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REVIEW

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An examination of the LPS-TLR4 immune response through the analysis of molecular structures and protein–protein interactions

Ruiqin Luo[1,2,3†], Yuexin Yao[1,2,3†], Zhuo Chen[1,2,3*] and Xiaoming Sun[1,2,3*]

Abstract

The LPS-TLR4 immune response is a critical mechanism in the body's defense against Gram-negative bacterial infections, yet its dysregulation can lead to severe inflammatory diseases. Lipopolysaccharide (LPS), a pivotal pathogen-associated molecular pattern (PAMP) on the surface of gram-negative bacteria, is recognized by Toll-like receptor 4 (TLR4), initiating a complex cascade of immune responses. This review delves into the intricate molecular structures and protein–protein interactions that underpin the LPS-TLR4 signaling pathway, offering a comprehensive analysis of both extracellular recognition and intracellular signal transduction. We explore the roles of key molecules such as LBP, CD14, MD-2, and TLR4 in the initial recognition of LPS, followed by the downstream signaling pathways mediated by MyD88-dependent and MyD88-independent mechanisms. The MyD88-dependent pathway primarily activates NF-κB and AP-1, leading to macrophage M1 polarization and the release of pro-inflammatory cytokines, while the MyD88-independent pathway triggers IRF activation and type-I interferon production. By elucidating the structural basis and functional interactions of these signaling molecules, this review not only enhances our understanding of the LPS-TLR4 immune response but also highlights its implications in both infectious and non-infectious diseases. Our findings underscore the potential of targeting this pathway for therapeutic interventions, offering new avenues for the treatment of inflammatory and immune-related disorders.

Keywords LPS, Immune response, Molecular structure, TLR4 Signal transduction, Pattern recognition receptor

Introduction

Gram-negative bacteria, ubiquitous in nature, encompass common species such as *Escherichia coli* (E. coli), *Klebsiella pneumoniae* (Kp), *Salmonella typhimurium* (St), *Helicobacter pylori* (Hp), and *Porphyromonas gingivalis* (Pg). A critical pathogenic component of their cell wall is lipopolysaccharide (LPS), also known as endotoxin, which is a complex glycolipid comprising a hydrophilic polysaccharide and a hydrophobic lipid A moiety [1]. During the initial phase of Gram-negative bacterial infection, LPS acts as a significant antigen and pathogen-associated molecular pattern (PAMP), influencing the transcription of multiple genes in macrophages and triggering diverse immune responses, resulting in systemic inflammation [2–5]. For instance, when *Pseudomonas*

†Ruiqin Luo and Yuexin Yao contributed equally to this work.

*Correspondence:
Zhuo Chen
chenzhuo@whu.edu.cn
Xiaoming Sun
151321820@163.com
[1] School of Basic Medical Sciences, Hubei University of Medicine, Shiyan 442000, China
[2] Biomedical Research Institute, Hubei University of Medicine, Shiyan 442000, China
[3] Hubei Key Laboratory of Embryonic Stem Cell Research, Hubei University of Medicine, Shiyan 442000, China

aeruginosa (Pa) invades the respiratory tract, its LPS prompts macrophages to release inflammatory factors like TNF-α and IFN-γ, contributing to pneumonia. Beyond macrophage interaction, LPS also induces the production of reactive oxygen species and gel-forming mucins in airway epithelial cells, leading to conditions such as asthma, chronic obstructive pulmonary disease, and cystic fibrosis [2]. In Pg-induced periodontitis, LPS can cause excessive activation of macrophages and osteoclasts, resulting in periodontal tissue damage and alveolar bone loss [3]. Moreover, LPS is implicated in other severe conditions, including sepsis [6] and shock [7], beyond its role in inflammation.

LPS can induce both nonspecific (or innate) immune responses and specific immune responses, significantly influencing Gram-negative bacterial infections [8–12]. In innate immunity, the TLR4-mediated signaling pathway is critical for LPS-induced inflammation. TLR4 signaling involving numerous signaling molecules, such as LPS-binding protein (LBP), CD14, myeloid differentiation factor-2 (MD-2), and myeloid differentiation primary response protein 88 (MyD88) [13, 14]. Upon LPS stimulation, the nuclear factor-kappa-B (NF-κB) signaling pathway in inflammatory cells (e.g., macrophages) is activated through TLR4-mediated signal transduction, which includes both MyD88-dependent and MyD88-independent pathways [14]. This activation ultimately leads to the release of various cytokines, including IL-1β, IL-6, IFNs, and TNF-α [15, 16]. These inflammatory mediators play a dual role: they aid in the immune system in combating bacterial infections while also causing tissue damage and fever, contributing to inflammatory diseases [17–19]. Consequently, targeting the LPS-TLR4 signaling pathway with pharmacological agents, such as TLR4 inhibitors [20] and MD-2 blockers [21], has emerged as a promising therapeutic strategy for treating Gram-negative bacterial infections. However, a comprehensive understanding of the LPS-TLR4 signaling mechanism and its intricacies remains essential for advancing this research direction.

This review provides an overview of each signaling molecule (including its structure and role in the LPS-TLR4 signaling pathway) involved in the LPS-TLR4 signal transduction. Meanwhile, the connection between the TLR4 signaling pathway and other non-infectious clinical diseases is briefly introduced in the end of this article. These will provide researchers in the field of biomedicine a deeper understanding of the LPS-TLR4 immune response. The protein 3D structures in this article are all sourced from PDB database (https://www.rcsb.org/) or AlphaFold database (https://www.alphafold.ebi.ac.uk/), and reconstructed into images using Pymol software (version 2.5.7). The relevant cartoon diagrams, signal pathway diagrams, and protein plane domain diagrams were drawn via FigDraw website (https://www.figdraw.com/), Powerpoint, and SAI software (ver.2 (64 bit). 2024). The information not found in the references in this work is sourced from the Uniprot protein database (https://www.uniprot.org/).

Related molecules for LPS extracellular recognition

LBP

LBP, a member of the LBP/bactericidal permeability-increasing protein (BPI) family within the lipid-transfer/LBP superfamily, was first identified in the late 1980s [22–25]. This serum glycoprotein, primarily synthesized in the liver, lungs, and gastrointestinal tract, circulates freely in the bloodstream [24]. Human LBP (AlphaFold ID: AF-P18428-F1) is a 53 kDa glycoprotein comprising 481 amino acids. As a pseudo-symmetric protein, LBP contains two structural domains (Fig. 1A), each featuring antiparallel β-strands surrounded by an α-helix, with a phospholipid molecule co-crystallized in the intervening pocket. The N-terminal domain (NTD) of LBP contains a cluster of cationic residues critical for LPS recognition and binding [23]. According to Ryu et al., LBP binds longitudinally to LPS micelles through its NTD tip, with alkaline patch A on the β2-β3 connecting ring playing a pivotal role in this interaction [26]. Upon LPS capture, LBP's C-terminal functional motifs and conserved alkaline patches interact with CD14's acidic concave patch through charge complementarity, facilitating LPS transfer to CD14. During bacterial infections, serum LBP levels can increase several-fold, peaking in the acute phase [24]. Additionally, albumin plays a crucial role in the LBP-LPS interaction by binding to LPS's lipid A portion, shielding its hydrophobic region from the aqueous environment, thereby enhancing LPS monomer extraction and transfer to specific receptors [27, 28]. In the absence of albumin, LBP-LPS binding becomes unstable and transient [29].

