

# TOLL-LIKE RECEPTORS AND THEIR CONTRIBUTION TO INNATE IMMUNITY: FOCUS ON TLR4 ACTIVATION BY LIPOPOLYSACCHARIDE

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*Summary:* Mechanisms of innate immunity are triggered as a result of recognition of evolutionarily conserved structures of microorganisms, named pathogen-associated molecular patterns. Their recognition is mediated by specialized receptors which initiate signalling cascades leading to expression of pro-inflammatory mediators and regulation of acquired immunity. Among several classes of such receptors, Toll-like receptors (TLRs) are extensively studied as they can sense an array of microbial cell wall and membrane components as well as single- and double-stranded RNA and DNA motifs typical for microorganisms. Each TLR consists of a ligand-binding domain containing leucine-rich repeats, a single transmembrane domain and a signalling TIR domain. After ligand binding, TLRs dimerize which facilitates the interaction of their TIR domains with adaptor proteins triggering signalling cascades. TLRs engage four common adaptor proteins, about ten signalling kinases, and a few transcription factors including NF $\kappa$ B, IRF and AP-1. In this review, special attention is paid to TLR4 activated by lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, since an exaggerated response to LPS may lead to potentially deadly septic shock. In recent years considerable progress has been made in the understanding of how the cooperation of several proteins, including CD14, TLR4/MD-2 complex and scavenger receptors, modulates the cell response to LPS. These studies have also revealed a dichotomy of signalling pathways triggered by TLR4 which depends on the participation of MyD88 and TRIF adaptor proteins and leads to the expression of genes encoding pro-inflammatory cytokines and type I interferons, respectively. The key event in the TRIF-dependent pathway is the internalization of activated TLR4.

*Key words:* Toll-like receptors, signal transduction, lipopolysaccharide, sepsis, innate immunity

## INTRODUCTION

A key role in the functioning of the vertebrate immune system is played by evolutionarily old mechanisms of innate immunity. These mechanisms allow for a rapid response directed against microbes which have successfully overcome the physical barriers protecting the body. They also participate in the mobilization and control of acquired immunity mechanisms, assuring maintenance of long-term immunological memory.

One of the most significant properties of the immune system is its ability to distinguish „foreign” microbe particles from „own” molecules of the host body. This distinction is important because of the need for early detection of pathogens and for avoiding the risk of developing autoimmune diseases. Another problem which must be confronted by the immune system is the need to recognize a large number of potentially hazardous species belonging to so variable microorganisms as bacteria, viruses, fungi and protozoa. This goal is fulfilled by *Pattern Recognition Receptors* (PRRs) present in cells such as monocytes, macrophages, neutrophils, dendritic and epithelial cells. These receptors are capable of recognizing evolutionarily conserved molecules typical for entire groups of microbes and absent from the host body. These molecules are called *Pathogen-Associated Molecular Patterns* (PAMPs) [39].

## PATTERN RECOGNITION RECEPTORS (PRRS)

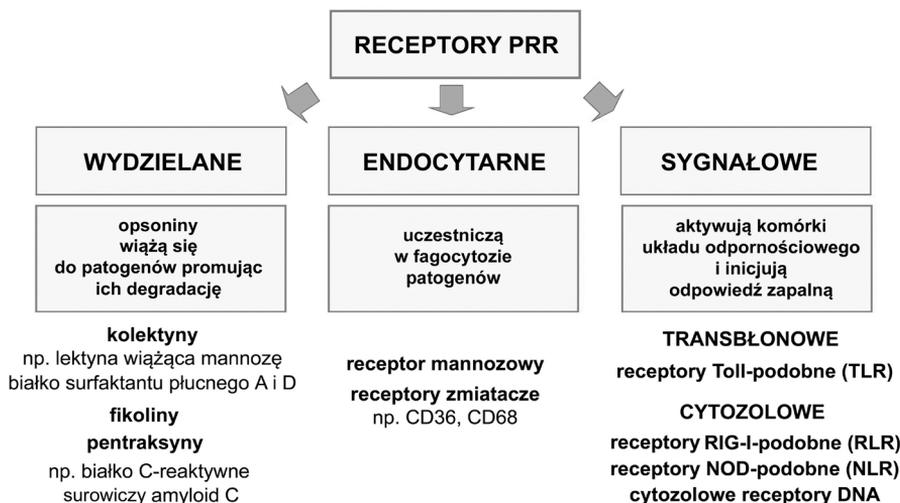
Typical examples of the molecular patterns recognised by PRRs are components of the bacterial cell wall and, in the case of Gram-negative bacteria, also components of the outer membrane of the bacteria. These structures contain molecules typical for bacteria, such as peptidoglycans, lipopolysaccharides and specific lipoproteins. Another significant group of ligands recognised by PRRs are nucleic acids. In this case their distinction from analogous host molecules is possible due formation of specific structures, the presence of specific sequences and differences in modifications of microbial nucleic acids (e.g., absence of methylated CpG motifs).

It is postulated that, apart from the recognition of pathogens, PRRs play an additional function by participating in responses to endogenous signals indicating tissue injury. Such signals can involve products of proteolysis of the extracellular matrix, which accumulate during tissue damage, and certain intracellular proteins, such as HMGB-1 (*High-Mobility Group Box-1*), released during cell apoptosis [84]. Such endogenous molecules capable of activating the immune system with an involvement of PRRs are termed DAMPs (*Damage/Danger-Associated Molecular*

*Patterns*) [3]. Nevertheless, it should be noted that according to some investigators the ability of DAMP molecules to directly activate PRRs is questionable. It is suggested that the endogenous factors just increase the sensitivity of cells to trace amounts of PAMPs [10].

