Endotoxin: Structure Source and Effects

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1.1 Endotoxin

The concept that endotoxin, an insoluble part of the bacterial cell, was a toxic substance able to evoke a typical picture of bacterial infection, even without the presence of living bacteria was introduced for the first time by Richard Pfeiffer in 1892 [1]. Subsequently, many years were needed to characterize the exact structure, function, and mechanism of action of endotoxin, nowadays recognized as lipopolysaccharide (LPS).

LPS is the major component of the cell wall of Gram-negative bacteria, recovering the 75% of the surface of the outer leaflet of the outer membrane of the cell wall. It is a glycolipid composed of a hydrophobic lipid part (lipid A) anchored in the outer leaflet and a hydrophilic polysaccharide part that extends outside the cell. The polysaccharide part is divided into two domains: the core region and the O antigen (also named O-chain). The O-chain is composed of several units of oligosaccharide and is tied to lipid A through the core region [2]. The main role of LPS molecules is to create a hydrophobic structure that results in a permeability barrier that protects bacteria from antimicrobial factors [3].

LPS is produced by most Gram-negative bacteria, with a few exceptions represented for example by Treponema pallidum [4]. Although the structure of LPS is

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well conserved, differences can be observed among species of bacteria. For example, an LPS without the O-chain is produced by some species of Gram-negative bacteria and it is called as "rough" LPS, as opposed to a "smooth" LPS, which includes the O-chain [5, 6]. LPS is a component of the bacterial wall essential for survival in a hostile environment. Indeed, Gram-negative bacteria that lack LPS or have LPS without an O-chain are more sensitive to antibiotics and, in general, to the host's defense mechanisms [3].

Among LPS components, lipid A deserves particular attention, as it is responsible for activating the immune system and for inducing pyrogenic and toxic effects. The structure of lipid A can differ among Gram-negative bacteria in the number and the length of fatty acid chains attached and the presence or absence of phosphate groups or other residues [3]. Generally, in most cases, LPS is constituted by a diglucosamine backbone phosphorylated at positions 1 and 4 and acylated with 5 or 6 fatty acyl chains. The most present fatty acyl chain is the 3-hydroxy-tetra-decanoinc acid. Studies demonstrated that alterations of lipid A can cause alterations in its biological activities. Indeed, the variable structure of lipid A determines its stimulatory or inhibitory action. For example, lipid A with a diglucosamine backbone, two phosphates, and six fatty acyl chains, is best sensed by the host's complex of myeloid differentiation factor 2 and the toll-like receptor 4 (MD-2-TLR4) [7].

LPS in the cell membrane of anaerobic Bacteroidales, which are present in the commensal microbiota of the human gut, has an under-acylated (tetra- or penta-acyl) lipid A that is a potent TLR4 inhibitor. Consequently, by silencing the TLR4 pathway, it facilitates the host's tolerance of gut microbes [8]. However, it is unknown if this phenomenon has any effect on the progression of infection [9]. In fact, the lipid A structure of Pseudomonas aeruginosa but also of many other Gram-negative bacteria does not possess six fatty acyl chains [7]. Yersinia pestis instead is able to produce hexa-acyl LPS at 21–27 °C and tetra-acyl LPS at 37 °C, and thus it is able to escape the host's first-line defense in mammals. Moreover, a genetically modified strain of Yersinia pestis which produces hexa-acylated LPS at 37 °C appeared to be avirulent, as it is able to facilitate the early recognition of infection and the effective onset of immune signaling [10]. During chronic infection, modifications of LPS molecules are possible and happen to facilitate the evasion of host immune defense and biofilm adaptation [11].

Gram-negative bacteria are a major part of the gut microbiota and are a source of LPS [12]. Normally, minor amounts of LPS can move into the bloodstream with the potential of triggering an immune response. However, to protect the host from a noxious over-activation of the immune system, several mechanisms exist for detoxification and elimination of LPS [13]. Among them, there is the rapid sequestration of LPS by lipoproteins, mainly high-density lipoproteins (HDL) in cooperation with the phospholipid transfer protein (PLTP). Lipoproteins transport LPS to the liver, where it is inactivated by enzymes such as acyloxyacyl hydrolase and alkaline phosphatase and, then, excreted in the bile [13].

Another mechanism of detoxification relies on the binding of LPS to the small form of HDL (called HDL3), which is produced by intestinal epithelial cells. In

particular, HDL3 by binding the LPS binding protein (LBP) captures the LPS and forms the HDL3-LBP-LPS complex. This complex hides LPS from liver macrophages, and instead induces its inactivation by favoring the effect of the plasmatic enzyme acyloxyacyl hydrolase (AOAH), thus protecting the liver from inflammation and fibrosis that may develop in the course of chronic exposure to LPS [14].