CD14

CD14, initially identified by Sanna et al. in the late 1980s, functions as both a monocyte differentiation antigen and a cell surface LPS receptor. This protein is expressed on various immune cells, including macrophages, neutrophils, and dendritic cells [30, 31]. Human CD14 (AlphaFold ID: AF-P08571-F1), encoded by a gene located in a growth factor-rich region on chromosome 5, consists of 375 amino acids. The protein exhibits a curved spiral structure comprising thirteen β-chains, with 11 chains overlapping the leucine-rich repeat (LRR) region (Fig. 1B). Its ligand-binding pocket contains hydrophobic residues in the first five positions and charged residues along the rim. The concave surface, formed by 11 parallel and two antiparallel strands, requires N-terminal disulfide bonds and glycosylation for proper folding and

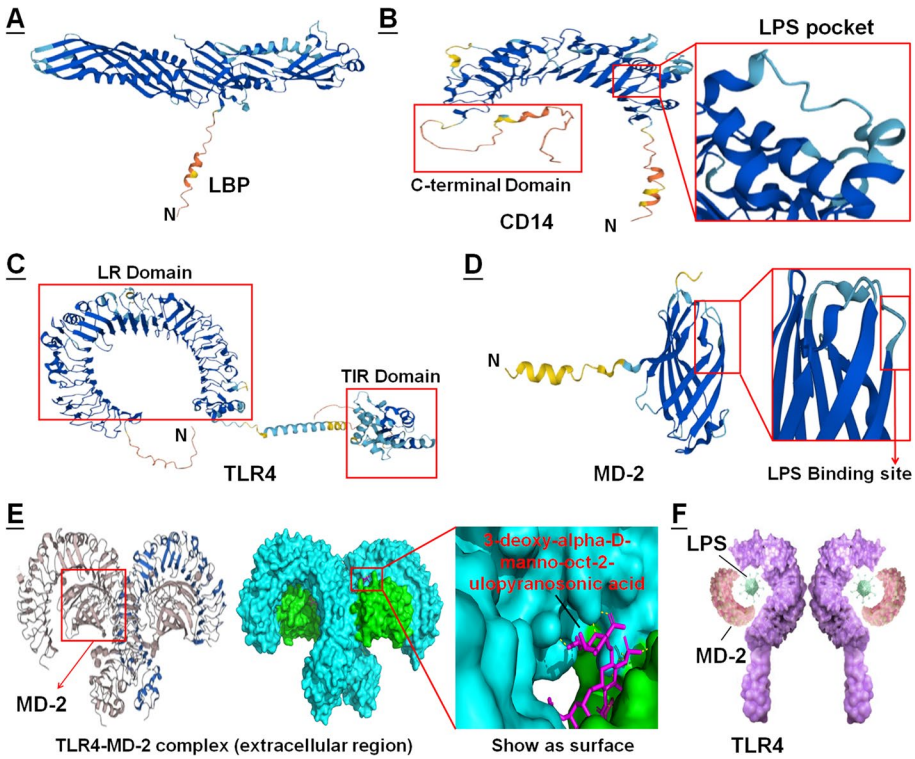

Fig. 1 The three-dimensional structural pattern diagram of LBP, CD14, TLR4, MD-2, and TLR4-MD-2 complex and the binding pattern diagram of LPS and TLR4-MD2 complex. **A** LBP comprises two structural domains, each featuring antiparallel β-strands surrounded by an α-helix. **B** CD14 contains thirteen β-chains, including eleven overlapping with LRR and two LPS-binding pockets at the LRR termini (one indicated in the diagram). **C–D** TLR4 consists of an LRR domain and a TIR domain (**C**), while MD-2 contains a TIR domain and an LPS-binding site (**D**). For enhanced visualization of CD14's LPS-binding pocket and MD-2's LPS-binding site, the orientation was adjusted during magnification. **E** Pattern diagram of the extracellular domain of TLR4-MD-2 complex. **F** The pattern diagram of LPS binding to TLR4-MD-2

signaling [31, 32]. Studies demonstrate that LPS binds to CD14's N-terminal hydrophobic pocket [33]. Interestingly, according to UniProt database annotations, the C-terminal domain (residues 290–375) is essential for LPS response, suggesting that there may be a more complex interaction mechanisms that warrant further investigation to fully elucidate CD14-LPS binding dynamics.

CD14 plays a pivotal role in the LPS-TLR4 signaling pathway by recognizing LBP-delivered LPS and presenting it to TLR4, making LPS the first and most extensively studied CD14 ligand. CD14 exists in two forms: a glycosylphosphatidylinositol (GPI)-anchored membrane glycoprotein and a soluble serum protein lacking the GPI tail, both capable of recognizing LBP-presented LPS. Upon LPS binding, CD14 delivers LPS to the TLR4-MD-2 complex through direct TLR4 interaction, subsequently promoting TLR4-mediated intracellular signaling. While CD14 functions as a co-receptor in this process, the precise molecular mechanisms underlying its facilitation of LPS recognition by TLR4 remain unclear [31, 32, 34, 35]. In vitro studies demonstrate that CD14 knockout macrophages show diminished LPS reactivity, highlighting CD14's importance in TLR4-mediated LPS recognition. Interestingly, CD14 knockout mice exhibit reduced bacterial growth and lower mortality following intranasal Burkholderia pseudomallei infection, with their macrophages and leukocytes retaining pathogen recognition and killing capabilities [36]. These findings suggest that CD14's role in specific inflammatory contexts requires further investigation.

TLR4 & MD-2

TLR4, a member of both the Toll-like receptor family and leucine-rich receptor (LR) family, represents an early-identified and extensively studied pattern recognition receptor (PRR) that mediates LPS-induced inflammatory immune responses [37]. When Ruslan et al. first described TLR4 in 1997, scholars also first recognized that TLR can function as PRR [35, 38]. Structurally distinct from typical LRR proteins, human TLR4 (AlphaFold ID: AF-O00206-F1) is a 95 kDa transmembrane protein composed of 839 amino acids. It features a LRR extracellular domain for ligand binding and a cytoplasmic Toll/IL-1 receptor (TIR) domain for downstream signaling

(Fig. 1C) [39]. The N-terminal and central domains of TLR4 exhibit charge complementarity, facilitating stable TLR4-MD-2 heterodimer formation [40]. Functionally, TLR4 serves as a crucial PRR in innate immunity, recognizing CD14-presented LPS through TLR4-MD-2 complex formation (PDB ID: 4g8a) to initiate downstream signaling [14, 35, 41]. Notably, the N-terminal region, particularly the first 20 amino acids, is essential for TLR4-MD-2 interaction [42].

MD-2, a member of the myeloid differentiation factor family, was first characterized by Shimazu et al. in 1999, with its cDNA sequence and function reported [43]. Human MD-2 (AlphaFold ID: AF-Q9Y6Y9-F1) is an 18 kDa protein comprising 160 amino acids. With the exception of avian MD-2, all known MD-2 homologs contain seven conserved cysteine residues and at least two glycosylation sites. Similar to TLR4, MD-2 possesses a TIR domain essential for TLR4 polymerization and LPS recognition [42, 44]. The LPS binding site is localized between residues 119 and 123 (Fig. 1D). On the cell membrane, MD-2 and TLR4 form a complex typically consisting of two TLR4 and two MD-2 molecules (Fig. 1E). Upon LPS binding, the ligand is sandwiched between TLR4 and MD-2 (Fig. 1F) [45]. Structural studies of MD-2 are challenging due to its tendency to aggregate, making monomer isolation difficult [42]. Notably, Shimazu et al. demonstrated that TLR4 transfection alone fails to induce LPS response in 293 T cells, whereas co-expression of TLR4 and MD-2 confers significant LPS responsiveness, underscoring MD-2's essential role in TLR4 function [43].

