TLR4 Ligands: Single Molecules and Aggregates

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Abstract Lipopolysaccharide (LPS, endotoxin) is an amphipathic glycolipid that undergoes self-aggregation. The physical state and 3D organization of LPS in the aggregated state has a high impact on the biological activity and pathophysiology. Here, the basis of aggregate formation and the role of aggregate properties are presented for bacterial LPS, LPS-mimetic, and TLR4-modulating compounds with a focus on the concept of the "endotoxic conformation". A network of sequentially interacting molecules is operative to enable a sensitive and targeted delivery of LPS from aggregates to the TLR4 receptor. The structural and thermodynamic aspects of the transport and the molecular recognition of LPS by TLR4/MD-2 are presented to provide a mechanistic understanding of TLR4 activation by its ligand. Furthermore, delivery mechanisms and activation of the cytoplasmic LPS receptors caspase-4/5/11 are discussed. These insights are important for the development of new classes of immune-modulating compounds by chemical synthesis and also for modern in silico approaches to identify new lead structures for the development of therapeutics.

Keywords LPS physico-chemistry · Supramolecular structure · Lipid transport · TLR4 activation · Caspase-4/5/11 activation

1 Basis of LPS Pathophysiology

Activation of the toll-like receptor 4 (TLR4) by bacterial lipopolysaccharide (LPS, endotoxin) is among the most sensitive responses of the human immune system. LPS is the main component of the outer membrane of Gram-negative bacteria and

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represents a central molecular trigger for the immunological recognition of an infection, the induction of inflammation, and the initiation of an antimicrobial immune response. Due to the complex physico-chemical nature of LPS, this recognition process is organized by a network of sequentially interacting molecules that have evolved to enable a sensitive and targeted delivery of LPS to cellular receptor systems. The peculiar physico-chemical behavior of LPS released from the cell wall of bacteria into aqueous environment and body fluids, the structural prerequisites for biological activity of LPS, and thermodynamic aspects of the process of molecular recognition are presented here.

The glycolipid LPS is a membrane component present exclusively in the cell envelope of Gram-negative bacteria. The cell wall of Gram-negative bacteria is organized in several layers, an inner cytoplasmic membrane, a thin peptidoglycan layer, and an outer membrane. The cytoplasmic membrane is composed of the phospholipids phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin. The outer membrane contains an inner leaflet solely of phospholipids, whereas the outer leaflet is composed of LPS. Thus, LPS is the major molecule presented on the microbial surface. Chemically, LPS is composed of a bis-phosphorylated diglucosamine backbone, which is acylated in amide- and ester-linkage with up to seven fatty acids. This amphiphilic part of the molecule, termed lipid A, is the membrane anchor of LPS. In rough mutant strains, lipid A is substituted with a head group of the unusual sugar 2-keto-3-deoxyoctonate (Kdo) and further sugar residues that are distinguished as an inner and an outer core region depending on the length of the sugar substitution. In wild-type strains, an O-specific chain is attached to the outer core composed of a large number of repeating units of additional sugars [1]. The lipid A part is responsible for the immunological recognition of LPS and is thus also termed the "*endotoxic principle*" of LPS [2]. Activation of the immune system by LPS is extremely potent. LPS concentrations in the range of picogram per ml are sufficient to induce activation of TLR4 in monocytes and macrophages. The downstream signaling cascades lead to activation of nuclear-factor-κB (NF-κB) or interferon-regulatory factor 3 (IRF3) and IRF7 responsive pro-inflammatory genes [3–5]. Among LPS-TLR4 related diseases, the most harmful ones are sepsis and septic shock, pathological conditions that are accompanied by a high rate of morbidity and mortality. Excessive activation of monocytes and macrophages by bacterial pathogens leads to a dysregulated immune response. Systemic overproduction of inflammatory cytokines such as interleukin-1 (IL-1), IL-6, tumor-necrosis-factor-α (TNF-α), chemokines, and lipid mediators initiate a cascade that culminates in life-threatening organ-dysfunction and death [6, 7]. Recent data derived from single cell RNA profiling of a sepsis patient cohort indicate that during bacterial sepsis a unique immune cell signature is generated in mononuclear cells that can be clearly distinguished from other disease entities [8].