Depending on their mode of functioning PRRs are divided into three classes: secreted receptors, receptors which mediate phagocytosis, and signalling receptors (fig. 1). The former comprise soluble molecules of opsonin type which bind to the surface of pathogens, facilitating their internalization and initiating classical and lectin pathways of complement activation. PRRs engaged in phagocytosis are transmembrane proteins which mediate internalization of pathogens, leading to degradation of the internalized particles and presentation of antigens released from them. The third class of PRRs comprises several receptor families with signalling domains which are capable of activating transcription of genes coding for pro-inflammatory proteins. They include:

- *Toll-Like Receptors* (TLRs) – the most thoroughly characterized family of PRRs described in greater detail below;
- *RIG-I-Like Receptors* (RLRs) – a family of three cytoplasmic helicases which detect double-stranded viral DNA. Two of them, RIG-1 and MDA-5, containing CARD (*Caspase Activation and Recruitment Domain*), induce a signalling pathway leading to the synthesis of type I interferons; the third member of the family, LPG, lacks the CARD domain and functions as a positive regulator of RIG-1 and MDA-5 [66]. RLRs have been shown to take part in recognition of viruses belonging to families of Flaviviridae (e.g., hepatitis C virus), Picornaviridae (e.g., Polio virus) and Paramyxoviridae (e.g., Newcastle disease virus);
- *NOD-Like Receptors* (NLRs) – large family of receptors (23 such receptors have been identified in humans and 34 in mice) which recognize PAMPs in the cytoplasm. The receptors may carry various effector domains, the most typical of which include CARD, PYD (*Pyrin Domain*) and BIR (*Baculovirus Inhibitor Repeats*) domains. The numerous ligands recognized by the receptors include peptidoglycan, its component  $\gamma$ -D-glutamyl-*meso*-diaminopimelic acid, muramyl dipeptide from the cell wall of Gram-positive bacteria, flagellin, and compounds released due to cell damage (e.g., uric acid) and certain bacterial toxins. Activation of these receptors can lead to formation of a multimolecular complex termed the inflammasome, responsible for promotion of inflammatory processes and capable of initiating a specific form of apoptosis, pyroptosis [4, 39];
- cytosolic receptors of DNA – a group of mostly unidentified proteins, responsible for initiation of production of type I interferons in response to plasmid, viral or bacterial DNA in the cytoplasm. It is postulated that the process progresses with an involvement of DAI, STING and ATG9a proteins [39].



**FIGURE 1.** Classification of pattern recognizing receptors depending on their function. The receptors mediating phagocytosis are located in the plasma membrane of phagocytic cells, such as macrophages and dendritic cells. TLRs are present also in other cells of immune system, as well as in epithelial and endothelial cells, and in fibroblasts. RLRs and some NLRs (e.g., NOD1) are present in the cytoplasm of most cell types

It is worth noting that despite the significant structural variability of both the ligands and the PRRs binding them, the scheme of the inflammatory response activation and its course remain similar. Typical for PRRs is the effect of narrowing the numbers of different molecules engaged in cell activation, starting at a few hundreds types of recognised PAMPs, through few tens of receptors and over ten adaptor proteins linked to them, to just about ten main kinases engaged in signal transduction and several transcription factors controlling large groups of genes. The main kinases involved in PRR signalling include those belonging to the IRAK (*Interleukin-1 Receptor-Associated Kinase*), TRAF (*TNF Receptor-Associated Factor*), MAPK (*Mitogen-Activated Protein Kinase*) and IKK (*I $\kappa$ B Kinase*) families.

Finally, the signalling pathways triggered by TLRs, RLRs and NLRs lead to activation of just a few main transcription factors: NF $\kappa$ B (*Nuclear Factor  $\kappa$ -light chain-enhancer of activated B cells*), AP-1 (*Activator Protein-1*) and IRF 3, 7 and 5 (*Interferon Regulatory Factors*). The first two mobilize expression of hundreds of genes responsible for production of cytokines, chemokines and pro-inflammatory enzymes. The most important compounds of this type include tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ), interleukins IL-1, -6, -8, -10, -15 and -18, and chemokines MIP-1, MCP1, 2 and 3. Transcription factors IRF3 and IRF7 regulate expression of genes coding for type I interferons, IFN- $\alpha$  and IFN- $\beta$ .

The main effect of expression of genes under control of these transcription factors is initiation and regulation of inflammatory responses as well as activation of the complement system, stimulation of phagocytes' activity, and apoptosis of infected cells. Moreover, the role of PRRs in activation and control of acquired immune responses has become increasingly better understood the recent decade [24, 67]. Activation of PRRs is known to positively affect expression of genes encoding molecules of the main histocompatibility complex and co-stimulatory molecules, such as CD80 and CD86, eventually promoting activation of T helper cells. On the other hand, cytokines released by cells in response to activation of PRRs induce differentiation of T lymphocytes into one of the subpopulations, e.g., Th1 or Th2, determining the subsequent immune reactions [46]. For example, activation of TLR4 stimulates secretion of interleukins, including IL-1, IL-12 and IL-18, promoting development of Th1-type response [67]. Activation of TLRs may also modulate the course of immune reactions through their effect on the functioning of regulatory cells, such as lymphocytes Treg CD4<sup>+</sup> CD25<sup>+</sup>FoxP3<sup>+</sup> [16].

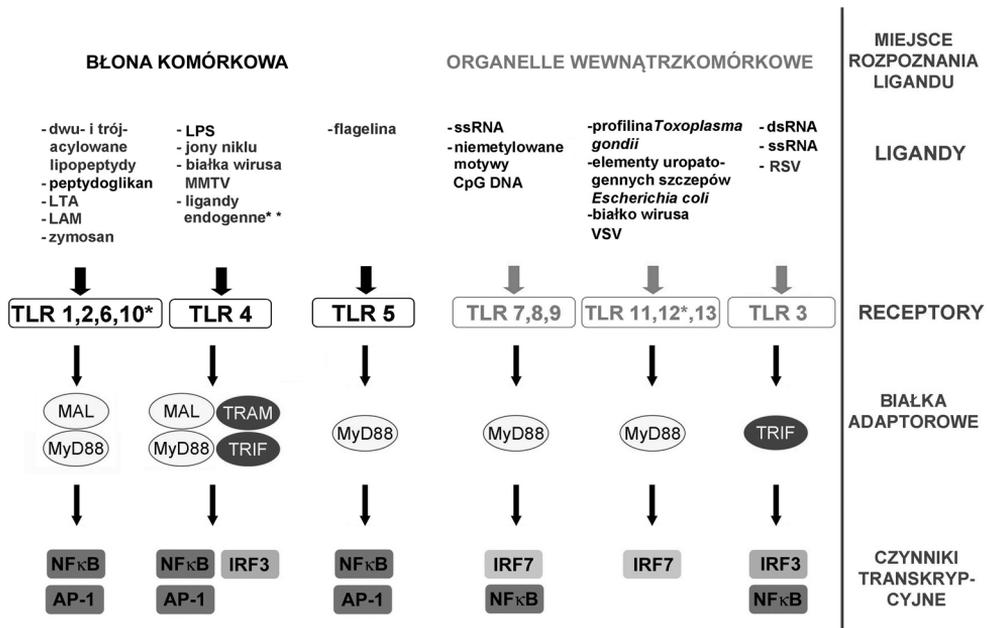
## TOLL-LIKE RECEPTORS (TLRS)