Related molecules for LPS-TLR4 mediated intracellular signaling transduction

The LPS-TLR4 intracellular signaling cascade primarily operates through two distinct pathways (Fig. 2). The MyD88-dependent pathway involves sequential engagement of MyD88, TIR domain-containing adapter protein

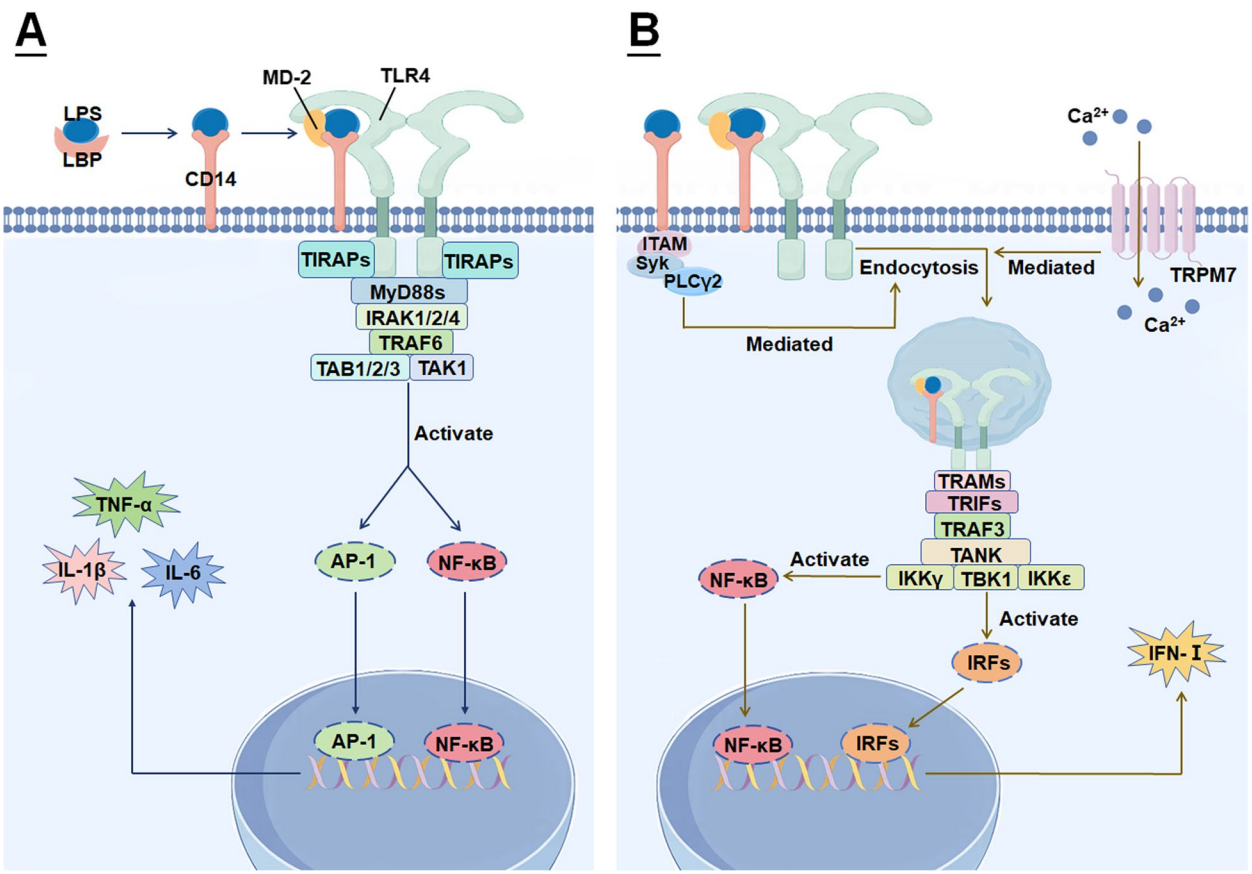

Fig. 2 LPS-TLR4 mediated signal transduction pathway. **A** In the MyD88-dependent signaling pathway, LPS stimulation triggers TLR4-MD-2 complex recruitment of MyD88 and TIRAP through its TIR domain, forming a signaling platform. This platform subsequently recruits IRAKs, TRAF6, TABs, and TAK1, completing the signal transduction cascade that activates AP-1 and NF-κB, leading to macrophage M1 polarization and subsequent inflammatory factor release. **B** In the MyD88-independent pathway, LPS stimulation induces TLR4-MD-2 complex endocytosis mediated by the CD14-ITAM-Syk-PLCγ2 complex and calcium influx, forming an endosome. This endosome recruits TRAM, TRIF, TRAF3, TANK, IKBKG, IKBKE, and TBK1, completing the signal transduction cascade that activates NF-κB and IRFs, ultimately mediating type-I IFN production

Table 1 Signaling molecules in MyD88-dependent signaling pathway

Signaling molecule	Function in LPS-TLR4 signal transduction	Reference
MyD88	Binding to the TIR domain of TLR4 and recruiting IRAKs	[46–49]
TIRAP	Combining with TIR domain of TLR4 and MyD88 to assist in recruiting IRAKs and mediating the phosphorylation of IRAK4	[37, 47–49]
IRAKs	Combining with MyD88 and recruiting TRAF6	[37, 50, 51]
TRAF6	Combining with IRAKs and forming K63/ml mutiple- ubiquitinated chains to recruit TABs and TAK1	[52–54]
TABs	Combining with K63/ml ubiquitinated chains and TAK1 to form a complex which mediates the activation and nuclear translocation of NF-κB and AP-1	[55–57]
TAK1	Combining with K63/ml ubiquitinated chains and TABs to form a complex that mediates the activation and nuclear translocation of NF-κB and AP-1	[57–59]

Table 2 Signaling molecules in MyD88-independent signaling pathway

Signaling molecule	Function in LPS-TLR4 signal transduction	Reference
TRAM	Interacting with TLR4 and recruiting TRIFs	[60, 61]
TRIF	Binding to TRAM through the TIR domain and interacting with TRAF3	[62]
TRAF3	Combining with TRIF and interacting with TANK to transmit signals, promoting activation of the type-I interferon response or inflammasome	[63, 64]
TANK	Binding to TRAF3, recruiting IKKγ, IKKε and TBK1	[65, 66]
IKKγ	Binding to TANK, activating NF-κB and promoting the activation of TBK1 and IKKε	[67–69]
IKKε	Combining with TANK to form a polymer platform which mediates the phosphorylation and nuclear translocation of IRFs and NF-κB	[68, 70–73]
TBK1	Combining with TANK, IKKγ to form a polymer platform which mediates the phosphorylation and nuclear translocation of IRFs and NF-κB	[71–73]

(TIRAP), interleukin-1 receptor-associated kinases (IRAKs), TNF receptor-associated factor 6 (TRAF6), TAK-binding proteins (TABs), and TGF-β-activated kinase 1 (TAK1) (Table 1), ultimately leading to NF-κB and activator protein-1 (AP-1) activation. Alternatively, the MyD88-independent pathway utilizes TRIF-related adapter molecule (TRAM), TIR domain-containing adapter molecule 1 (TICAM1, or TRIF), TNF receptor-associated factor 3 (TRAF3), TRAF family member-associated NF-κB activator (TANK), inhibitor of nuclear factor kappa-B kinase subunits γ/ε (IKKγ/ε), and TANK-binding kinase 1 (TBK1) (Table 2) to activate NF-κB and interferon regulatory factors (IRF), thereby initiating type-I interferon responses [14].