The molecular recognition of LPS by TLR4 requires its release from the bacterial cell surface. Upon cell division and cell death, endotoxin is naturally shed from the bacterial cell wall. Bacterial killing by antimicrobial immune responses of the host, mediated, for example, by complement or antimicrobial effector molecules, will liberate endotoxin from the cell wall and release it into the circulation. Of note,

also the antimicrobial activity of antibiotics can lead to massive release of endotoxin into the bloodstream resulting in exaggerated immune responses that may contribute to the development of sepsis [9–12]. Various experimental animal models have demonstrated that injection of pure LPS into the blood stream or the peritoneum is sufficient to induce sepsis [13]. Galactosamine-sensitized mice represent a well-established and widely used model for the investigation of endotoxemia-associated pathology that requires low amounts of LPS in the range of 0.05–0.01 μg per animal to induce lethal effects [14]. Challenge of human volunteers with highly purified LPS is a clinical model allowing to perform highly controlled studies to investigate LPS-induced systemic inflammation in vivo [15]. These experimental model systems all demonstrate severe pathophysiological effects of purified LPS in vivo.

2 Physico-Chemistry of the TLR4 Ligand LPS

To understand the biology of LPS, its complex physico-chemistry has to be considered. LPS is an amphipathic glycolipid that similar to phospholipids undergoes thermodynamically driven self-aggregation to reduce the contact of the hydrophobic acyl chains with water. Thus, purified LPS and LPS released from the bacterial cell wall will spontaneously form aggregates to minimize the Gibbs-free energy [16]. The concentration at which molecular aggregation starts is termed the critical micellar concentration (CMC). Above the CMC, with increasing LPS concentration, the monomer concentration remains constant or is even reduced in the case of negatively charged amphiphiles, and additional molecules are incorporated into the aggregated form [17] (Fig. 1a, b). Published values of the CMC for LPS and lipid A are, however, contradictory and span a wide range of concentrations [18–22]. This may be at least partially due to limitations of the methodological approaches used. For review see [23]. Evaluating the published data, the value of the CMC for lipid A can be approximated in the concentration range $< 10^{-9}$ M.

The physical state and 3D organization of biological lipid aggregates is determined by the molecular conformation of the aggregate forming molecules. Geometric models of lipid aggregation allow an estimation of the aggregate structure based on the shape parameter $S = v / (a_0 \cdot l_c) = a_h / a_0$ (v = volume of the hydrophobic moiety, l_c length of the fully extended hydrophobic moiety, a_0, a_h cross-sectional areas of the hydrophilic and hydrophobic moiety, respectively) introduced by Israelachvili [17]. For $S < ½$ micellar structures are adopted, and in particular cases an H_I phase can also be formed. Between $S = ½$ and 1, unilamellar and multilamellar bilayer structures are favored. Whether a particular glycolipid adopts a uni- or a multilamellar structure is a complex problem, which depends, among others, on geometrical constrains, the presence of charges in the head group, the kind of counter ions, and the hydration properties of the glycolipid [16]. For $S > 1$, inverted structures such as inverted hexagonal (H_{II}) or cubic (Q) structures are formed in which the acyl chains are directed outward, and the hydrophilic moiety inward [24, 25]. In the range around $S = 1$, various phases may coexist and phase transition may be induced by

