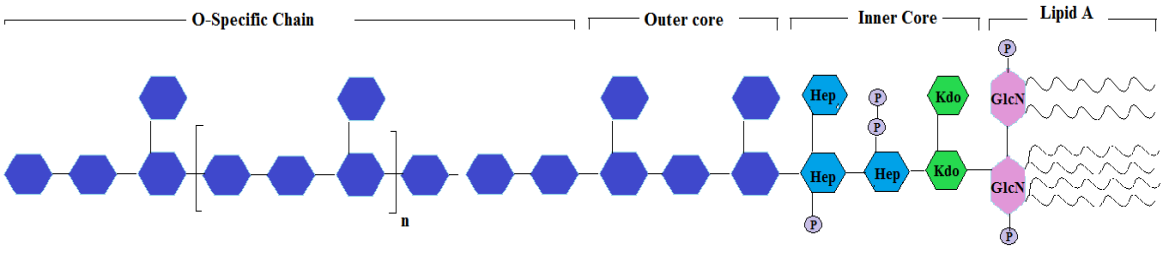

Fig.1. General Structure of LPS, showing the different units.

Abbreviations used: Hep; heptoses, kdo; keto-deoxyoctulosonate, GlcN: glucosamine, Ⓟ; phosphate, blue hexagons; polysaccharides, and wavy lines; fatty acyl-groups.

Although structurally less variable but variations exists in length, position and number of fatty acids (Fig.1). Minimal requirement for bioactivity is lipid A of E.coli having two Gluco-configurated hexosamine residues, two phosphoryl groups and six fatty acids. Deficiency of any of the components results in decrease of the activity (Kovach et al., 1990; Loppnow et al., 1986). However the requirement differ from species and also depends on the subtype of Toll-like receptor 4 [TLR4] (Lien et al., 2000; Poltorak et al., 2000). Physical structure of endotoxin is also a requirement for bioactivity as LPS tends to aggregate above critical micellar concentration [CMC] and predominate in the concentration required for bioactivity (Takayama et al., 1990; Takayama et al., 1994). The signaling cascade starts with the binding of LBP to LPS aggregates, which then transfers monomers to CD14. However the immunogenicity difference between LPS monomers and aggregates is still not clear (Mueller et al., 2005; Shnyra et al., 1993; Takayama et al., 1990). Also, activation of TLR4 by smooth-form LPS requires the cooperation of accessory proteins (CD14, LBP), while rough-form LPS can trigger the TLR4/MD-2 directly thus rough-form LPS activates a broader spectrum of TLR4/MD-2-positive cells and with a higher potency than smooth-form LPS (Huber et al., 2006).

3. LPS RECOGNITION

LPS stimulation of mammalian cells occurs through a series of interactions with several proteins including the LPS binding protein (LBP), CD14, MD-2 and TLR4. LBP, a 58-60 KDa serum glycoprotein, is an acute phase protein. LBP belongs to the lipid transfer/LBP family and is the first host protein involved in recognizing the LPS. LBP has a dual role in interactions with LPS, depending on stiochometric ratio. At low concentrations it enhances the LPS signaling by extracting it from the bacterial membranes (Vesy et al., 2000) and transferring the monomers of LPS to the CD14 at the rate of 150 molecules/min (Yu and Wright, 1996), while at high concentrations it inhibits the LPS signaling by shuttling the LPS to the serum lipoproteins and by formation of aggregates with LPS (Gutsmann et al., 2001; Wurfel et al., 1995). Increased secretion of LBP stimulated by LPS found in the serum of septic patients gave an insight of regulatory role of LPS.