These mechanisms of detoxification are insufficient in case of disruption of the intestinal barrier, and an increased quantity of endotoxin enters the bloodstream. This is likely when the intestinal epithelium, formed by only one layer of cells, is damaged by hypoperfusion, inflammation, or dysregulation of commensal flora, resulting in an increased gut-barrier permeability and LPS translocation into the blood [15–17].

1.2 Pathway of LPS

LPS can stimulate extracellular and intracellular pathways that lead to the activation of the immune response.

1.2.1 Toll-Like Receptor 4-Myeloid Differentiation Protein 2 (TLR4-MD-2) Pathway

The TLR4 is the main receptor for LPS and one of the pattern recognition receptors responsible for the early detection of microbes by the innate immune system. TLR4 is expressed on the surface of monocytes, neutrophils, macrophages, dendritic, and epithelial cells, as well as within endosomes, forming the front line of the host's defense mechanisms against Gram-negative bacteria.

LPS molecules in the bacterial cell wall and also soluble LPS-aggregates can bind the LBP that in turn forms a complex with either a soluble or membrane-bound cluster of differentiation-14 (CD14), which is subsequently transferred to the TLR4/MD-2 complex. This promotes the TLR4/MD-2 dimerization and then the activation of intracellular MyD88 (myeloid differentiation factor 88) pathway, which determines the early activation of nuclear factor κB (NFκB), leading mainly to the production of proinflammatory cytokines (TNF-α, IL1B, IL-6, IL12B), or the TRIF (Toll-like receptor domain adaptor inducing interferon-β) pathway, which, on the other hand, is involved in the late phase of transcriptional activation of cytokines (IL-10) and in the development of endotoxin tolerance [18, 19]. The hyperactivation of the immune system triggered by pathogens and the subsequent cytokine storm leads to organ damage, multiorgan failure, and death [20].

However, the progress in research on LPS recognition systems led to important discoveries of TLR4-independent pathways sensible to LPS that may also play a central role in the pathophysiology of infection and related mortality.

1.2.2 Transient Receptor Potential (TRP) Ion Channels

TRP ion channels are membrane-bound channels that act as cellular sensors of environmental and intracellular stimuli. LPS can bind TRP channels present in neurons and airway epithelial cells [21]. Specifically, the activation of the subtype TRPA1 channels in nociceptive neurons by the LPS induces pain during inflammation [22]. The activation of the TRPV4 channels in the airway epithelium instead boosts ciliary beat frequency and the production of bactericidal nitric oxide, which facilitates the pathogen clearance from the airways. TRP channels by recognizing LPS provide an immediate response to invading pathogens, which is faster and independent of the canonical TLR4 pathway [21].

1.2.3 Intracellular LPS Pathways

LPS can enter the cytosol as LPS/outer-membrane-vesicle (OMV)-high mobility-group-box-1 (HMGB1) complex internalized through the receptor for advanced glycation (RAGE). When LPS enters the cytoplasm of macrophages, as well as endothelial and epithelial cells, it is sensed by inflammatory caspases such as caspase-4/5 in humans. The activation of caspases plays a crucial role in intracellular pathogen detection and defense. Indeed, caspases can lead to the induction of pyroptosis, an inflammatory form of cell death. Moreover, activated caspases can cause pore formation in the cell membrane with subsequent cell lysis and release of proinflammatory cytokines (IL-1β and IL-18) [23]. Inflammasome activation and pyroptosis are important mechanisms of the innate immune response against pathogens that are able to invade the cytosol and have a major role in the pathophysiology of sepsis. Caspases such as caspase-11 is also responsible for bacterial clearance of Klebsiella pneumoniae and Acinetobacter baumannii, as well as Burkholderia lung infections [23]. Furthermore, caspases may be responsible for sensing penta-acylated LPS, which is not detected by TLR4 [24]. Caspase-mediated pyroptosis of endothelial cells has a fundamental role in the host's defense and immune surveillance functions of the microvasculature [25]. Finally, an over-activation of pyroptosis can cause excessive cell death and inflammation leading to organ failure and septic shock [26].

1.2.4 Endotoxic Shock and Organ Damage Caused by LPS

Endotoxic shock is a severe, generalized inflammatory response caused by high bloodstream levels of LPS. A large amount of LPS triggers an extensive, uncontrolled systemic inflammation that leads to multi-organ failure and death. Patients typically present with fever and refractory hypotension. Organ failure secondary to hypoperfusion is common and patients may have oliguria, lactic acidosis, acute alterations in mental status, and disseminated intravascular coagulation (DIC). The pathological modifications induced by endotoxin in several organs contribute to the fatal outcome and are shown in Fig. 1.1.