MyD88-dependent signaling transduction

MyD88

Initially identified by Lord et al. in 1990 for its role in IL-6-induced myeloid differentiation [74, 75], MyD88 was subsequently recognized as a crucial adaptor protein in TLR signaling and a central regulator of inflammatory responses [76, 77]. Human MyD88 (AlphaFold ID: AF-Q99836-F1) is a 33 kDa protein consisting of 296 amino acids with three functional domains: a death domain (DD, residues 54–109), an intermediate domain (ID, 110–155), and a TIR domain (159–296) (Fig. 3A) [74]. Upon TLR4 activation, MyD88's TIR domain mediates interactions with TIRAP and TLR4, while its DD recruits IRAKs to form a multiprotein complex. This multiprotein platform, comprising multiple MyD88 molecules, TIRAP, and IRAK family proteins, initiates downstream TLR4 signaling [46–49]. Notably, TLR4 signaling persists partially in MyD88-deficient conditions through TIRAP-mediated pathways [78]. Beyond LPS-TLR4 signaling, MyD88 also participates in TLR2 signaling and HMGB1 recognition through TIRAP interactions. Given its central role in TLR signaling, MyD88-deficient mice serve as valuable models for studying TLR deficiencies and exhibit increased susceptibility to various bacterial and parasitic pathogens [74, 78].

TIRAP

The identification of TIRAP, also known as MyD88-adaptor Like (MAL), emerged from Tiffany et al.'s

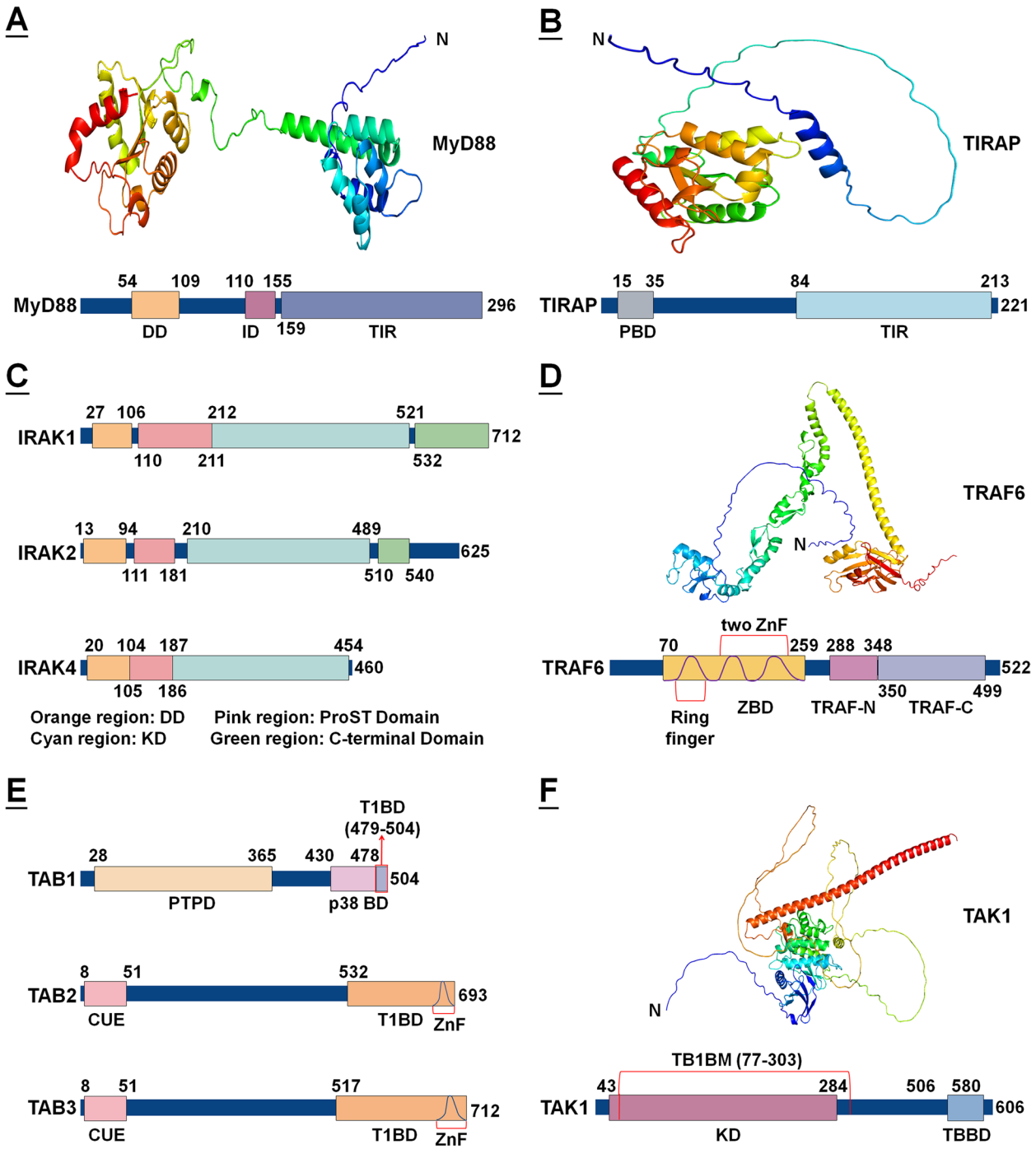

Fig. 3 The structural domains pattern diagram of MyD88, TIRAP, IRAKs, TRAF6, TABs, and TAK1. **A** MyD88 is composed of a DD, an ID, and a TIR domain. **B** TIRAP contains a PBD and a TIR domain. **C** IRAK1, IRAK2, and IRAK4 each have a DD, a ProSTD, and a KD. However, IRAK1 and IRAK2 also possess a C-terminal domain, which is absent in IRAK4. **D** TRAF6 features a ZBD, which includes a RING finger and two zinc ZnFs, along with a TRAF-N domain and a TRAF-C domain. **E** TAB1 is equipped with a PTPD, a p38 BD, and a T1BD. In contrast, TAB2 and TAB3 both contain a CUE domain and a T1BD that includes a ZnF. **F** TAK1 consists of a KD, a TBBD, and a TB1BM

2001 genome-wide search for TIR domain-containing sequences, highlighting the importance of this domain in TLR4 signaling [78]. Human TIRAP (AlphaFold ID: AF-P58753-F1) is a 24 kDa protein comprising 221 amino acids with two functional domains: a phosphatidylinositol 4,5-bisphosphate (PIP2) binding domain (PBD,

residues 15–35) that localizes TIRAP to PIP2-rich membrane regions, and a TIR domain (84–213) (Fig. 3B) [79]. TIRAP plays a pivotal role in MyD88-mediated signaling by bridging TLR4 and MyD88 through TIR domain interactions, facilitating IRAK4 phosphorylation and signal propagation [47–49]. Notably, TLR4 recruits two TIRAP homodimers, each capable of binding four MyD88 molecules, creating an eight-MyD88 signaling complex that amplifies downstream signals. While this amplification mechanism is universal, its efficiency varies across cell types due to differential TIRAP and MyD88 expression levels. Furthermore, evolutionary selection of the TIRAP S180L variant confers protection against septic shock, with global epidemiological data showing an inverse correlation between S180L prevalence and sepsis incidence [37]. Although the precise mechanism remains unclear, we hypothesize that S180L may attenuate NF-κB signaling, thereby reducing systemic inflammation. Beyond TLR4 signaling, TIRAP participates in multiple pathways, including phosphatidylinositol 3-kinase p85, B-cell adaptor for phosphoinositide three kinase, protein kinase C-δ, p38 MAPK, and c-Jun signaling [79].