CD14 is a glycoprotein, which exists either as a membrane form or as a soluble form in serum (Kirkland and Viriyakosol, 1998). Membrane form is GPI-anchored and is found in association with Toll-like receptors (TLRs) on the surface of monocytes, macrophages, neutrophils, and hepatocytes (Wright et al., 1990). CD14-LPS complex has low stiochiometry and binding is facilitated by LBP (Hailman et al., 1994; Tobias et al., 1995) then LPS is transferred to TLR4/MD-2 complex. CD14 knock-out mice showed a significant response to LPS (Haziot et al., 1998) as CD14 only increase the LPS responsiveness and is not essential for signaling. However in the absence of CD14 smooth LPS cannot activate TLR4 at all, whereas rough LPS or Lipid A activates TLR4 via MyD88/Mal pathways. In the presence of CD14, both smooth and rough LPS can recruit both MyD88/MyD88 adapter like protein (Mal) and TIR-domain containing adapter inducing interferon-β/TRIF-related adapter molecule (TRIF/TRAM) pathways. Recent studies show that CD14 is associated with src family kinase and heterodimeric G proteins in lipid rafts (Lentschat et al., 2005) thereby acting as a signal amplifier by moving TLRs into kinase-rich environments of lipid raft microdomains (Finberg and Kurt-Jones, 2006).

MD-2 a secreted glycoprotein and is classified into the ML [MD-2-related lipid-recognition] superfamily of lipid-binding protein (Inohara and Nunez, 2002). MD-2 occurs in soluble form (sMD-2) or in complex with ectodomain of TLR4, and is indispensable for the signaling of LPS as TLR4 by itself cannot recognize the LPS (Fig.2). A variety of in-vitro studies demonstrated LPS hyporesponsiveness in cells expressing TLR4 alone or TLR4 with mutant MD-2 (C95Y) (Nagai et al., 2002; Schromm et al., 2001). Structural studies of MD-2 showed a deep hydrophobic cavity sandwiched between two antiparallel β sheets, in which four acyl chains of LPS can be

accommodated, with open region of pocket having positively charged residues which stabilize the binding of LPS (Ohto et al., 2007). MD-2 can also bind to amphipathic ligands i.e Eritoran (a synthetic lipid-A analog) and Lipid IVa (a lipid-A precursor). These ligands provided an insight for LPS signaling however do not initiate signaling thereby showing complexity of LPS binding. These antagonists are good targets for sepsis therapy like eritoran is currently in phase III clinical trials for the treatment of severe sepsis. TLR4/MD-2 complex has 10 fold higher affinity for LPS than with MD-2 alone, although little is known about the role of TLR4 in the recognition of LPS. MD-2 forms covalent oligomers but LPS monomers transferred by CD14 bind MD-2 monomers, the reverse order of the LPS transport has also been seen i.e., from MD-2 to CD14 but not to LBP (Mullen et al., 2003; Visintin et al., 2001). This transfer requires a 1000-fold molar excess of CD14 to endotoxin-MD-2 and is supposed to play a regulatory role.