### GENERAL CHARACTERISTICS

TLRs form a family of transmembrane proteins with a similar, three-domain structure and high homology to the Toll protein of *Drosophila melanogaster*. „Toll” in German means „fantastic”, „mad” and was used for the first time by Cristina Nüsslein-Volhard and Kathryn Anderson to denote a gene whose mutation in mother leads to a disturbed development of *Drosophila* embryo along its dorso-ventral axis [1]. Subsequent studies in the Jules A. Hoffmann's group demonstrated that the Toll protein is of key importance for the resistance of the fruit fly to fungal infections [41]. A protein of the Toll family was linked for the first time with vertebrate immunity at the end of 1990s owing to studies conducted, among others, by the Bruce Beutler's team [48, 59]. For their discoveries on the activation of innate immunity J.A. Hoffmann and B. Beutler received the Nobel prize in medicine or physiology for 2011. Subsequent screening of sequenced vertebrate genomes has led to the identification of about 100 TLR genes. Genes encoding TLRs have been also detected in the genomes of invertebrates as diverse as the nematode *Caenorhabditis elegans*, the horseshoe crab *Tachypleus tridentatus*, and the sea urchin *Strongylocentrotus purpuratus*. Since proteins structurally and functionally similar to TLRs have been also detected in plants, these receptors seem to represent one of the most ancient component of the host defence mechanisms [42, 14].

Until now, thirteen TLRs have been identified and described in mammals. Twelve of them are expressed in mice and ten in humans. They were grouped into six families on the basis of amino acid sequence homology (fig. 2). In general, each subfamily of the receptors recognize one vast class of PAMPs and most of vertebrates carry at least one receptor of each subfamily. TLRs are present in cells of the immune system, epithelial cells of the intestines (TLR4 and 5), urinary bladder, lungs, liver and kidneys (TLR11). Recently, receptors of the TLR11-13 group were detected in cells of the mammalian central nervous system [64, 50]. In humans the entire TLR11-13 subfamily is represented only by a pseudogene, which is supposed to condition the sensitivity of humans to infections with uropathogenic strains of *Escherichia coli*. In mice, on the other hand, the gene coding for TLR10 is inactive [18, 86].

TLRs can also be classified into two groups based on their location within a cell (fig.2). Receptors of subfamilies TLR1 (TLR1, 2, 6 and 10), TLR4 and TLR5 subfamilies are present mainly in the plasma membrane. Due to their localization on



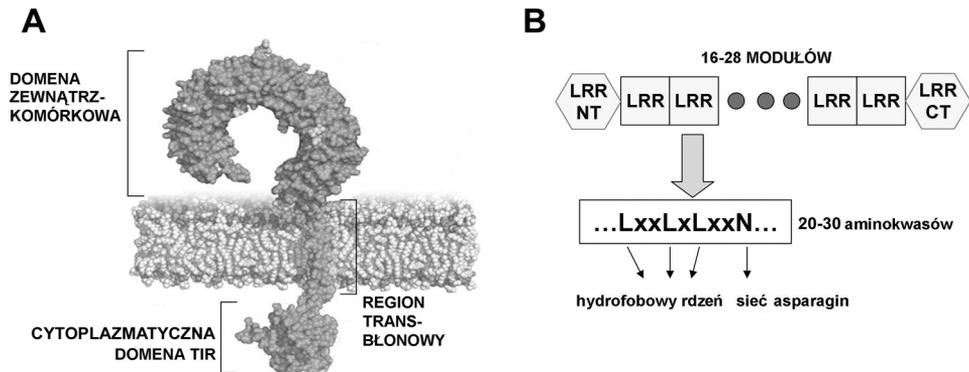
**FIGURE 2.** Classification and properties of mammalian TLRs. \*Ligands of TLR10 and TLR12 have not yet been identified. \*\*Some studies indicate that TLR4 and TLR2 are able to bind endogenous molecules generated upon tissue and cell damage. A putative list of such molecules includes heat shock proteins, nuclear proteins and components of the extracellular matrix [3]. Other studies argue against this possibility and posit that endogenous molecules can increase sensitivity of cells toward minute amounts of PAMPs [10]. LTA – lipoteichoic acid, LAM – lipoarabinomannan, RSV – respiratory syncytial virus, MMTV – mouse mammary tumour virus, VSV – vesicular stomatitis virus, ssRNA – single-stranded viral RNA, dsRNA – double-stranded viral RNA. Based on [34, 39]

the cell surface they recognize elements of the cell walls, capsules and motility apparatus of pathogenic microbes. On the other hand, receptors of subfamilies TLR3, TLR7-9 and TLR11-13 are located in intracellular organelles, such as endosomes, and take part mainly in the detection of nucleic acids of pathogens. Until recently, TLR11 was thought to be a surface receptor but further studies have confirmed its intracellular localization [58].