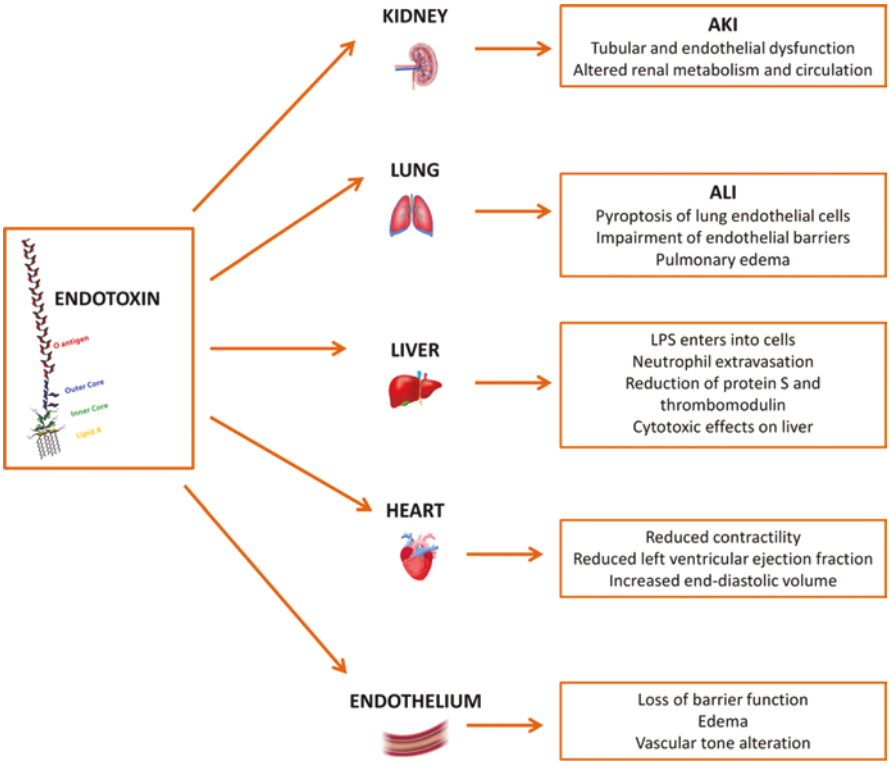

Fig. 1.1 Organ damage induced by lipopolysaccharide (LPS). *ALI* Acute lung injury, *AKI* Acute kidney injury

1.2.5 The Kidney

Acute kidney injury (AKI) is reported in at least 40–50% of patients with shock and is associated with significantly higher mortality [27, 28]. AKI is also characterized by metabolic and fluid abnormalities, which necessitate adjustments in volume and pharmacotherapy, most notably limiting antimicrobial choice. The pathophysiology of septic AKI is complex and, in addition to hypoperfusion, involves the interaction between vascular, tubular, and inflammatory factors. The exact mechanism underlying septic renal dysfunction is unknown, but experimental evidence is supporting the role of the TLR4, which is expressed in the kidney [29]. Specifically, TLR4 is located in the tubular epithelium, in the vascular endothelium and glomeruli. LPS is indeed filtered in renal glomeruli and internalized by S1 proximal tubules through TLR4 receptors. TLR4 activation causes the release of cytokine and chemokine; infiltration of leukocytes, which results in endothelial dysfunction; tubular dysfunction and altered renal metabolism and circulation [30]. In this way, there is a development of severe oxidative stress and damage also to the near S2 segments [30, 31]. Among other effects, TLR4 can directly block bicarbonate absorption in the medullary-thick ascending limb, reduce renal sodium, chloride, and glucose

transporters, induce luminal obstruction, and decrease tubular flow [30]. Other factors that contribute to septic AKI are endothelial activation and alterations to glomerular glycocalyx and the deposit of neutrophil extracellular traps (NETs) in the renal tissue [32, 33]. Direct renal damage by LPS can explain the occurrence of AKI, even when hemodynamic parameters are well-preserved [31]. In fact, it was shown that protocolized hemodynamic resuscitation did not influence either the development or the course of AKI in patients with septic shock [28]. As a result, the concept of acute tubular necrosis attributed to ischemia from hemodynamic changes in AKI was replaced by the theory of the interplay between inflammation, oxidative stress, and microvascular dysfunction [34].

1.2.6 The Lung

Histological alterations induced by LPS in the lungs are thickening of the septum, edema, congestion, and high leukocyte infiltration into the interstitium, which correlated with a significant increase in the serum concentrations of NETs and the extent of lung injury [33]. The inflammatory response is characterized by the release of prostaglandins, platelet-activating factors (PAF), leukotrienes, and thromboxanes, which can cause the respiratory distress syndrome by increasing the vascular permeability and contractions of smooth muscle cells in the lung. Lung injury was also attributed to the LPS-triggered pyroptosis of the endothelial cells. Specifically, LPS via caspase-4/5/11 mediated pyroptosis that led to disruption of the endothelial barrier resulting in pulmonary edema, the release of proinflammatory cytokines, fluid protein leakage, and a massive infiltration of leukocytes [25].