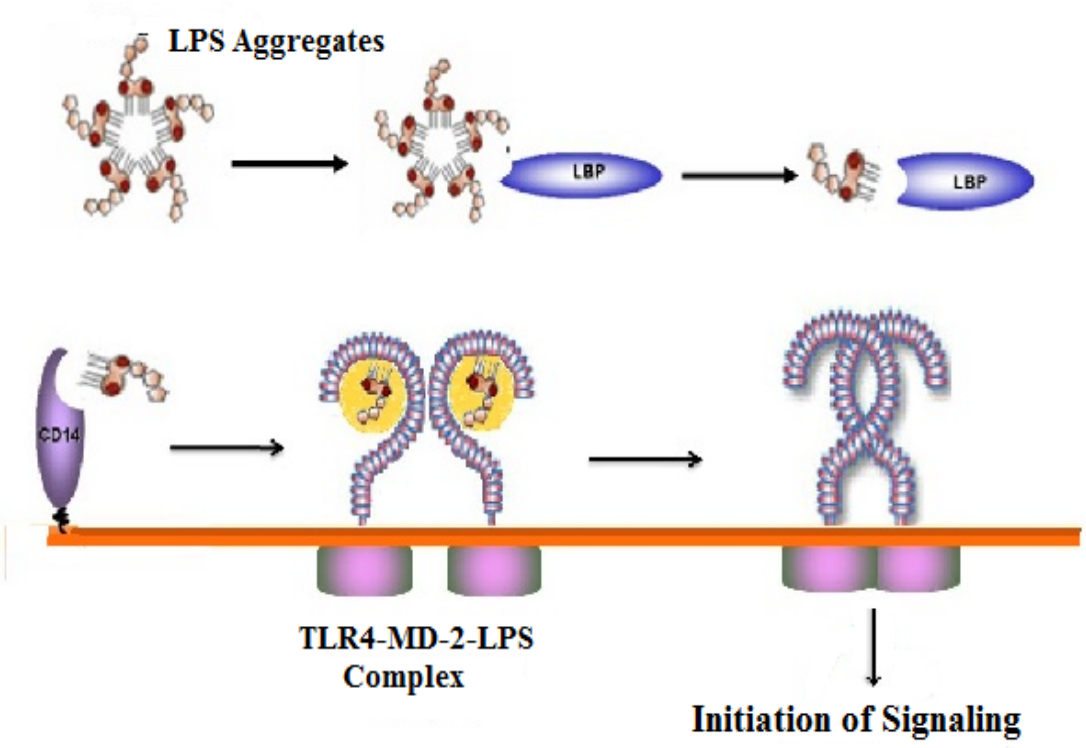

Fig.2. Recognition of LPS by CD14 and MD-2

LPS initially recognized by LBP, eventually leads to formation of TLR4-MD-2-LPS complex via CD14 on membrane, resulting in dimerization of TLR4 subunits triggering TLR4 pathway. Yellow circle represent MD-2 and '?' represents the extracellular part of TLR-4 (i.e., leucine rich repeat, LLR) and the cylindrical portion represents the intracellular Toll/IL-1 receptor (TIR) domain of TLR-4.

3.1 TLR4 AND ITS ACTIVATION

All TLRs have same basic structure and possess extracellular leucine rich repeats (LRR), a single transmembrane region and intracellular Toll/IL-1 receptor (TIR) domain. Different TLRs differ in extra and intracellular regions, recognizing different ligands and giving rise to distinct responses. TLR4 differs from other TLRs in interacting with its ligands indirectly via, MD-2 which acts as co-receptor. The LRR domain interacts with MD-2 while the TIR domain is involved in interacting with adapter proteins inside the cell. Besides structure, normal expression of TLR4 to cell surface is essential for proper signaling. Normal surface expression of TLR4 requires chaperones present in ER. Protein associated with TLR-4 (PRAT4A) and gp96 are two such chaperones whose

decreased level of expression changes the response of TLR4 towards its ligands (Takahashi et al., 2007; Yang et al., 2007). TLR4 in addition to LPS also recognizes lipotechoic acid (LTA), fibronectin, taxol (a plant diterpene) which is structurally unrelated to LPS but exhibiting LPS mimetic effects on murine cells (Kawasaki et al., 2000). TLRs are activated by ligand induced dimerization. TLR4 like other TLRs, exist as homomultimers or heteromultimers even without their ligands (Ozinsky et al., 2000) but these cannot induce signaling because of inappropriate orientation of TIR domain. Dimerization of the extracellular domains creates a proper TIR-TIR interface (TIR domains) which then leads to recruitment of adaptor proteins and initiation of intracellular signaling (Weber et al., 2005).

LPS induced dimerization of TLR4/MD-2 complex is well known but the mechanism is not so clear because TLRs have been refractory to crystallographic analysis. However recently Kim et al proposed a model for LPS induced TLR4/MD-2 dimerization (Kim et al., 2007). The crystal structure (using hybrid LRR technique) of TLR4 as TLR4-MD-2 and TLR4-MD-2-eritoran complex showed that other two TLR4 molecules form a ‘‘m’’-shaped complex with binding MD-2 in the two hooks. MD-2-eritoran complex failed to activate the dimerization of TLR4 but eritoran showed no contact with TLR4. Although TLR4-MD-2 still remains to be co-crystallized with its agonist ligand LPS, mutational studies however have shown that residues of MD-2 that are not involved in direct contact with either LPS or TLR4 play a key role in homodimerization of the TLR4-MD-2 complex in the presence of LPS. LPS, is thus proposed to induce conformational changes in MD-2 that promotes interaction between MD-2 and the central and/or C-terminal domain of the second TLR4. TIR domain is involved in intracellular signaling; a single point mutation in the TIR domain abolishes the response to LPS (Xu et al., 2000).