Individual TLR subfamilies also differ between each other in their preference for adaptor proteins, which interact with the receptors and allow for generation of signalling pathways (fig.2). Until now, five adaptor proteins used by TLRs have been identified: MyD88 (*Myeloid Differentiation primary response gene 88*), TIRAP/Mal (*TIR domain-containing Adaptor Protein/MyD88-adaptor-like*), TRIF/TICAM-1 (*TIR domain-containing adaptor inducing IFN- $\beta$ /TIR-Containing Adaptor Molecule 1*), TRAM/TICAM2 (*TRIF-Related Adaptor Molecule/TIR-Containing Adaptor Molecule 2*) and SARM (*Sterile  $\alpha$ - and HEAT/armadillo motif-containing protein*), of which the first four participate in various combinations in signal generation while SARM is a negative regulator of the TRIF-dependent pathway [77, 5]. Except TLR3, most TLRs mobilize signalling pathways with participation of MyD88, while TRIF is involved in signalling by TLR3 and TLR4 only [77, 34]. The binding of distinct adaptor proteins by TLRs leads to the generation of distinct signalling pathways, which provides one of the mechanisms allowing a better adjustment of the cellular response to the arising danger.

## STRUCTURE OF TLRs

TLRs are type I glycoproteins with a single transmembrane helix which links the N-terminal domain responsible for ligand binding with the signalling domain at the C-terminus of the receptor (fig. 3A). Analysis of the amino acid sequences and crystal structures of several TLRs pointed to a similar, evolutionarily conserved scheme [7, 27, 36, 45, 57]. The domain located at the N-terminus of the receptors is usually termed “ectodomain” but it should be remembered that in cases of intracellular receptors the domain is directed toward the lumen of the organelles. In all TLRs this domain consists of 16-28 leucine-rich repeats (LRRs) [47] (Fig. 3B). One of the best characterized is TLR4 receptor, activated by lipopolysaccharide (LPS), which contains 22 such motifs. The LRR motif consists of 20-30 amino acids of an evolutionarily conserved sequence LxxLxLxxN (where L represents leucine, N – asparagine, x – any amino acid) and a variable region. The extracellular domain, containing a few to several dozens of LRR motifs, has been detected in over 6000 proteins. For around 50 of these proteins, including a few TLRs, their crystal structures has been solved: all of them have a similar horseshoe-like shape (Fig.3A). The LxxLxLxxN sequences are located on the concave side of the horseshoe-like struc-



**FIGURE 3.** Structure of Toll-like receptors. **A** – Model of TLR monomer in the plasma membrane exemplified by TLR3 (acc. to [45], modified). **B** – Extracellular domain of TLRs containing leucine-rich repeats LRR (acc. to [31], modified). The LxxLxLxxN sequences form parallel  $\beta$ -strands building the concave part of the domain. The convex part consists of variable residues of the LRR motifs (not shown) which form helices and loops

ture within the parallel  $\beta$ -strands that form it; the variable regions of LRRs, on the other hand, form the outer, exposed surface of the horseshoe and can play significant functions in interactions with ligands. The leucine residues together with the other hydrophobic amino acids of LRR motifs form a continuous hydrophobic core of the TLR ectodomain. The domain structure is additionally stabilized by numerous hydrogen bonds of the asparagine residues of the LRR motifs [26, 38].

Ectodomains of several TLRs conform to such a „typical” structure of LRR motif-containing proteins. In the case of TLR4, however, and also the entire TLR1 subfamily, the amino acid sequences of the LRR motifs in the central portion of the ectodomain diverge from the evolutionarily conserved pattern: the motifs lack the typical asparagine network which stabilizes their structure, and moreover, they manifest variability also in the number of amino acids forming the motifs. The differences in the structure of the LRR motifs result in the fragmenting of the receptor ectodomain into three subdomains and in the exposure of inner pockets of a hydrophobic character [27, 32, 36]. At present, the structural divergences are postulated to play a role in adaptation of the receptors to binding of ligands of a specific chemical character. Receptors with a typical structure, such as TLR3 and TLR7-9, bind hydrophilic ligands, such as nucleic acids, using the outer surface of the ectodomain. On the other hand, receptors with the distinct three subdomains are capable of binding hydrophobic ligands using the exposed inner hydrophobic pockets. In such a way TLR1, 2 and 6 recognize bi- and tri-acylated bacterial lipopeptides and lipoproteins [32]. TLR4 is the only receptor of the TLR family that does not bind its ligand LPS directly but requires an assistance of a co-receptor, MD-2 protein, for

the binding [35, 52, 55]. The hydrophobic pockets present in TLR4 ectodomain are thought to allow for dimerization of the receptor through binding of one of the six lipid chains in LPS by one of the receptors while the remaining lipid chains of LPS remain buried in a hydrophobic pocket of MD-2 protein associated with a neighboring TLR4 molecule [61].

It should also be added that TLRs, similarly to other proteins containing LRR motifs, carries at their amino- and carboxyl-termini so called LRRNT and LRRCT domains (fig. 3B). These domains contain no LRR motifs but include cysteine residues forming disulphide bonds, in this way additionally stabilizing the structure of the ectodomain and protecting its hydrophobic core from exposure to the environment. Another feature of TLRs is the presence of evolutionarily conserved amino acid sequences in the extracellular domain (mainly on its surface), where N-glycosylation takes place. This modification is believed to be important for ligand recognition and localization and transport of the receptors [70, 78].

The cytoplasmic domain of TLRs, responsible for initiation of signal generation, is the TIR (*Toll/IL-1receptor homology*) domain, also present in receptors for interleukins and in adaptor proteins of TLRs; due to its presence these three protein families are considered to form a single superfamily. The TIR domain contains around 200 amino acid residues. They form a centrally located  $\beta$  sheet of five strands  $\beta$ A- $\beta$ E, surrounded by five helices,  $\alpha$ A- $\alpha$ E. Loops which link the elements protrude over the domain surface and most probably play a role in its multimerization required for the formation of a signalling complex [54, 81].

Analysis of the crystal structures of several TLRs from various subfamilies in complexes with their ligands [27, 32, 45, 57] or antagonists [36, 55] permitted not only the identification of properties common for all TLRs but also suggested a common model for activation of these receptors [26, 31]. The binding of a ligand induces local subtle changes of the receptor conformation allowing for its dimerization, which, with the exception of the TLR1-TLR2 and TLR2-TLR6 complexes, is homodimerization. Despite the differences in the ligand-binding sites between various TLRs, the arising complex has basically the same shape, resembling the letter M, due to bringing together of the ectodomains in the juxtamembrane region. This, in turn, leads to an approximation of the cytoplasmic domains of the two receptors and their arrangement in an orientation which promotes their dimerization. This is accompanied by conformational alterations in the receptor TIR domain, allowing for their association with the TIR domains of adaptor proteins [53].