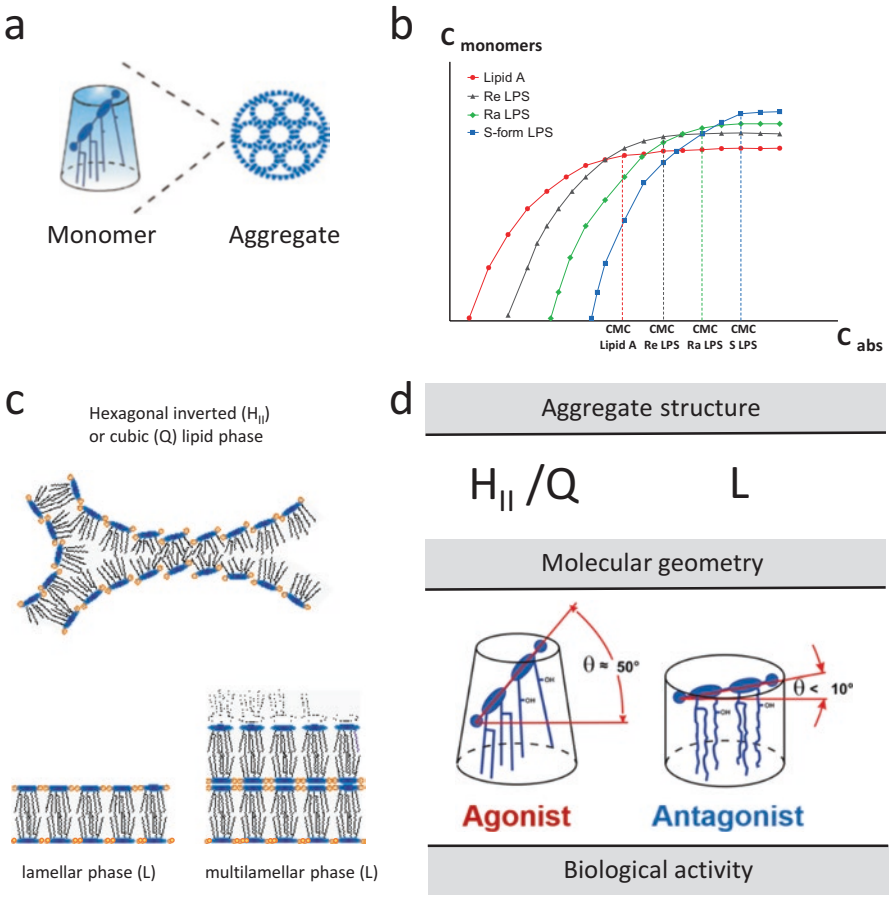

Fig. 1 Aggregation behavior, lipid phases, and molecular conformation of the TLR4 ligand LPS. (**a**) In aqueous environment, LPS undergoes aggregation to form supramolecular assemblies. (**b**) Dependence of the threshold concentration of LPS aggregate formation (CMC, critical micellar concentration) on the carbohydrate content of LPS. Theoretical concentration monomers versus absolute concentration C_{abs} for different endotoxin chemotypes. Above the CMC, the aggregate concentration increases and the monomer concentration remains constant. (**c**) Aggregate structures adopted by enterobacterial lipid A and LPS are complex hexagonal inverted (H_{II}) or cubic (Q) lipid phases. Lamellar bilayer or multilamellar (L) lipid phases are frequently observed for LPS with a reduced number of acyl chains. (**d**) Concept of the endotoxic conformation: Correlation of the aggregate structure and biological activity. Biological activity depends on the occurrence of hexagonal inverted or cubic lipid phases with a conical molecular geometry and a positive tilt angle of lipid A. LPS and lipid A with a cylindrical geometry and low or no backbone tilt angle form lamellar bilayer structures. These lipids do not activate the signaling receptor cascade but may be potent antagonists of TLR4 activation

small extrinsic changes such as hydration, ions, or temperature. For a determination of the aggregate structures, physical techniques such as small-angle scattering with X-rays (SAXS) or neutrons (SANS) must be applied.

The state of fluidity of the acyl chains directly affects the space coverage of the hydrophobic moiety and thus has a large impact on the occurrence of cubic and inverted aggregate structures that require conical molecules. Basically, two states of acyl chain fluidity can be adopted, the gel (β) and the liquid-crystalline (α) phase. In the gel phase the ordered acyl chains are in the all-trans configuration, while the liquid-crystalline (fluid) phase is much less ordered due to the introduction of increasing amounts of *gauche*-conformers. Between these phases, a (pseudo) first-order transition can be observed at a glycolipid-specific temperature T_m. The value of T_m is governed by various parameters such as the length and the degree of saturation of the hydrocarbon chains, the head group and further substitutions, hydration of the glycolipid, and solution properties such as pH, ionic strength, and the presence of divalent cations. Phase transition can take place while maintaining the aggregate structure, but phase transition can also induce conversion of the aggregate structure. Of note, not all aggregate structures can occur in both phases, in particular the H_{II} and Q structures are commonly not observed below T_m.

The structural polymorphism of endotoxins is described for lipid A, LPS Re, and other rough mutant LPS as well as wild-type LPS from *Salmonella minnesota* and *Escherichia coli* presenting complete phase diagrams by varying the concentration of water, Mg^{2+} as important physiological cations, and temperature [26–28]. All enterobacterial LPS exhibit a gel to liquid crystalline phase transition at T_m = 30 to 36 °C, depending on the length of the carbohydrate chain, with the lowest values for LPS Re. Of note, enterobacterial lipid A has the highest T_m with values around 45 °C. Most importantly, the aggregate structure of LPS adopts mainly non-lamellar organizations, which can be assigned to aggregates with inverted hexagonal H_{II} or cubic symmetry, in particular for endotoxins with short carbohydrate chains (lipid A, LPS Re). From these data, a conformational concept of endotoxins was deduced: the lipid A part of LPS adopts a conical shape with a cross-section of the hydrocarbon chains being higher than that of the hydrophilic part. This concept is valid for LPS with hexaacylated lipid A. In contrast, in penta- und tetraacylated lipid A the cross-sections of both molecular parts are nearly identical, forming multilamellar aggregates (Fig. 1c).

3 Role of Aggregates in Biological Activity

A question that has been discussed quite controversially in the literature is the question of the biologically active unit of LPS, whether this is the aggregate or the monomer. Several lines of evidence support that aggregates are the physical entities that are targeted by the immunological LPS-binding proteins in serum and that are required in the first place for the activation of TLR4 downstream of the transport chain. An experimental approach that strongly supports that aggregates are a prerequisite for biological recognition is the separation of aggregates and monomers in a diffusion chamber. Challenge of mononuclear cells with monomeric or aggregated LPS demonstrated that monomers were not able to induce cell activation, whereas

aggregated LPS at the same concentrations showed robust cytokine induction. This result was observed in the absence as well as in the presence of human serum or in the presence of LPS-binding protein [29]. Other studies showed that a lower state of LPS aggregation is associated with largely reduced mortality in a model of galactosamine-sensitized mice [30]. From these data, it can be concluded that LPS in the aggregated state is required for biological recognition.