TLR4 is the only TLR that uses all four adapters and activates both the MyD88- and TRIF-dependent pathways (Fig. 3). On dimerization, TIR domain of TLR4 recruits the TIR domain containing adapter proteins i.e. TIRAP/Mal, MyD88, TRIF, TRAM and SARM (McGettrick and O'Neill, 2004). TIRAP/Mal and TRIF are two main adapters responsible for the downstream signaling of LPS i.e. MyD88 dependent and TRIF dependent, others have a regulatory role. Recent studies show, TLR4 activates the MyD88-dependent pathway earlier than the TRIF-dependent pathway. TLR4 initially recruits Mal at the plasma membrane and subsequently facilitates the recruitment of MyD88 to trigger the initial activation of NF-κB and MAPK (Kagan and Medzhitov, 2006). However, TRIF-dependent pathway is activated after subsequent TLR4 endocytosis and and is trafficked to the endosome, where it forms a signaling complex with TRAM and TRIF, rather than Mal and MyD88, that leads to IRF3 activation as well as the late-phase activation of NF-κB and MAPK (Kagan et al., 2008; Rowe et al., 2006; Tanimura et al., 2008). Thus complete induction via TLR4 signaling needs activation of both the MyD88- and TRIF-dependent pathways which is in contrast to other TLRs. The MyD88 dependent pathway is initiated by all TLRs (except TLR3) and MyD88 independent pathway is peculiar to TLR3 and TLR4.

3.1.1 MyD88 DEPENDENT PATHWAY

MyD88 dependent pathway so called because of MyD88 which was first adapter characterized to play a crucial role in LPS induced TLR4 pathway. The MyD88 interaction with TLR was presumed from the homologous IL-1 receptor pathway which proved to be correct (Wesche et al., 1997). MyD88 is indispensable for proper innate response, because in MyD88 deficient mice most of TLRs i.e., TLR4, TLR9, TLR5 and TLR7 did not respond to their corresponding ligands (Hacker et al., 2000; Hemmi et al., 2002). But no effect was seen on TLR3 response to its ligand as it requires TRIF, another adapter. In TLR4 pathway, TRIF is involved in MyD88 independent pathway and induces Type I interferons. LPS induced dimerization of TLR4 intiates cascade of intra-cellular events by recruiting adapters, starting with MyD88. MyD88 is essential for TLR4 signaling pathway, as MyD88 knock-out mice are resistant to LPS induced sepsis (Kawai et al.,

1999). MyD88 deficient macrophages showed delayed activation of NF-κB without proinflammatory cytokines production after LPS stimulation. MyD88 has TIR domain at C-terminal which is separated via a short linker from an N-terminal death domain. The MyD88 death domain is involved in interaction with other proteins in contrast to the death domain of FAS and TNF.