IRAK

In 1996, Cao et al. identified IRAK in the IL-1R signaling pathway, characterized by its structural similarity to Pelle, a kinase crucial for NF-κB homolog activation [80]. The IRAK family comprises four serine/threonine kinases (IRAK1–4) ranging from 460 to 712 amino acids, which are highly conserved across vertebrates [50]. These proteins share four characteristic domains: a DD, a proline-serine-threonine-rich domain (ProSTD) serving as the primary phosphorylation site, a kinase domain (KD), and a C-terminal domain (CTD) containing TRAF6-binding motifs (TBMs) (Fig. 3C). Notably, IRAK4 lacks the C-terminal domain present in IRAK1 and IRAK2. Within the LPS-TLR4 signaling pathway, IRAK1, IRAK2, and IRAK4 play essential roles [50, 51].

Following the formation of the MyD88-TIRAP platform, MyD88's DD interacts with IRAK4's DD, creating a complex comprising six to eight MyD88 and four IRAK4 molecules. This stable interaction facilitates IRAK2 binding in a 4:4 stoichiometric ratio with IRAK4. Upon MyD88 binding, IRAK4 undergoes trans-autophosphorylation, subsequently recruiting and phosphorylating IRAK1 and IRAK2. This phosphorylation cascade triggers IRAK1/2 auto-hyperphosphorylation in their ProSTD, activating their kinase activity and inducing conformational changes that expose TRAF6-binding sites. This multi-protein complex, termed "myddosome", typically consists of six MyD88, four IRAK4, and four IRAK1 subunits, and is essential for NF-κB activation [37]. However, immunoprecipitation studies in mouse macrophages reveal preferential IRAK2 binding to the MyD88-IRAK complex, with IRAK1 detectable only during early complex formation or under low IRAK4 kinase activity conditions, suggesting complex regulatory mechanisms requiring further investigation. Notably, Tollip (Toll-interacting protein) serves as a key negative regulator of MyD88-IRAK complex formation. Through its C2 domain, Tollip binds IRAK1 to inhibit its phosphorylation and activity, while its Tollip-binding domain interacts with MyD88's TIR domain, disrupting complex assembly [50, 51].

TRAF6

In 1996, Cao et al. identified TRAF6 as a key signaling molecule in the IL-1 pathway through homology searches for TRAF2's TRAF-C domain on the NCBI database [81]. Human TRAF6 (AlphaFold ID: AF-Q9Y4K3-F1) is a 59 kDa protein comprising 522 amino acids with three structural domains: a zinc-binding domain (ZBD, residues 70–259) containing a RING finger and two zinc fingers, a TRAF-N domain (288–348), and a highly conserved TRAF-C domain (350–499) (Fig. 3D) [52]. Following IRAK phosphorylation in the MyD88-IRAK complex, TRAF6 is recruited through interactions mediated by its ZBD and TRAF-C domains. As an E3 ubiquitin ligase, TRAF6 collaborates with E2 enzymes Uev1A and Ubc13 via its ZBD to generate K63-linked polyubiquitin chains. These chains, when bound by linear ubiquitin assembly complexes, form K63/M1 hybrid chains that recruit TAK1-TAB kinase complexes, initiating downstream signaling cascades. Beyond TLR signaling, TRAF6 mediates pathways through interactions with CD40, RANK, and other receptors. Notably, among TRAF knockout mice (TRAF1–6), only TRAF6 deficiency results in osteopetrosis, highlighting its critical role in osteoclastogenesis. Furthermore, TRAF6 is essential for dendritic cell (DC) function, as TRAF6-deficient DCs exhibit severely impaired inflammatory cytokine production (e.g., IL-6 and IL-12) compared to wild-type counterparts [52–54].

TABs & TAK1

In 1994, Takahisa et al. identified TAK1 as a novel nuclear receptor superfamily member using polymerase chain reaction [82]. Two years later, Hiroshi S. et al. discovered TAB1 through yeast two-hybrid screening, demonstrating its direct interaction with TAK1 [55], marking the beginning of TAB family and TAK1 functional studies in TLR4 signaling. The TAB family, comprising TAB1, TAB2, and TAB3, plays essential roles in TLR4 signal transduction [14]. Human TAB proteins range from 504 to 712 amino acids, with molecular weights of 54 kDa (TAB1), 76 kDa (TAB2), and 78 kDa (TAB3).

TAB1 contains three domains: a PPM-type phosphatase domain (PTPD), a p38 binding domain (p38 BD), and a C-terminal TAK1 binding domain (T1BD). All TAB family members share a CUE domain (residues 8–51) and a ZnF-containing T1BD, except TAB1 (Fig. 3E), which is crucial for protein–protein interactions [55–57]. Human TAK1 (AlphaFold ID: AF-O43318-F1), existing primarily as the TAK1a splice variant, is a 67 kDa protein with 606 amino acids. Its structure includes a kinase domain (KD, 43–284) and a C-terminal TAB2/3 binding domain (TBBD, 506–580) containing a TAB1 binding motif (TB1BM, 77–303) (Fig. 3F) [58].

Upon formation of K63/M1-linked polyubiquitin chains by TRAF6, Uev1A, and Ubc13, TAK1 and TAB1/2/3 are recruited through their respective N- and C-terminal domains, assembling into a large signaling complex [57, 59]. This complex orchestrates IκB kinase and MAPK signaling cascades, ultimately regulating NF-κB and AP-1 activation [83]. Activated NF-κB and AP-1 then translocate to the nucleus, inducing M1 polarization and inflammatory gene expression (e.g., CD44) in macrophages, leading to inflammatory cytokine production [84–89]. The TABs-TAK1 complex is tightly regulated by multiple post-translational modifications that control its activity, stability, and assembly, including phosphorylation, acylation, ubiquitination, O-GlcNAcylation, methylation, and sumoylation. Phosphorylation of specific serine/threonine residues, particularly in TAK1's activation loop (Thr178, Thr184, Thr187, and Ser192), is crucial for NF-κB and AP-1 activation. Inflammatory stimuli trigger TAK1 autophosphorylation at Thr187 and Ser192, facilitating TAB2/3 binding. Notably, while TAB1 deficiency significantly impairs TAK1 kinase activity, it minimally affects Thr187 phosphorylation or TNF/IL-1-induced NF-κB nuclear translocation [59].

MyD88-independent signaling transduction

TRAM

Similar to Tiffany et al.'s approach, Katherine et al. identified TRAM in 2003 through a genome-wide search for TIR domain-containing genes [90]. That same year, its function as an adaptor in the TLR4-mediated MyD88-independent pathway was established [91]. Human TRAM (AlphaFold ID: AF-Q86XR7-F1), alternatively named TIRP or TICAM2, is a 27 kDa protein comprising 235 amino acids. Its structure includes an N-terminal domain (NTD, residues 1–39) featuring a myristoylation site at glycine-2 and a C-terminal TIR domain (73–229) (Fig. 4A) [37].