Analysis of the aggregate structures of a large number of lipid A and LPS, including preparations isolated from bacteria and analogs generated by chemical synthesis, by small-angle X-Ray diffraction (SAXS) have revealed a striking correlation of aggregate structure and biological activity. The three-dimensional organization of lipid aggregates is tightly connected with their ability to activate or antagonize cell activation. Thus, lipid structures with a conical molecular shape that assemble into complex H_{II} or Q lipid phases are correlated with high biological activity. In contrast, lipid A structures with a cylindrical molecular shape form lamellar or multilamellar lipid phases that do not express biological activity, however, several of these compounds express antagonistic activity, that is, they are able to inhibit cell activation by endotoxins [31]. This finding is supported by data for a variety of lipid A samples from different enterobacterial strains, lipid A in different salt forms, monophosphoryl lipid A (MPLA), lipid A from non-enterobacterial sources, and synthetic lipid A variants [32–34].

An important aspect of LPS aggregation states is the accessibility of the phosphate groups that have been shown to be of particular importance for the expression of biological activity [32, 35]. The bis-phosphorylated diglucosamine backbone does not align perpendicular to the membrane normal but adopts a tilt angle, which can be determined by FTIR spectroscopy using attenuated total reflectance analysis with polarized light [36]. The tilt observed in the lipid A backbone leads to exposure of the 1-phosphate group to the water phase, whereas the 4′-phosphate group is tilted downward pointing to the hydrophobic core of the membrane. The different degrees of hydration of the two phosphates are reflected by the infrared absorption peaks of the anti-symmetric stretching vibrations of the PO_2^- groups that can be determined in the wavenumber range of 1300–1260 cm^{-1}. Comparing the biological activities of different lipid A and LPS aggregate preparations, a strong correlation of a positive tilt angle of ≥35° with the expression of biological activity of lipid A is observed, whereas lipid A aggregates with a low tilt angle of the backbone around 10–15° express low or no biological activity [23]. Thus, a conical molecular conformation with a tilted glucosamine backbone exposing one phosphate group is the optimal structure for the expression of biological activity. Interestingly, the phosphate groups of LPS can be replaced by carboxymethyl groups without changing its bioactivity, but a negative charge is mandatory [34].

According to these findings, the term “endotoxic conformation” was coined, which relates the aggregate structure, the molecular conformation of individual molecules within the aggregates, and the biological activity to activate cells via TLR4 [37] (Fig. 1d). Of note, this correlation could also be confirmed for another group of TLR ligands, the bacterial lipopeptides, which activate host cells through the TLR2 receptor. Although the di- or triacylated lipopeptides show a considerably

lower degree of acylation than the lipid A portion of LPS, their biological activity could also be correlated to the molecular conformation within the aggregated state with conical molecules expressing high TLR2 activity and cylindrical molecules expressing antagonistic activity [38]. These findings underline the fundamental validity of the importance of aggregation structure in biological lipid recognition by TLR receptors. New developments in the biological and chemical synthesis of lipid A molecules will provide the opportunity to generate a wide variety of new structures [39, 40]. Such pipelines open the opportunity to feed a larger number of compounds into structure activity relationship (SAR) studies and may thus expedite the identification of inhibitors with optimized physical and biological behavior. This is especially important in view of the need for new lead structures for therapeutics to cope with inflammatory diseases.

Interaction of LPS aggregates with components in the blood circulation has been demonstrated to modulate the structure and also the harmful pathophysiology of endotoxin [41]. Thus, binding of aggregated LPS to lipoproteins represents an important pathway of detoxification. An interaction of LPS with lipoprotein particles in blood was already discovered long before TLR4 was identified as the LPS receptor. Studies on the LPS-binding protein (LBP) and soluble CD14 (sCD14) revealed that sequential interaction of LBP and soluble CD14 (sCD14) catalyze a transfer of LPS molecules from aggregates to high density lipoproteins (HDL) in serum [42, 43]. This lipid transport is achieved by the activity of LBP, by extraction of LPS molecules from aggregates and catalyzing the occurrence of an intermediate stage of LPS-sCD14 complexes. LBP shuttles LPS from LPS-sCD14 complexes to a variety of lipoprotein particles present in the circulation, such as HDL, low density lipoproteins (LDL), very low density lipoproteins (VLDL), and chylomicrons. These microparticles are targeted from the circulation to the liver with subsequent neutralization of the endotoxic activity [44]. Analysis of smooth (S)-LPS and rough (R)-LPS glycoforms demonstrated a rapid removal of R-LPS aggregates from the circulation, whereas S-LPS aggregates showed prolonged residence time in the serum in vivo [45]. Protective effects with increased survival have been reported for application of reconstituted HDL particles in murine models of polymicrobial sepsis induced by cecal ligation and puncture, in intraperitoneal sepsis induced by injection of *Escherichia coli*, as well as in a model of *Pseudomonas aeruginosa*–induced pneumonia. These in vivo studies demonstrate the potent anti-inflammatory and endotoxin detoxifying effects of HDL particles [46, 47].