Recent studies suggest that TLR4 differs in this respect from the other TLRs. The cytoplasmic and transmembrane domains of TLR4 have an intrinsic capacity to dimerize, in contrast to other receptors of the family [56]. The role of the TLR4 ectodomain is to inhibit the interactions until the specific ligand, LPS, is bound. It seems that such a system leads to a much more potent cell activation than that of the other TLRs. Studies on a constitutively active TLR4 in which the extracellular domain

was substituted by the corresponding domain of CD4 protein have demonstrated that TLR4 induces expression of pro-inflammatory genes with several-hundred-fold higher potency than the remaining receptors of the family [17]. Since septic shock, a potential effect of infection with Gram-negative bacteria, poses a serious threat to patients of intensive care units, both TLR4 and the mechanisms of its activation by LPS are particularly intensely studied by groups investigating the mechanisms of innate immune responses.

## **TLR4 TRIGGERS INFLAMMATORY RESPONSE IN REACTION TO LPS**

### **RECOGNITION OF LPS BY TLR4**

Lipopolysaccharides are a group of compounds with a similar structure, which constitute the main component of the outer membrane of Gram-negative bacteria [69]. Due to their crucial role in the maintenance of the bacterial cell structure, the LPS structure is highly conserved in evolution, providing a model example of PAMP. LPS strongly activates cells of the immune system, inducing an inflammatory response. An excessive reaction to LPS and presence of high LPS concentrations may lead to a systemic inflammatory reaction termed sepsis [65]. Severe sepsis is linked to life-threatening multiple organ dysfunction and the subsequent refractory hypotension provides grounds for diagnosis of septic shock, which in over 30% of cases leads to the patient's death.

The structure of LPS isolated from several types of bacteria and its modifications have been revealed in detail and described in earlier reviews [11, 60, 62]. LPS consists of three components: the polysaccharide chain termed the O antigen, the core and lipid A which anchors the LPS molecule in the outer membrane of Gram-negative bacteria. The greatest variability is observed in the composition and number of sugar residues forming the O antigen. Over 60 types of sugar residues and 30 non-sugar elements modifying them have been identified as components of the O antigen. The most frequently encountered sugars include hexoses such as glucose, fructose, galactose, mannose and rhamnose. In certain species of bacteria or in specific growth conditions the O-specific chain may be absent or expressed in a truncated form. This results in a „rough” phenotype of bacterial colonies in contrast to the typical „smooth” colonies. It is usually considered that the length and sugar composition of the O antigen do not affect the biological activity of LPS and its high variability make it unlikely to provide an easy target for PRRs. Nevertheless, an absence of the O-specific chain modulates the process of LPS recognition by cells of the immune system, as noted in cases of LPS originating from *Brucella*

*sp.* and *Salmonella sp.* and also from *Escherichia coli* [8, 25, 63]. Rough LPS, in contrast to LPS with a complete O-specific chain, activates certain cell reactions without an involvement of the CD14 protein [25, 21].

The core of the LPS molecule is also an oligosaccharide and contains a residue of 3-deoxy-D-manno-octulosonic acid (KDO) linked to the proximal glucosamine residue of lipid A. The KDO is usually substituted by subsequent residues of KDO and by residues of L-glycero-D-mannoheptose (Hep). The set of 1-3 KDO and 2 or 3 Hep constitutes the so called „inner core” to which three hexoses are typically attached (the „outer core”). The structure of this part is significantly less variable than that of the O antigen but may still contain various modifications. The core oligosaccharide is thought to be insignificant for the full biological activity of LPS but may exert some modulatory effect [11].

Lipid A is the most evolutionarily conserved part of LPS. It is the part recognised by receptors of the immune system and, thus, it is responsible for the pro-inflammatory activity of LPS [62]. The basal structure of lipid A is formed by two glucosamine molecules linked by a  $\beta$ -1,6 glycosidic bond and to this sugar skeleton, 4 to 7 fatty acid residues (depending on bacterial species) of a variable length are attached. The number and length of the fatty acid residues are the main source of lipid A heterogeneity among bacteria and also are the most significant factor affecting the biological activity of LPS. The presence of six chains, each comprising 12 to 14 carbon atoms, affords maximal immune cell activation while addition or removal of just a single chain reduces the pro-inflammatory activity of LPS by over two orders of magnitude [11, 62]. Another factor which determines the ability of LPS to activate the immune system involves phosphorylation of lipid A glycosamines [62]. These two structural properties determine the affinity with which LPS binds MD-2 and TLR4 proteins.

The recognition of LPS is a multi-stage process and it requires co-operation of a number of proteins, the LPS-binding protein (LBP), the CD14 protein and the TLR4/MD-2 complex. LBP is a 60-kDa glycoprotein which participates in detaching LPS monomers from micelles or directly from the bacterial outer membrane and allows for their transport to the CD14 protein. The closely related and structurally similar bactericidal/permeability increasing (BPI) protein binds LPS with a much higher affinity but does not transfer it to CD14. BPI is postulated to be important in LPS detoxification [79]. LBP and CD14 allow for concentration of LPS molecules before transferring them to the TLR4/MD-2 complex assuring a pronounced and rapid cellular response even to low concentrations of LPS (1 ng/ml and even lower) in the environment.