The activation of TRAM signaling requires endocytosis of the TLR4-MD-2 complex [92]. Upon LPS stimulation, the TLR4-MD-2 complex undergoes endocytosis with the cell membrane, forming an endosome. This process, termed "inflammatory endocytosis," depends on CD14 and calcium ion influx, mediated by tyrosine kinase Syk and phospholipase Cγ2 (PLCγ2) activation. It is further regulated by TYRO protein tyrosine kinase-binding protein (DAP12), integrin alpha-M (ITAM), and high-affinity immunoglobulin gamma Fc receptor (FcγR) [93, 94]. Notably, the membrane molecule TRPM7 also mediates calcium ion influx and participates in TLR4-MD-2 complex endocytosis [95]. Following LPS stimulation and endosome formation, TRAM detaches from the cell membrane and interacts with the TLR4 TIR domain on the endosome membrane via its N-terminal myristoylation site, recruiting TRIF via the TIR domain to initiate downstream signaling [60, 61].

TRIF

TRIF was initially identified through database screening of TIR domain-related sequences in the expression sequence tag database by Masahiro et al. in 2002 and subsequently characterized as a crucial adaptor in TLR signaling in 2003 [96, 97]. Structurally, human TRIF (AlphaFold ID: AF-Q8IUC6-F1) comprises 712 amino acids with a molecular weight of 76 kDa. The protein contains three functional domains: an N-terminal domain (NTD, residues 1–153) involved in auto-regulation, a TIR domain (residues 387–545), and multiple adaptor-binding motifs including a TBK1-binding motif (TBBM), TRAF6-binding motif (TF6BM), TRAF2-binding motif (TF2BM), and a C-terminal RHIM (receptor-interacting protein homotypic interaction motif) (Fig. 4B) [62].

Functionally, TRIF plays an essential role in NF-κB activation and IFN-β production within the MyD88-independent signaling pathway. The C-terminal region of TRIF regulates both NF-κB activation and apoptosis initiation. Upon activation of the TLR4-mediated MyD88-independent pathway, TRIF is recruited to endosomes by TRAM through TIR domain interactions. Subsequently, TRIF engages with other adaptor molecules (e.g., TRAF3) via its NTD to initiate and modulate downstream signaling. Notably, the NTD also functions as a negative regulatory element in this pathway by preventing the binding of downstream signaling molecules to TRIF [62].

TRAF3

In the 1990s, due to the unclear downstream signaling pathway of CD40, Hu et al. identified a novel TRAF family member, TRAF3, through yeast two-hybrid screening, demonstrating its interaction with the CD40 cytoplasmic domain [98]. Initially characterized for

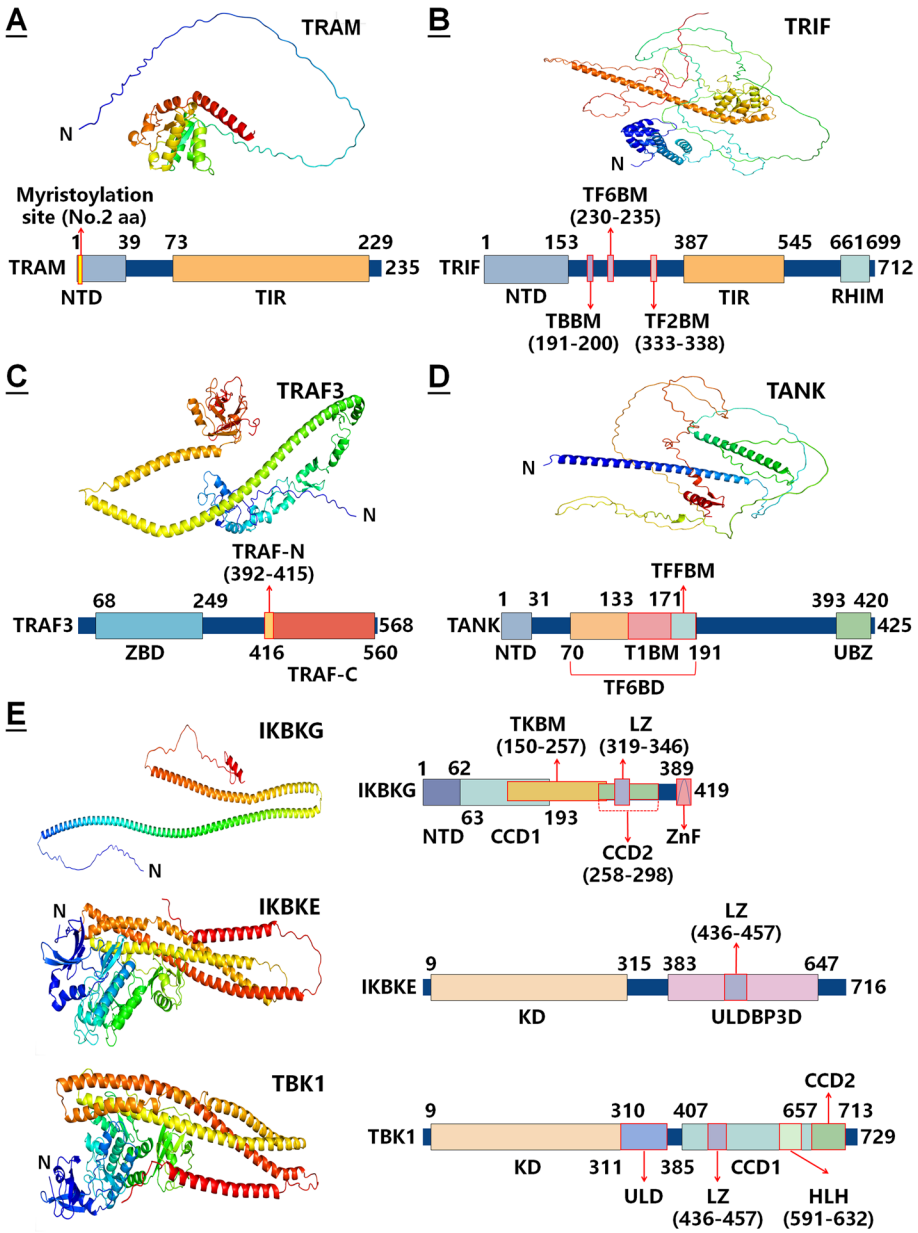

Fig. 4 The structural domain pattern diagram of TRAM, TRIF, TRAF3, TANK, and IKK family (IKBKG, IKBKE, and TBK1). **A** TRAM consists of an (NTD harboring a myristoylation glycine site and a TIR domain. **B** TRIF comprises an NTD, a TIR domain, and multiple functional motifs, including TBK1M, TF6BM, TFBM, and RHIM. **C** TRAF3 contains a ZBD, a TRAF-N domain, and a TRAF3 domain, similar to TRAF6. **D** TANK is composed of an NTD, a TF6BD, a UBZ, a TIBM, and a TFFBM. **E** IKBKG includes an NTD, two coiled-coil domains (CCD1 and CCD2), a TKBM, a (LZ, and a ZnF domain. IKBKE contains a KD and a ULDBP3D, which incorporates an LZ. TBK1 possesses a KD, a ULD, and two CCDs (CCD1 and CCD2), with CCD1 containing one LZ and one HLH motif

its role in CD40-mediated T lymphocyte-dependent immune responses and B lymphocyte activation [99, 100], TRAF3 was subsequently found to participate in interferon production [70], leading to its emerging recognition in TLR4 signaling. Structurally, human TRAF3 (AlphaFold ID: AF-Q13114-F1) consists of 568 amino acids with a molecular weight of 64 kDa, featuring three characteristic domains: an N-terminal zinc-binding domain (ZBD) containing a RING finger and two zinc finger (ZnF) structures, a C-terminal TRAF-N domain, and a highly conserved TRAF-C domain (Fig. 4C), sharing structural similarity with TRAF6 in the MyD88-dependent pathway. During TRIF signaling activation, TRAF3 is recruited by TRIF through its N-terminal

domain, potentially via the TRAF-C domain, facilitating IRF3 phosphorylation and subsequent signal transmission through TANK interaction [63]. Functionally, TRAF3 serves as an E3 ubiquitin ligase through its ZBD, promoting K63 activation and Lys-63-linked polyubiquitin chain formation to initiate type-I interferon responses. Additionally, TRAF3 negatively regulates the MyD88-dependent NF-κB pathway, but the precise mechanisms underlying this inhibitory effect require further investigation [63, 64].