Protein interaction with endotoxin aggregates has also been demonstrated to modulate biological activity by directly changing the 3D structure of LPS aggregates. In this context, biophysical analysis of the mode of action of antimicrobial peptides (AMP) has shown the capacity of AMPs to convert the physical organization of LPS to multi-lamellar aggregates, an effect that is directly correlated to the endotoxin-neutralizing capacity [48, 49]. Mechanistic insights were revealed in particular from studies on the LPS-neutralizing polypeptide Aspidasept and variants thereof [50–52]. A very interesting finding, first observed by Jack Levin and coworkers, is the intriguing capacity of hemoglobin (Hb) to increase

endotoxin-induced biological activities [53]. The addition of cross-linked Hb to penta-acylated LPS and lipid A preparations with a very low biological activity led to a drastic increase in cytokine secretion, such as TNF-α in human mononuclear cells [54]. Structural analysis demonstrated that Hb converts LPS aggregates from a non-lamellar structure to cubic symmetry. This was accompanied by a considerable reduction of the size and number of the original aggregates. Similar effects were also observed for TLR2 activating lipopeptides, suggesting a general molecular mechanism of Hb on aggregated lipids [55, 56]. It must be emphasized that Hb does not change the chemical structure of LPS or lipid A. An interesting aspect of the Hb activity is the observation that Hb itself shows membrane activity toward host cell membrane models. Thus, besides the direct effects of Hb on the aggregation structure of LPS, effects of Hb on the organization of the host cell membrane and the assembly and activation of the TLR4 receptor complex might also be considered for further mechanistic studies.

Natural LPS preparations derived from bacteria express quite a complex composition. Biological LPS aggregates are heterogeneous mixtures of diverse chemical LPS structures, containing different chemotypes ranging from deep-rough (Re) over rough mutant (Rb-Ra) LPS up to S-LPS [57]. The lipid A structure of the different chemotypes present in S-LPS preparations from wild-type bacteria displays considerable heterogeneity with respect to the acylation patter. Thus, in S-LPS from *Salmonella abortus equi*, the rough fraction was found to contain the expected acylation pattern of ester- and amide-bound 3-OH-14:0, whereas in the smooth fraction, a significant part of ester- as well as amino-linked acyl chains was absent [58]. An important observation in this context is the finding that the immunological active fraction of S-LPS is the R-chemotype fraction of LPS [59, 60].

Another aspect of the frequently observed presence of under-acylated lipid A structures such as pentaacyl and tetraacyl lipid A in natural LPS preparations is the low impact of these lipid species on the overall biological activity of the preparation. Surprisingly, these molecular species do not appear to express their antagonistic potential even when being present in amounts of up to 20% in the aggregates of biologically active LPS. Instead, mixing experiments demonstrated that the admixture of 10–20 Mol% of the synthetic antagonist 406 in aggregates composed of the synthetic lipid A compound 506 rather enhanced the biological activity. For the antagonistic glycolipid cardiolipin, similar results were obtained with up to 50 mole% of cardiolipin [29]. When the antagonists were not present in the lipid A aggregates but applied separately before stimulation with lipid A, complete inhibition of cell activation was observed. The finding that antagonistic compounds enhance endotoxic activity when present in the same aggregate indicate that the presentation of molecules in the aggregated state plays a decisive role for the molecular interaction of binding proteins and receptors.

The presented data support that in biological systems, LPS in the aggregated state is the physico-chemically relevant molecular state that is targeted by the participating transport molecules of the sequential recognition chain, which is discussed in Sect. 4.