CD14 is a 56-kDa protein containing LRR repeats in its extracellular part, in which it resembles TLRs. CD14 forms homodimers and in such a form binds LPS through a hydrophobic pocket located at the outer surface of the dimer [49, 37]. CD14 contains no transmembrane domain and is incorporated in the outer leaflet of

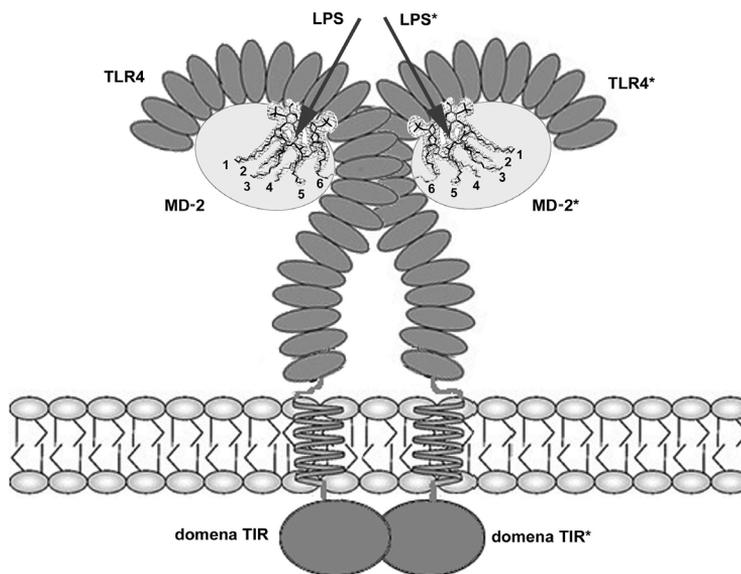
the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor attached to the C-terminus of the protein. The extracellular phospholipase C may hydrolyse the ester bond linking the acyl group which anchors CD14 in the plasma membrane with the remaining fragment of the GPI anchor. This leads to a release of CD14 in its soluble form (sCD14), which allows for recognition of LPS by cells devoid of CD14 in the membrane-bound form (mCD14). The presence of the GPI anchor in the membrane form of CD14 points to the association of the protein with so called lipid rafts. These are plasma membrane microdomains enriched in sphingolipids and cholesterol, distinguished from the surrounding more liquid regions of the plasma membrane containing mainly glycerophospholipids. Current views on the raft structure assume that they are dynamic, nano-scale accumulations of lipids and few proteins which during activation of immunoreceptors such as T cell receptor form more stable platforms facilitating the arrangement of protein complexes taking part in signal transduction [44]. The anchoring of CD14 in lipid rafts suggests the existence of a similar mechanism in the assembly of the CD14/TLR4/MD-2 signalling complex [71]. Of particular interest are experimental data which confirm the assumption, originating from recent investigations based on manipulation of the lipid composition of the rafts in the plasma membrane: enrichment of the plasma membrane in cholesterol due to a mutation of ABCA1 transporter or incorporation of saturated fatty acids to the membrane induced association of TLR4 with the raft fraction, dimerization of the receptor and expression of genes for pro-inflammatory cytokines [80, 87].

Commonly, CD14 and LBP together are assumed to play a role of sensors which effectively capture LPS and transfer it to the TLR4/MD-2 complex. However, CD14 may also have other functions in the process of cell stimulation by LPS. In macrophages and dendritic cells isolated from mice devoid of CD14 protein one of two signalling pathways triggered by TLR4 was found to be impaired (see below). The impaired pathway is dependent on the involvement of TRIF adaptor protein, is linked to endocytosis of activated TLR4 and leads to production of type I interferons [85]. CD14 participates also in LPS internalization by cells along a pathway which fails to activate them but allows for removal of LPS from the environment and for its intracellular detoxification [9].

Within the pathway which activates the pro-inflammatory response of cells, the transfer of LPS from CD14 to the TLR4/MD-2 complex represents the last stage of recognition of the molecule and begins the signal transduction process. MD-2 has a hydrophobic cavity surrounded by two  $\beta$ -sheets, which forms a broad pocket binding the lipid part of the LPS molecule [55]. MD-2 protein is linked to the outer surface of TLR4 by hydrogen and ionic bonds. The binding of LPS to the MD-2 pocket results in formation of a complex consisting of two TLR4/MD-2 heterodimers (fig. 4). In this process the LPS molecule plays a role of a bridge in the case of the optimal LPS structure containing six fatty acid residues, of which five fill the

MD-2 pocket while the sixth is partially uncovered and interacts with the hydrophobic pocket of the neighbouring TLR4 molecule. The linkage is additionally stabilised by two phosphate groups of lipid A which form ionic and hydrogen bonds with lysine and arginine residues on the surface of the neighbouring TLR4 and MD-2 molecules [57]. Despite their proximity, direct interactions between the two TLR4 molecules remain weak. This model explains the significance of the role played by lipid A structural modifications for biological activity of LPS, since an absence of the phosphate groups or deviations from the optimum number of fatty acid residues do not allow the formation of a stable signalling complex.

TLR4 was not known to initiate signalling cascades in response to LPS until 1998 [59]. Earlier studies pointed to an involvement in this process of other receptors, such as integrins CD11/CD18b, complement regulatory protein CD55, and scavenger receptors [12]. Studies conducted in the recent decade, based on an analysis of fluorescence resonance energy transfer between labelled proteins and on biochemical analysis of cellular fractions have demonstrated that LPS-induced cell activation leads to formation of complexes in the plasma membrane including