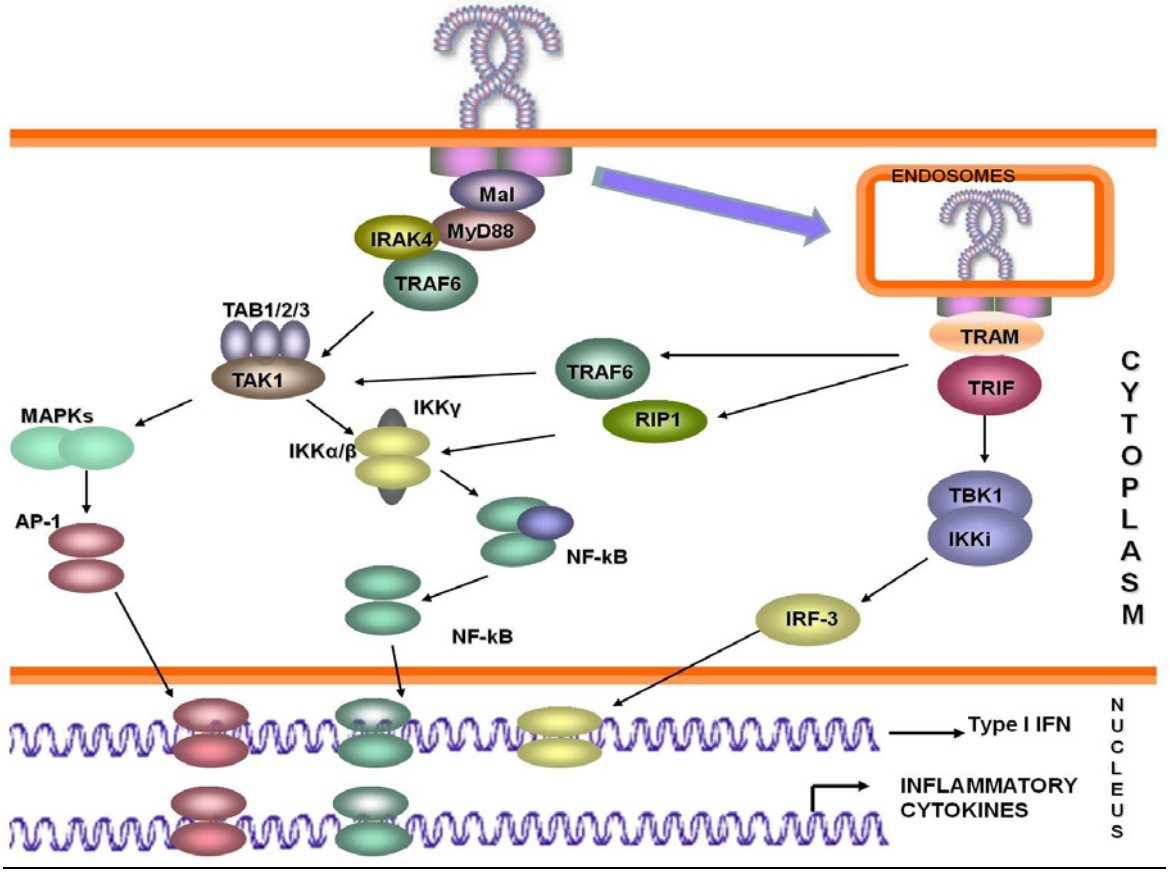

Fig.3. TLR4 Pathway

Dimerization of TLR4 leads to cascade of pathway leading to activation of NF-κB, AP-1 and IRF3 via MyD88 dependent and TRIF dependent pathway.

Abbreviations used: MyD88; myeloid differentiation protein 88, Mal; MyD88 adapter like protein, IKK α/β/γ; inhibitor of kappa B kinase α/β/γ. IKKγ is also called NF-kappa-B essential modulator (NEMO), IKKi; inducible inhibitor of kappa B kinase, NF-kB; nuclear factor kappa B, TAK-1; TGF- β activated kinase, TAB1/2/3; TAK-1 associated protein 1/2/3, TRIF; TIR-domain containing adaptor inducing interferon β, TRAM; TRIF-related adapter molecule, IRAK-4; IL-1 receptor associated kinase-4, TRAF-6; TNF receptor associated factor 6, MAPKs; mitogen activated protein kinases, AP-1; activator protein-1, RIP-1; receptor activated protein-1, TBK-1; TANK-binding kinase-1, IRF-3; interferon regulatory factor-3.

However another adapter TIRAP/Mal is required for recruitment of MyD88 in TLR4 pathway (Fitzgerald et al., 2001). Mal (MyD88 adaptor-like), cloned through a computer-based search for proteins containing TIR domains (Horng et al., 2002), is localized in plasma membrane because of its PIP2 binding domain. Mal-knockout mice/macrophages displayed resistance to lethal effects of LPS with no effect on activation of IRF-3 and expression of IFN-inducible genes. Mal form