4 From Aggregates to TLR4 Receptor Interaction

Biological recognition of LPS by TLR4 in the context of infections requires the extraction of the molecule from the bacterial cell envelope or from endotoxin aggregates to enable receptor binding. Within the family of Toll-like receptors, TLR4 has evolved the most complex cascade of using accessory proteins to enable the sensitive recognition of its ligand endotoxin. The accessory proteins comprise the LPS-binding protein (LBP), which is expressed by hepatocytes as an acute-phase protein and is highly upregulated upon infections [61, 62] and the soluble form of CD14, which is expressed as a glycosylphosphatidylinositol-anchored surface antigen mCD14 on monocytes and macrophages, and in lower amounts also on dendritic cells and neutrophils. CD14 is shed from the cell surface into serum, secreted from intracellular pools, and also produced as an acute-phase protein in the liver [63]. LBP binding to intact bacteria enhances phagocytosis. LBP can extract LPS monomers from the bacterial membrane [64, 65] and from endotoxin aggregates in solution [66]. A major function of the combined action of LBP and CD14 is to enable a highly sensitive activation of mononuclear cells [67]. Using ^{14}C- or ^{3}H-lipooligosaccharide (LOS) and LPS a sequential transport chain of LPS molecules from LBP to CD14, and subsequently to MD-2, reducing the binding affinity of the TLR4/MD2 complex to picomolar concentrations, was described [68, 69]. Of note, while metabolic radioactive labelling of LPS enables the sensitive detection of monomeric LPS, the emitted high radiation doses may induce cellular stress responses and could thus enhance also the cellular responsiveness. Reconstruction of the cascade by high resolution microscopy recently demonstrated on a molecular level that LBP acts as an accelerator of the initial process by allowing multiple rounds of LPS transfer to CD14 [70], further contributing to the extremely sensitive LPS recognition by the immune system.

From a biophysical perspective, thermodynamic considerations are highly relevant for the aggregation, disaggregation, and transport of LPS. Important insights into the transfer path of LPS were provided by molecular dynamic simulation analyses. The individual steps of LPS interaction were analyzed in a set of computational models of the bacterial outer membrane, the LPS aggregate, and complexes of CD14/LPS, MD-2/LPS, and CD14/TLR4/MD-2/LPS. The data obtained from these in silico studies revealed that channeling of the ligand along the receptor proteins binding lipid A with increasing affinity generates a thermodynamic funnel. The resulting energy gradient culminates in a terminal transfer of LPS to spontaneously assembled CD14/TLR4/MD-2 receptor complexes on the model of the host cell membrane [71]. In the bacterial membrane model, lipid A was retained with about 310 kJ mol^{-1} affinity, providing a high energy barrier for extraction. Divalent counterions such as Ca^{2+} and Mg^{2+} bridging the phosphate groups on the bacterial membrane contribute to this high energy barrier by tightly linking the negatively charged headgroups. The authors propose that LBP interaction disrupts this counterion barrier and thereby reduces the kinetic barrier for LPS extraction.

The TLR4/MD-2 complex is the signaling receptor for LPS at the cytoplasmic membrane [72–74]. Crystallographic data of complexes of the extracellular domain of TLR4 and MD-2 are the basis for the model that the receptor activation is enabled by the binding of a monomeric LPS molecule into the hydrophobic binding pocket of the accessory receptor protein MD-2, leading to the formation of TLR4/MD-2 heterodimers. Receptor dimerization is stabilized by the exposure of the acyl chain at position 2 at the lipid A at the surface of the MD-2 binding pocket, which forms together with the amino acid residue Phe126 of MD-2 a hydrophobic interaction patch for the adjacent TLR4 ectodomain. The complex is further stabilized by ionic interaction of the lipid A phosphate group in 4′ position with a cluster of positively charged residues in TLR4 and MD-2 [75, 76]. The dimeric receptor state is the platform that activates intracellular signaling via the engagement of adaptor protein myeloid differentiation primary response protein 88 (MYD88) assembly to large signaling platforms, the "Myddosome" complex activating the NF-kB pathway. Association of the TIR domain-containing adaptor protein inducing IFNβ (TRIF) to the TIR domains assembles the "Triffosome" platforms activating the interferon-regulatory factor (IRF)7-pathway [5]. Single molecule data obtained from quantitative super-resolution microscopy studies provide refined data about the receptor assembly. Krüger et al. revealed that the cell surface receptor complex of TLR4/MD-2 is present to about 50% in a monomeric state TLR4/MD-2 and to 50% in a dimeric state in unstimulated cells, demonstrating an intrinsic propensity of TLR4/MD-2 to dimerize. This ratio was dramatically shifted upon stimulation with LPS, leading to a large increase of the fraction of dimeric receptor state to about 75% [77]. A limitation of such investigations that has to be considered is the overexpression of TLR4_mEos2 receptors that were transfected in hamster embryonic kidnek cell line HEK293 in order to enable their detection in super resolution microscopy. The observation of dimerization of a significant pool of receptors was associated with a substantial degree of NF-κB activation even in the absence of LPS stimulation, supporting that dimerization and activation of TLR4 are closely related processes. Of note, data from molecular dynamic simulations on the thermodynamics involved in receptor ligand interaction suggest that the final step of LPS binding occurs to the preassembled TLR4/MD-2 dimeric receptors. This is in contrast to the mechanistic model that LPS induces receptor dimerization. Instead, it would be in accordance with the assumption that LPS binding leads to a stabilization of spontaneously formed receptor dimers [71].