TANK

In CD40 or type II TNFR-mediated signaling pathways, TRAF family members play crucial roles. In 1996, Cheng et al. identified a novel TRAF-associated protein, TANK, through yeast two-hybrid screening of TRAF3-interacting partners [101]. Human TANK (AlphaFold ID: AF-Q92844-F1) comprises 425 amino acids with a molecular weight of 48 kDa, featuring three structural domains: an N-terminal domain (NTD), a TRAF6-binding domain (TF6BD) containing a TRAF family binding motif (TFFBM) and a TBK1&IKKε-binding motif (TIBM), and a C-terminal ubiquitin-binding zinc finger (UBZ) domain (Fig. 4D).

During TRIF-mediated TRAF3 activation, TANK interacts with TRAF3 through its TF6BD or TFFBM, subsequently recruiting IKKε and TBK1 via TIBM [65]. While TANK can interact with IKKγ to mediate NF-κB and IRF3 activation, the specific TANK domain responsible for IKBKG binding requires further investigation. Additionally, TANK regulates TRAF function by maintaining TRAF2 in a latent state, preventing its binding to LMP1 and inhibiting LMP1-mediated NF-κB activation. Similar to TRAF3, TANK negatively regulates NF-κB signaling by reducing IKKγ and TRAF6 polyubiquitination, as evidenced by Wang et al.'s findings showing enhanced TRAF6 ubiquitination and hyperinflammatory responses in TANK$^{-/-}$ B cells, macrophages, and mice [66]. These regulatory functions position TANK as a potential therapeutic target for overcoming cancer treatment resistance and preventing autoimmune diseases.

IKKγ

In the late twentieth century, researchers recognized that IKK complex activation (IKKα and IKKβ) required phosphorylation, although the underlying mechanisms remained unclear. This prompted investigations into IKK complex composition and subunit functions. Using immobilized anti-IKKα immunoaffinity chromatography and other techniques, David et al. identified a novel IKK complex subunit, IKKγ (also termed IKBKG or NEMO (NF-κB essential modulator)) [102]. Human IKBKG (AlphaFold ID: AF-Q9Y6K9-F1) consists of 419 amino acids with a molecular weight of 48 kDa, featuring five structural domains: an N-terminal domain (NTD, residues 1–62), two coiled-coil domains (CCD1: 63–193; CCD2: 258–298), a Leucine zipper (LZ) domain (319–346), and a C-terminal zinc finger (ZnF) domain (389–419) crucial for ubiquitination, phosphorylation, and ubiquitinated protein recognition [67]. Additionally, IKBKG contains several binding motifs, including a TANK-binding motif (TKBM, 150–257) within the CCDs and a ubiquitin-binding motif (UBM, 242–350) (Fig. 4E). Functionally, IKBKG serves as a critical regulatory subunit in NF-κB signaling. Through its NTD, it binds to the NEMO-binding domain (NBD) of IKKα and IKKβ, forming a trimeric complex essential for NF-κB pathway signal transduction [67, 68]. In TLR4 signaling, IKBKG interacts with TANK via TKBM, subsequently activating NF-κB through ZnF-mediated phosphorylation while recruiting and activating TBK1 and IKKε via its CCDs [67, 69].

IKKε & TBK1

Robert et al. identified IKKε (also termed IKKE or IKBKE) in 2000 through sequence analysis of the previously discovered cDNA KIAA0151, demonstrating over 30% homology and structural similarity with IKKα and IKKβ [103]. The human IKBKE gene (AlphaFold ID: AF-Q14164-F1), located on chromosome 1q, encodes multiple splicing variants (IKBKEv1, IKBKEv2, and IKBKEv3) [68], with this review focusing on the full-length IKBKEv1 isoform. This 716-amino acid protein (molecular weight: 80 kDa) contains two functional domains: an N-terminal kinase domain (KD) and a C-terminal ubiquitin-like DEAD-box protein 3 interaction domain (ULDBP3D) featuring a LZ and helix-loop-helix (HLH) structure (Fig. 4E) [68]. TBK1, a novel IKK-related serine/threonine kinase first reported by Joel et al. in 1999 [104], shares 64% sequence homology with IKBKE at the primary structure level. The human TBK1 protein (AlphaFold ID: AF-Q9UHD2-F1) comprises 729 amino acids (molecular weight: 84 kDa) with four structural domains: an N-terminal KD, a ubiquitin-like domain (ULD), and two coiled-coil domains (CCD1 and CCD2). Notably, CCD1, also referred to as the scaffold dimerization domain (SDD), contains both LZ and HLH structures (Fig. 4E) [71].

Mature IKBKE is predominantly localized in the cytoplasm and phosphorylates multiple signaling molecules, including IκBα, IRF3, and IRF7. Upon TANK activation, IKBKE forms a complex with TBK1, which binds to the TIBM of TANK and IKBKG through CCD2, creating a polymeric platform. This platform mediates the phosphorylation and nuclear translocation of IRF3, IRF7, and NF-κB via its ULD and KD domains, ultimately triggering

type-I IFN responses [68, 70–73]. While the specific IKBKE domain interacting with TANK remains unclear, TBK1 is known to bind TANK through its CCD2. Additionally, SUMOylation of TBK1 at its C-terminal K694 induces spatial exclusion of certain adaptor proteins, enabling TBK1 to interact with other protein complexes and thereby enhancing its innate immune activation capabilities [71].

The relationship between TLR4 signaling transduction and other non-infectious clinical diseases

The TLR4 signaling pathway is implicated in diverse clinical conditions beyond infectious diseases. For instance, TLR4 has been associated with Parkinson's disease pathogenesis and plays roles in tumor growth and immune evasion [105, 106]. Downstream TLR4 signaling molecules also contribute to non-infectious diseases. In Tilstra et al.'s study, MyD88 regulates lupus progression in mice [107], and macrophage-specific MyD88 deficiency protects against non-alcoholic fatty liver disease-induced liver damage [108]. MyD88 also influences cardiovascular disease development [109]. TRAF6, whose knockout leads to osteosclerosis in mice, represents a potential therapeutic target for osteoporosis as mentioned above in part TRAF6. Similarly, TRAF3 contributes to inflammatory bowel disease pathogenesis [110]. In amyotrophic lateral sclerosis (ALS), TRIF deficiency accelerates disease progression more severely than MyD88 deficiency [111], while the TRIF-IFN pathway exacerbates Helicobacter pylori-induced gastric cancer [112]. TBK1 dysregulation or mutation underlies various neurodegenerative disorders, including frontotemporal dementia and Huntington's disease, and promotes tumor development in chronic and acute myeloid leukemia (AML). Inhibition of TBK1/IKBKE in AML cells reduces their activity, proliferation, and MYC oncogene expression. Furthermore, TBK1/IKBKE facilitates tumor immune escape and epithelial-mesenchymal transition [113]. TANK also contributes to tumorigenesis through NF-κB pathway regulation.