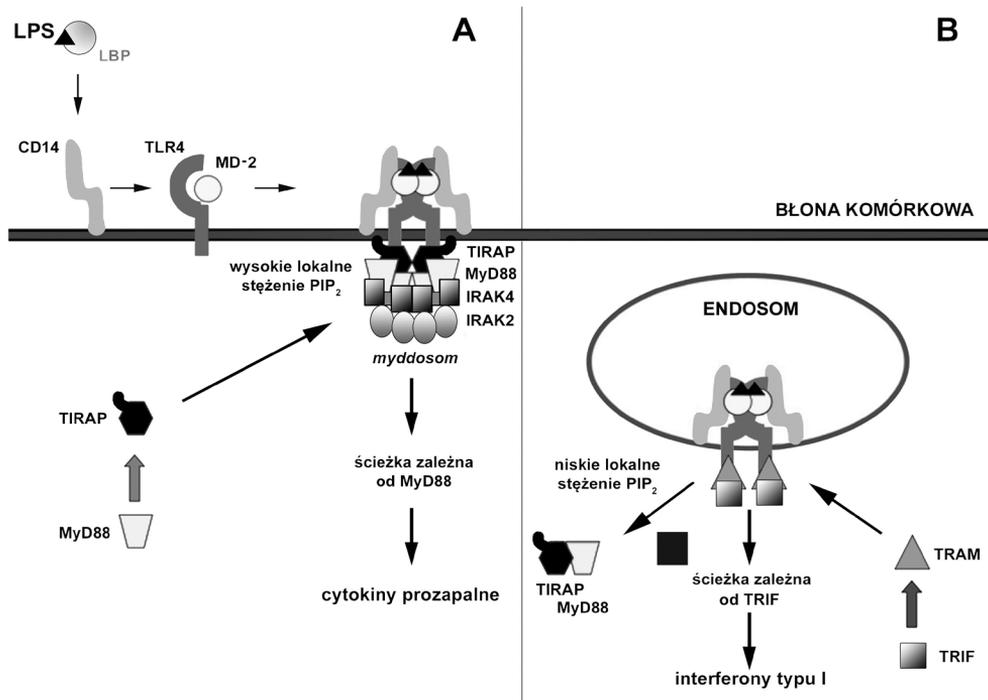


**FIGURE 4.** Structure of TLR4/MD-2 complex after LPS binding. Five of six fatty acid residues of LPS molecule are buried in the hydrophobic pocket of MD-2 while the sixth residue interacts with a hydrophobic fragment of an adjacent TLR4 molecule. This interaction induces dimerization of TLR4 which brings into proximity cytoplasmic signalling TIR domains of the two TLR4 molecules, facilitating their interactions with adaptor proteins. The complex of two TLR4/MD-2 heterodimers with LPS bound resembles letter M. R-LPS is shown devoid of the O-specific chain. Based on [55-57]

a number of proteins accompanying CD14 and TLR4 such as heat shock proteins, CD55 and the chemokine receptor, CXCR4 [72]. Formation of the protein complexes in response to LPS may be facilitated by accumulation of the proteins within the earlier-mentioned lipid rafts. Selection of proteins forming such complexes reflects the structural properties of LPS and can modulate the LPS-induced signalling pathways [73]. Studies on the mechanisms of LPS-induced cell stimulation are currently dominated by investigations of the role of TLR4 in this process. Nevertheless, research interest persists regarding the role of scavenger receptors in inflammatory response and current studies uncover a complex pattern of their involvement in the cellular reactions to LPS [28]. Data are available that indicate that members of the class B scavenger receptor family, such as CD36 and SR-B1/Cla-1, induce production of pro-inflammatory cytokines following activation by LPS, independently of TLR4 involvement [2, 40]. In turn, a class A scavenger receptor, SR-A1, seems to play a protective role against excessive cell stimulation by LPS. This reflects the ability of the receptor to internalize high doses of LPS using a pathway which does not induce synthesis of pro-inflammatory cytokines but leads to detoxification of LPS [19, 51].

## SIGNALLING PATHWAYS OF TLR4

TLR4 is the only member of the TLR family that interacts with four adaptor proteins containing TIR domains initiating two signalling pathways, the MyD88- and TRIF-dependent one (fig. 5). Interaction of the MyD88 protein with TLR4 occurs with an involvement of an additional adaptor, TIRAP/Mal protein [20]. Apart from the TIR domain allowing for the interaction of MyD88 with TLR4 and TIRAP protein, MyD88 contains in its carboxy-terminal fragment the so called death domain (DD) through which MyD88 interacts with the IRAK4 and IRAK1 kinases (the latter may be substituted by IRAK2 kinase). Crystallographic analyses have shown that six to eight molecules of MyD88 plus four IRAK4 and four IRAK2 (or IRAK1) kinases associate in a hierarchic manner, forming a signalling complex termed the myddosome [15, 43]. The formation of the myddosome assures close proximity of the IRAK kinases, allowing for phosphorylation of IRAK1 (or IRAK2) kinases by IRAK4. Following the phosphorylation, IRAK1 dissociates from the receptor complex and interacts with TRAF6 (*Tumour necrosis factor Receptor Associated Factor 6*) which functions as an E3 ubiquitin ligase and activates the subsequent signalling kinase TAK1 (*Transforming growth factor  $\beta$ -Associated Kinase-1*) [83]. The TAK1 kinase activated in this manner is capable of initiating IKK (I $\kappa$ B kinase) and MAPK (mitogen-activated kinase) pathways. This ultimately leads to expression of pro-inflammatory cytokine genes using two transcription factors, NF $\kappa$ B and AP-1. The main pro-inflammatory cytokine produced along the pathway is TNF- $\alpha$  [34].



**FIGURE 5.** Sequential activation of TLR4 signalling pathways dependent on its internalization. **A** – Binding of LPS leads to an assembly of a complex containing CD14, TLR4 and MD-2. A high local concentration of PI(4,5)P<sub>2</sub> allows for binding of the pleckstrin homology domain of TIRAP adaptor protein. On the other hand, the TIR domains of TIRAP molecules interact with TIR domains of TLR4 and MyD88 protein. To the complex, four IRAK4 kinases and four IRAK2 kinases bind via DD domains present also in MyD88. A myddosome is assembled, triggering MyD88-dependent signalling pathway. Between six and eight molecules of MyD88 have been found in the myddosome suggesting a possibility of the assembly of higher-order clusters of activated TLR4 [15, 43]. **B** – After internalization of TLR4, the concentration of PI(4,5)P<sub>2</sub> in the endosome membrane decreases, leading to dissociation of TIRAP-MyD88 complexes. Adaptor proteins TRAM and TRIF bind to TIR domains of TLR4 triggering the signalling pathway independent of MyD88. Based on [29, 34]

The signalling pathway dependent on the MyD88 adaptor protein is typical for almost all TLRs except TLR3 (fig. 2). On the other hand, the other signalling pathway, dependent on the TRIF adaptor protein, can be initiated only by TLR4 and TLR3. In order to be recruited, TRIF requires another adaptor protein, TRAM, which mediates its interaction with TLR4 [82]. The TRIF protein, together with a group of accessory proteins, interact with the TBK1 (*TANK Binding Kinase-1*) and IKKi kinases which phosphorylate transcription factors IRF3 and, to a lower extent, IRF7 [13]. Inactive forms of the IRFs are present in the cytoplasm but following phosphorylation in their C-terminal regions they undergo homodimerization

and translocation to the nucleus, where they activate transcription of genes coding for type I interferons. Along the TRIF-dependent pathway, apart from phosphorylation of IRF3 and IRF7, also the NF $\kappa$ B transcription factor becomes activated. The TRIF-dependent pathway is suggested to be responsible for late-phase activation of NF $\kappa$ B, while the MyD88-dependent pathway leads to early, rapid activation of NF $\kappa$ B directly after LPS binding. Nevertheless, signal transduction along both pathways is required for a full activation of pro-inflammatory cytokine production in response to LPS [34].