5 Non-LPS Ligands of TLR4: Aggregate States and Biological Activity

Targeting of TLR4 by non-endotoxin compounds is of particular interest for therapeutic immune modulation of the receptor. The strong pathophysiology that can be elicited by TLR4 activation and the lack of treatment options for patients has

attracted particular attention for the development of inhibitors. A successful line of development is based on lead structures of endotoxins with a naturally occurring low endotoxicity. LPS from the non-pathogenic bacterium *Rhodobacter sphaeroides* (Rs) LPS was discovered as a potent inhibitor of TLR4 activation by LPS. Rs lipid A became a lead structure for synthetic TLR4 antagonists. The compound Eritoran tetrasodium (E5564), generated by EISAI Inc. (Andover, USA), showed high antagonistic activity in murine and human cells while demonstrating improved stability, pharmacology, and pharmacokinetics [78–80]. The crystal structure of Eritoran bound to the mouse TLR4/MD-2 complex revealed the geometry, hydrophobic volume inside the binding cavity of MD-2 and the location of electrostatic interactions of the phosphate groups with positively charged patches at the rim of the hydrophobic pocket and the adjacent TLR4 molecule [81]. The visualization of the molecular geometry and the localization of the molecular interfaces greatly increase our understanding of the mode of action of the antagonist. One very interesting approach by EISAI Inc. was the generation of non-LPS compounds that mimic the physico-chemical characteristics of lipid A structures. They synthesized phospholipids with six acyl chains and two phosphates linked by a serine-like backbone, the latter with a spacer allowing to vary the length and the volume of the molecular backbone. By this strategy, lipid A mimicry molecules with different critical packing shapes were generated. Structural analysis of the molecular geometry of the compounds in the aggregates state revealed a conical molecular geometry for the compound with the smallest backbone (EISAI 803022), which expressed high biological activity. In contrast, compounds with a long spacer at the backbone expressed cylindrical molecular shape and were found more or less devoid of biological activity. The applicability of this structure activity correlation for molecules with different chemistry is referred to as “the generalized endotoxic principle” [38, 82, 83]. These findings clearly demonstrate the potency to develop non-LPS compounds as modulators for TLR4 by physico-chemical mimicry.

New approaches of targeting TLR4 pursue a strategy of simplified molecules for drug development. Synthetic disaccharide-based anionic amphiphiles were reported by Borio et al. as inhibitors of LPS-induced inflammation. The compounds were designed on the basis of optimizing the orientation and torsion of the MD-2 interacting groups. They show potent antagonistic activity at micromolar concentrations in human and murine macrophages. Structure–activity relationship studies and molecular dynamic simulation of the interaction with MD-2 were used to select two compounds with optimized properties as lead structures for future studies [84]; however, information on the molecular geometry and aggregation behavior is not yet available. Another example of synthetic TLR4 modulators is based on monosaccharide scaffolds. IAXO102 is a cationic antagonist inhibiting TLR4 activation in human cells and in an in vivo model of murine sepsis [85]. The FP series of anionic monosaccharide-based synthetic compounds was developed by computational approach to optimize docking of the lipids to the hydrophobic pocket of MD-2. For compound FP7 an insertion of the acyl chains into the hydrophobic cavity of MD-2 is demonstrated [86]. Antagonistic activity on LPS-induced activation of cytokines and chemokines is shown in human monocytes and dendritic cells [87]. A set of FP7

variants with variations of length of the acyl chains was analyzed in detail for physico-chemical properties such as solubility, structure, and phase-behavior in the aggregated state. The occurrence of mixed lamellar/nonlamellar or pure lamellar aggregate structures was observed, consistent with their low biological activity. FP compounds bind to MD-2 at μmolar concentrations and the most potent antagonistic activity on LPS-mediated TLR4/MD-2 activation in HEK cells was reported for FP7 with an IC50 of 2.0 μM and FP12 with 0.63 μM [88]. In addition, FP7, like Eritoran, demonstrated benefit in non-LPS inflammation in a murine model of lethal influenza infection, supporting that targeting TLR4 is not restricted to LPS-driven bacterial pathologies [87, 89].