dimers with MyD88, thereby facilitating the interaction between MyD88 and TIR domain of TLR4. MyD88 recruits the serine threonine kinase IRAK by homotypic interaction via death domain. So far four IRAKs i.e. IRAK-1, IRAK-2, IRAK-4 and IRAK-M are identified (Cao et al., 1996; Kobayashi et al., 2002; Li et al., 2002; Wesche et al., 1999) but only IRAK-1 & IRAK-4 are characterized as adapters in MyD88 dependent pathway while IRAK-M plays regulatory role. On LPS stimulation, MyD88 recruits IRAK-4 and IRAK-1 which are then sequentially phophorylated and dissociated from MyD88. IRAK-4 knockout mice are seen resistant to LPS induced septic shock, also IRAK-$4^{-/-}$ macrophages are defective in LPS induced response. The IRAK4 mutations are reported in patients with recurrent infections and poor inflammatory response (Picard et al., 2003). Thus IRAK-4 is essential for TLR4 signaling both in mouse and humans. Recent evidences show IRAK1 and IRAK4 can phosphorylate Mal, promoting ubiquitination and degradation thereby playing a regulatory role (Dunne et al., 2010).

Downstream of IRAK4/1 is another adapter TRAF6, member of TNFR associated factor (TRAF) family which are involved in cytokine signaling. TRAF6 has TRAF domain, for interaction with other molecule and RING domain which function as E3 ubiquitin domain ligase. On stimulation RING domain interacts with the ubiquitin conjugating complex (E2) Ubc13/Uev1 (Picard et al., 2003) resulting in the synthesis of polyubiquitin through lysine63 to the TRAF6. The ubiquitinylated TRAF6 complexes with heterotrimer of the protein kinase TAK1 and the adaptor molecules TAB1, TAB2 and TAB3. TAB2 and TAB3 bind lysine 63 linked polyubiquitin chains and activate TAK1 (Chen, 2005). TAK1 is a member of MAPK kinase kinase family and and acts as a common activator of NF-κB and AP-1. In unstimulated cells, NF-κB is sequestered in the cytoplasm as an inactive form by interacting with inhibitor of NF-κB (IκB) proteins. Upon stimulation TAK1 activates IKK complex consisting of IKKα and IKKβ protein kinases and a regulatory molecule, IKKγ/NEMO, which phosphorylates IκB (ser32 and ser36). The phosphorylation targets IkBs for ubiquitination and degradation by the 26S proteasome, allowing NF-κB to be released into the nucleus and to bind to the κB site which controls various immune genes including proinflammatory cytokines. TAK1 simultaneously phosphorylates various members of the MAP kinase kinase family, MKK3 and MKK6, which subsequently activate MAP kinases (JNK, ERK and p38) which then activate AP-1.

3.1.1.1 MyD88 INDEPENDENT PATHWAY

Subset of LPS response is mediated by MyD88 independent pathway which was unrevealed by the study of MyD$88^{-/-}$ mice. MyD88 knockout mice did not completely lack LPS response and showed delayed activation of NF-κB and JNK without production of inflammatory cytokines, along with expression of type I interferons and interferon inducible genes (Kawai et al., 1999). The complete pathway was elucidated after some time because of the delay in discovery of adapters. The first being TRIF, identified by a database search (Yamamoto et al., 2002) is a TIR domain containing protein consisting of 712 amino acids in humans. The first evidence came from the investigations of Yamamoto (Yamamoto et al., 2003) who used TRIF deficient mice. The activation of NF-κB in response to LPS in these mice was normal, but when the cells were deficient in both TRIF as MyD88, the NF-κB response to LPS was totally abolished. Also dominant negative TRIF inhibited TLR3 induced activation of the IFN-β promoter and RNAi mediated knockout of TRIF caused impairment in TLR3 ligand induced IFN-β expression. However different from TLR3, TRIF interacts with TLR4 indirectly via TRAM which is myristoylated and is localized near membrane (Rowe et al., 2006). TRAM acts as bridge, similar to Mal and has no role in LPS signaling but is as essential as TRIF. LPS induces upregulation of TLR4-association with TRAM and their subsequent translocation into endosome/lysosome. TRAM phosphorylation is seen essential for this purpose and is mediated by protein kinase-epsilon (Kagan et al., 2008; Tanimura et al., 2008). TRIF mediates the downstream signaling via different adapters i.e. RIP, TRAF6 (both activating NF-κB) and TRAF3 (activating IRF3). The

internalized signaling complex consisting of TLR4 and TRAM colocalizes with TRAF3, in endosome/lysosome (Tanimura et al., 2008).