An interesting modification of the pathway was detected in the case of TLR7 and TLR9 of dendritic cells, which employ MyD88 for induction of type I interferon synthesis in response to viral infection. In this case the multi-molecular enzymatic complex formed around MyD88 allows for phosphorylation of the IRF7 transcription factor by IRAK1 and IKK $\alpha$  kinases, which induces its translocation to the nucleus and induction of expression of genes coding for type I interferons [33].

For several years TLR4 was assumed to trigger both signalling pathways as a plasma membrane protein. This was regarded a unique property of TLR4 since all other PRRs (including the remaining TLRs, see fig. 2) capable of activating interferon production initiate signal transduction from membranes of intracellular organelles or from the cytoplasm. An alternative model of activation of signalling pathways by TLR4 has been proposed by Kagan and collaborators [29, 30]. This model assumes that the activation of the signalling pathways dependent on MyD88 and on TRIF is temporally and spatially separated (fig. 5). According to the suggested model the membrane receptor complex consisting of CD14 and TLR4/MD-2 is assembled in plasma membrane regions rich in phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P<sub>2</sub>]. The presence of a PI(4,5)P<sub>2</sub>-binding domain (so called pleckstrin homology domain) in the TIRAP adaptor protein allows for its association with the plasma membrane which, in turn, promotes interaction of the TIR domains of this protein, MyD88 and TLR4. Activation of TRIF-dependent pathway is followed by internalization of TLR4. In the membrane of the nascent endosome the concentration of PI(4,5)P<sub>2</sub> drops significantly which results in dissociation of MyD88-TIRAP from TLR4. This, in turn, allows for association of the second set of adaptor proteins, TRAM and TRIF, and activation of the TRIF-dependent pathway [29]. It is worth noting that TRAM protein is devoid of the PI(4,5)P<sub>2</sub>-binding domain and, therefore, does not interact with the plasma membrane and does not compete with MyD88 for the binding of the TIR domains of TLR4 [30]. Thus, the TIRAP and TRAM proteins play a role of „sorting adaptors”, securing generation of signalling cascades from distinct cellular compartments.

This model leaves unresolved the questions of factors controlling the internalization of TLR4 and the endocytosis pathway along which it may take place. Some data point to an involvement of dynamin and clathrin in this process, which suggests endocytosis of TLR4 by a classical pathway, within clathrin-coated vesicles, similarly as in the case of transferrin receptor. At first the data were based on a mi-

croscopic analysis of the co-localization of TLR4, LPS and transferrin and on the inhibitory effect exerted by mutated forms of dynamin or clathrin heavy chain on the internalization of TLR4 [22]. Recently, inhibitors of dynamin enzymatic activity and compounds blocking association of clathrin with its accessory proteins were employed. The inhibitors repressed internalization of activated TLR4 and significantly reduced phosphorylation of IRF3 factor and synthesis of interferon  $\beta$  [30, 76]. On the other hand, a report by Zanoni and co-workers showed that TLR4 is internalized via macropinocytosis which is controlled by the tyrosine kinase Syk, phospholipase  $C\gamma 2$  and by influx of  $Ca^{2+}$  ions [85]. According to that report, the increase in cytoplasmic  $Ca^{2+}$  ion concentration dependent on the activity of phospholipase  $C\gamma 2$  controls activation of IRF3 and expression of its target genes [6]. The primary factor which controls internalization of TLR4 seems to be its co-receptor, the CD14 protein. In macrophages isolated from mice devoid of CD14, internalization of TLR4 and production of interferon  $\beta$  were negligible [85], although the mechanisms of such a relationship remain unknown.

TLR4 also appears in the membrane of phagosomes within which *Escherichia coli* are internalized. This process initiates the TRIF-dependent signalling pathway and synthesis of type I interferons [23]. Authors of that report indicate that TLR4 is transported to the phagosome membrane from early endosomes and that this stage of intracellular TLR4 transport remains under control of a GTPase from the Rab family, Rab11. This notion is additionally supported by other reports, according to which the TLR4/MD-2 complex is constitutively present in endosome membranes and in the Golgi apparatus of macrophages, from which it may be incorporated to the plasma membrane but it can also recognize LPS and initiate both the TRIF- and MyD88-dependent signalling pathways [68, 75].

The endocytic pathway participates also in terminating the activation of TLR4. Following binding of LPS, TLR4 translocates to late endosomes and lysosomes and becomes degraded. This process involves another Rab-family GTPase, the Rab7b protein, which associates with the lysosome membrane. Activity of this protein plays a significant role in promoting degradation of TLR4 and in negative regulation of TLR4 signalling [74]. The negative control of LPS-induced pro-inflammatory response includes also the internalization of high LPS doses described above, dependent on the participation of scavenger receptors [51].

## SUMMARY

Studies on the mechanisms of signal transduction by TLRs conducted within the last 15 years using diverse genetic, crystallographic and immunologic techniques have significantly broadened our understanding of the immune system functioning. Particular attention was focused on TLR4 due to the high mortality of septic shock

victims. A breakthrough in the field resulted from studies demonstrating the role of endocytosis in activation of one of TLR4 signalling pathways. However, those discoveries failed to translate into practical application of the accumulated knowledge for controlling sepsis, and the fate of drugs designed on the basis of that knowledge, such as the TLR4 antagonist eritoran, remains uncertain (see: <http://www.eisai.com/news/enews201108pdf.pdf>). A more complex approach needs to be applied in studies on TLR4 functioning in the context of other receptors and proteins engaged in immune reactions, such as scavenger receptors and more attention should be paid to their interactions. This type studies should provide novel data on the mechanisms of negative regulation of TLR4 signalling, particularly valuable for diminishing the potentially lethal inflammatory reaction.

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