6 LPS Aggregates in the Activation of Non-TLR4 Receptors

TLR4-independent recognition of LPS is a recently discovered alternative pathway enabling an immunological response to cytosolic LPS. Fifteen years after the identification of TLR4 as the LPS receptor [72], a role of the human caspases-4/-5 and the murine caspase-11 in sensing LPS in the cytoplasm of host cells was reported. These caspases were demonstrated to induce non-canonical inflammasome activation in response to LPS, leading to the production of the cytokines IL-1β and IL-18 and the induction of a pro-inflammatory type of lysis-induced cell death named pyroptosis [90, 91]. Activation of the intracellular caspase pathway by LPS is accompanied by a strong pathophysiology. Studies in TLR4-deficient mice provided evidence for TLR4 independent sepsis driven via non-canonical inflammasome activation [91]. LPS recognition by caspases was shown to involve direct binding of LPS to caspase-4/5/11 in vitro [92]. The binding of LPS to the caspase is mediated by the N-terminal caspase-activation and recruitment domain (CARD) domain. LPS-binding to the CARD domain induces oligomerization and is required for catalytic activity and biological activity of the caspase complexes. The molecular mechanisms involved in caspase activation by LPS are however not yet fully understood. A central question to be resolved is the pathway of cytosolic LPS delivery. Preparations of purified LPS that are highly potent in activating TLR4/MD-2 do not initiate the inflammasome pathway; however, specific delivery of the LPS into the cytosol is required. This delivery can be achieved experimentally by electroporation of cells or by transfection of cells using lipophilic cationic chelating reagents to complex LPS, both techniques representing non-physiologic artificial pathways to induce caspase-dependent inflammasome and IL-1β/IL-18 activation. Alternatively, in tissue culture settings the delivery of LPS by co-administration with cholera toxin B (CTB) mediates uptake via ganglioside M-1 (GM-1) and enables inflammasome activation [91, 92]. The discovery that LPS as part of outer membrane vesicles (OMVs) released from Gram-negative bacteria activate the non-canonical inflammasome pathway provided first evidence for a physiologic system of LPS transport to the intracellular compartment. The release of OMVs is a process observed in basically all Gram-negative bacteria and represents a vital sign of living

bacteria [93, 94]. The uptake of OMVs involves phagocytosis and endosomal uptake [95, 96]. The mechanism of transfer or release of LPS from the vesicles into the cytosol is, however, unclear. Biophysical studies on OMVs demonstrated that in contrast to LPS, the LPS-containing bacterial vesicles can fuse with host cells, suggesting an intrinsic property for entry into the host cell cytoplasm [97]. Recent reports also indicate specific pathways for transport of LPS into the cytosol, candidates including the LPS-binding protein [98] and the high mobility group box 1 (HMGB-1) protein that mediates LPS transport via the RAGE receptor [99]. Involvement of a TLR4 – TIR-domain containing adapter-inducing interferon-β (TRIF) – guanylate-binding protein 2 (GBP2) pathway has been shown to enable the release of OMV-delivered LPS into the cytosol-inducing caspase activation in a murine model [100–102]. Since caspase regulation and activation displays a high diversity between different cell types and between murine and human cell systems, further studies will be needed to specify the role of different pathways. Considering the complex regulation of the TLR4 activation cascade, it is highly likely that not one but several pathways for LPS delivery may be in action to ensure the sensitive recognition of LPS.

The biophysical entity of caspase activation is also under debate. Biochemical studies demonstrated high affinity binding of purified caspase-4 to highly aggregated LPS and LPS-containing bacterial membrane vesicles. The resulting complexes did not indicate 1:1 molecular complexes but rather suggested an assembly of the caspase-4 at the LPS-containing membrane surfaces as a mechanism of activation [103]. Oligomerization of caspase by LPS as a critical step for caspase activation was also demonstrated in an independent study [92]. In contrast, another study showed by analytical ultracentrifugation and electron microscopy that binding of caspase-4 to large LPS aggregates induces disaggregation of LPS to low molecular weight caspase-4/LPS complexes [104]. Interestingly, for the caspase activation by particular LPS structures, also species-specific differences between human and murine caspases were reported [105]. Also in this relatively new field of LPS recognition by cytoplasmic caspases, it is apparent that the interaction of LPS with host cell membranes, transport proteins, and intracellular receptors is highly governed by biophysical mechanisms. Knowledge on the role of the aggregation state, the molecular geometry, and presentation of chemical groups of LPS to this group of receptors will be important to fully reveal their mode of activation.

7 Conclusions

The physico-chemistry of endotoxin is highly important for a mechanistic understanding of TLR4 activation by its ligand and also the basis for the development of new classes of immune-modulating compounds. A complete understanding of the molecular process of TLR4 activation has to consider the complex supramolecular structure, the variety of phase states and phase transitions occurring under different conditions, as well as the surface forces and thermodynamics governing the process

of LPS recognition by TLR4. Future approaches for the discovery of new TLR4-interacting compounds should therefore consider not only the optimal receptor docking but also the physico-chemistry involved in LPS recognition.

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