The C-terminal domain of TRIF contains Rip homotypic interaction motif (RHIM) which on LPS stimulation interacts with RIP1, leading to activation of NF-κB (Meylan et al., 2004). Cells doubly deficient for MyD88 and RIP1 fail to activate NF-κB in response to LPS, suggesting that RIP1 is selectively involved in the TRIF dependent NF-κB activation. TRIF possesses three typical TRAF6-binding domains in the N-terminal region, which mediate interaction with TRAF6 (Sato et al., 2003) thereby acting as a connecting link between the two pathways. TRIF/TRAF-6 interaction is responsible for delayed activation of NF-κB and MAPK in LPS signaling as seen by studies on MyD88-deficient mice (Kawai et al., 1999). TRIF–RIP1 and TRIF–TRAF6 pathways might converge at the IKK complex to induce maximum activation of NF-κB.

TRAF3 recruitment by TRIF activates type I interferons and interferon-inducible genes which is activated by TRIF-dependent pathway only. $TRAF3^{-/-}$ cells were seen defective in Type I interferon induction on LPS stimulation (Hacker et al., 2006; Oganesyan et al., 2006). On recruitment TRAF3 associates with TANK (TRAF family member-associated NF-κB activator), TBK1 (TANK binding kinase 1) and IKKı which then activates interferon regulatory factor (IRF). TBK1 and IKKı belong to IKK family and phosphorylates IRF3 at serine residues which leads to its dimerization and translocation (Fitzgerald et al., 2003). IRF3, along with several transcriptional factors NF-κB, ATF2/c-jun, and IRF-7 (Theofilopoulos et al., 2005) bind to the interferon sensitive response element (ISRE), inducing type I interferon genes including IFN-β.

While IRF3 is constitutively expressed, expression of IRF7 is weak in unstimulated cells and dramatically induced by stimulation with LPS, type I IFN or virus infections. Thus, initial induction of IFN-β is largely dependent on IRF3 activation. Secreted IFN-β acts on neighboring cells and activates the JAK–STAT pathway via type I IFN receptor to induce IRF7 expression, which results in the amplification of type I IFN induction through a positive feedback mechanism. IFN-β, in turn, activates Stat1, leading to the induction of several IFN-inducible genes (Doyle et al., 2002; Toshchakov et al., 2002). IRF3 can then bind to the interferon sensitive response element (ISRE), inducing a subset of genes including IFN-β133. While IRF3 is constitutively expressed, expression of IRF7 is weak in unstimulated cells and dramatically induced by stimulation with LPS, type I IFN or virus infections. Thus, initial induction of IFN-β is largely dependent on IRF3 activation. Secreted IFN-β acts on neighboring cells and activates the JAK–STAT pathway via type I IFN receptor to induce IRF7 expression, which results in the amplification of type I IFN induction through a positive feedback mechanism. IFN-β, in turn, activates Stat1, leading to the induction of several IFN-inducible genes. The induction of Type I interferons and interferon-inducible genes are important for antiviral and anti-bacterial responses (Bowie and Haga, 2005; Perry et al., 2005).

4. CONCLUSION

Although LPS has been recognized as a main culprit in pathogenesis of sepsis and LPS/TLR4 pathway been investigated from years, the strategies targeting LPS, TLR4 and inflammatory cytokines proving to be promising in preclinical studies have failed in clinical environment. Our challenge for the future is to redirect our thinking to evaluate anti-endotoxin therapies in patients with endotoxemia, rather than in patients with the ill-defined syndrome of sepsis, and then to determine in which approach might result in LPS neutralization as beneficial, as opposed to potentially harmful. Other microbial mediators found in Gram-positive bacterial and viral and fungal pathogens are now appreciated to activate many of the same host defense networks induced by LPS. This information is providing novel interventions in the continuing efforts to improve the care of septic patients.

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