These findings demonstrate TLR4 signaling's involvement in multiple disease processes across cardiovascular, neurological, immune, skeletal, and oncological systems. Therefore, deeper molecular understanding of TLR4 signaling holds significant potential for advancing clinical research and therapeutic development.

Discussion and outlook

LPS plays a crucial role in the pathogenesis of various Gram-negative bacterial infections. The recognition of LPS during infection is complex, involving both extracellular receptors and intracellular processes [1]. As a key PAMP, LPS may interact with additional PRRs beyond TLR4 that remain to be identified. This review focuses on the extracellular recognition and inflammatory mechanisms mediated by LPS-TLR4 signaling, including the structural features of relevant signaling molecules (noting that structural data from UniProt may differ from literature reports). Finally, we briefly discuss the involvement of TLR4 signaling in non-infectious diseases.

TLR4 serves as a critical PRR for LPS, playing a pivotal role in LPS-induced inflammatory responses. The LPS-TLR4 signaling cascade can be summarized as follows: Upon Gram-negative bacterial invasion, serum LBP captures LPS and transfers it to macrophage CD14. CD14 then presents LPS to the TLR4-MD-2 complex through physical proximity, triggering two distinct signaling pathways: MyD88-dependent and MyD88-independent signaling. In the MyD88-dependent pathway, TIRAP binds to the TLR4 TIR domain, recruiting MyD88 to form a signaling platform. This complex recruits IRAKs, inducing their phosphorylation and subsequent interaction with TRAF6. TRAF6, in conjunction with Uev1A and Ubc13, generates K63/M1-linked polyubiquitin chains. These chains mediate the assembly of a larger complex containing TABs and TAK1, ultimately leading to NF-κB and AP-1 nuclear translocation. This process promotes macrophage M1 polarization and the release of inflammatory factors, contributing to disease pathogenesis. The MyD88-independent pathway involves CD14-ITAM-Cy2-Syk and TRPM7-mediated calcium influx, leading to TLR4-MD-2 complex endocytosis and TRAM recruitment. TRAM binds to the TLR4 TIR domain, recruiting TRIF (though the TRAM-TRIF stoichiometry remains unclear). TRIF then interacts with TRAF3 and TANK, forming K63 polyubiquitin chains that activate TANK. This complex recruits IKBKG, TBK1, and IKBKE, mediating NF-κB and IRF3/7 phosphorylation and nuclear translocation, resulting in type-I IFN production. Notably, signaling molecules like TRIF and TRAF3 provide negative feedback regulation to prevent excessive immune responses. TLR4 signaling components also participate in multiple other pathways, including IL-1R signaling, TLR2/3/5/7/8 signaling, skeletal muscle regulation, and TGF-β signaling [62, 114, 115].

Given the critical role of LPS-TLR4-mediated immune responses in Gram-negative bacterial infections, the LPS-TLR4 signaling pathway and its downstream molecules represent promising therapeutic targets for inflammatory diseases. Recent advances in this field, as outlined in the introduction, underscore the importance of understanding LPS-TLR4 signaling for developing anti-inflammatory treatments. This review provides a

comprehensive analysis of LPS-TLR4 signaling molecules, offering insights for future research directions and potential therapeutic strategies. The structural details of these signaling molecules may facilitate investigations into protein–protein interactions (PPIs) and protein-molecule interactions (PMIs), potentially leading to the discovery of novel signaling components. For instance, TIRAP, TRAM, and TRIF were identified through screening for TIR domain-containing sequences in the human genome, while the yeast two-hybrid system has proven valuable for identifying new PPI targets. The findings presented here also have implications for treating various systemic diseases. TRAF6, essential for osteoclastogenesis, represents a potential target for osteoporosis therapy. Similarly, TIRAP and its S180L mutant, which respectively promote and negatively correlate with sepsis progression, offer insights into sepsis mechanisms and treatment strategies. In cancer research, TANK's negative regulation of NF-κB suggests its potential in overcoming cancer cell resistance and inhibiting proliferation and migration. This review not only suggests multiple research directions for inflammatory and non-inflammatory diseases but also provides a detailed framework for understanding PPI/PMI mechanisms in relevant signaling pathways, establishing a solid foundation for exploring related therapeutic targets.

Conclusion

In conclusion, this review provides a detailed examination of the LPS-TLR4 immune response, focusing on the molecular structures and protein–protein interactions that drive this critical signaling pathway. By dissecting the roles of key molecules such as LBP, CD14, MD-2, and TLR4 in LPS recognition, and the subsequent MyD88-dependent and MyD88-independent signaling cascades, we have elucidated the mechanisms underlying the inflammatory and immune responses to Gram-negative bacterial infections. Our analysis highlights the dual nature of the LPS-TLR4 pathway, which, while essential for host defense, can also contribute to the pathogenesis of various inflammatory and immune-related diseases when dysregulated. The insights gained from this review not only deepen our understanding of the molecular basis of the LPS-TLR4 immune response but also pave the way for the development of targeted therapeutic strategies. By identifying potential drug targets within this pathway, we offer new possibilities for the treatment of a wide range of diseases, from infectious conditions to chronic inflammatory disorders. This comprehensive overview underscores the importance of continued research into the LPS-TLR4 signaling pathway, with the ultimate goal of improving clinical outcomes for patients affected by these diseases.

Abbreviations

AP-1	Transcription factors activator protein-1
CCD	Coiled-coil domain
CUE	Coupling of ubiquitin conjugation to endoplasmic reticulum degradation
DD	Dead domain
HLH	Helix-loop-helix
ID	Intermediate domain
IKK	Inhibitor of nuclear factor kappa-B kinase subunit
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
KD	Kinase domain
LRR	Leucine-rich repeat region
LZ	Leucine zipper
MD-2	Myeloid differentiation factor-2
MyD88	Myeloid differentiation primary response protein 88
NTD	N-terminal domain
PBD	Phosphatidylinositol 4,5-bisphosphate (PIP2) binding domain
ProSTD	Proline-serine-threonines domain
PTPD	Protein phosphatase Mg^{2+}/Mn^{2+}-dependent (PPM)-type phosphatase domain
p38 BD	P38 binding domain
RHIM	Receptor-interacting protein homotypic interaction motif
TAB	TAK-binding protein
TAK1	TGF-β-activated kinase 1
TANK	TRAF family member-associated NF-κB activator
TBBD	TAB2/3 binding domain
TBBM	TBK1 binding motif
TBK1	TANK-binding kinase 1
TB1BM	TAB1 binding motif
T1BD	TAK1 binding domain
TFFBM	TRAF family binding motif
TF2BM	TRAF2 binding motif
TF6BD	TRAF6 binding domain
TIBM	TBK1&IKKε-binding motif
TIRAP	TIR domain-containing adapter protein
TKBM	TANK binding motif
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adapter molecule
TRIF	TIR domain-containing adapter molecule 1
UBZ	Ubiquitin-binding ZnF
ULD	Ubiquitin-like domain
ULDBP3D	Ubiquitin-like (UL) DEAD-box protein 3 interaction domain
ZBD	Zinc-binding domain
ZnF	Zinc finger

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Authors' contributions

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare no competing interests.

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