1.2.7 The Heart

TLR4 is also expressed in cardiomyocytes and its activation induces an inflammatory response with the production of cytokines and chemokines that have a negative effect on cardiac contractility [35]. LPS may trigger heart multiple caspase activation and cytochrome c release from the mitochondria causing myocardial cells apoptosis. Moreover, caspase-3 activation may also directly induce changes in calcium myofilament response, in troponin T cleavage, and in sarcomere disorganization, without determining death of myocardial cells [36]. In healthy volunteers, increased endotoxin levels resulted in a reduction of left ventricular ejection fraction and an increase of end-diastolic volume [37]. In the experimental model, the administration of LPS determined significant pathological changes such as myocardial bundles, congestion of capillaries with leukocytes attached to the endothelium, and histological changes of cardiomyocytes [33]. Other studies also indicated that LPS-associated cardiac dysfunction was also mediated by TLR4 activation [38].

1.2.8 The Liver

The liver is an important participant in the body's reaction to endotoxemia. Experimental studies demonstrated that LPS uses both TLR4 and caspase-11/gasdermin D pathways to induce the release of the nuclear protein high mobility group box 1 (HMGB1) from hepatocytes [39]. Complexes of HMGB1 and LPS are internalized via RAGE into the cytosol of macrophages and endothelial cells, where LPS activates caspase-11 and induces pyroptosis and cell death [40]. The intracellular effect of LPS is considered to play a central role in the pathogenesis of sepsis [23].

In the liver, LPS affects the architecture of the sinusoidal endothelium and blood flow velocities, which leads to extravasation of neutrophils, interaction of neutrophil and hepatocyte, decrease of protein S and thrombomodulin, which contributes to a procoagulant state and has a cytotoxic effect directly on hepatocytes [32]. Histological changes induced by LPS in the liver included enlarged sinusoids, increased volume of endothelial cells, high number of leukocytes in the lumen, hypertrophy and hyperplasia of Kupffer cell, along with the presence of leukocytes close to periportal areas and congestion of the central vein with swollen hepatocytes [33].

1.2.9 The Vascular Endothelium

Endothelial cell dysfunction is considered a key factor for the progression to organ failure [32]. The presence of LPS in the blood causes shedding of the glycocalyx lining of the vascular endothelium that leads to the loss-of-barrier function, the formation of edema, and the dysregulation of vascular tone [32].

The stimulation of endothelial cells with LPS determines the upregulation of several adhesion molecules (E-selectin, P-selectin, intercellular adhesion molecule-1, etc.), cytokines (IFN-α, INF-γ, IL-6), and chemokines (CCL2, CCL3, CCL5). Moreover, LPS decreases the expression of thrombomodulin, tissue-type plasminogen activator, and heparin, while increasing the expression of tissue factor (TF) and plasminogen activator inhibitor 1 (PAI-1) [36]. Moreover, LPS can induce the activation of the Hageman factor that stimulates the intrinsic pathway of coagulation that leads to the conversion of fibrinogen in fibrin. These effects, together with the activation of the extrinsic pathway, determine the shift of the hemostatic balance from an anticoagulant to a procoagulant state and induce endovascular thrombosis and the occurrence of DIC.

Furthermore, LPS can induce the release of nitric oxide (NO) and reactive oxygen species that cooperate in increasing endothelium damage and permeability. Endothelial damage determines the attachment of neutrophils, which further amplify the oxidative response. The activated Hageman factor can induce the stimulation of the kinins system by converting the pre-kallikrein into kallikrein that, in turn, catalyzes the conversion of kininogen into bradykinin, a vasoactive peptide that determines vasodilation and increases vascular permeability. LPS can also activate the complement cascade through the classic or alternative pathways, further

contributing to the increased permeability and chemotaxis of polymorphonuclear leukocytes. Finally, LPS can trigger caspase-dependent pyroptosis in endothelial cells resulting in the disruption of the endothelial barrier and fluid leakage [25].

1.3 Evaluation of Endotoxin-Induced Shock

There is no doubt that a clinical diagnosis of endotoxin-induced shock cannot be established by using only merely diagnostic tools, but it also needs the recognition of signs by clinicians. However, the prompt identification of clinical criteria to use in this setting has become over the years increasingly important since they have an impact on mortality and morbidity. In this context, the recognition of the stage from early inflammation to multi-organ dysfunction is fundamental.

Among clinical criteria, there is the use of Acute Physiologic Assessment and Chronic Health Evaluation (APACHE) score, Sequential Organ Failure Assessment (SOFA), and quick SOFA score. All scores evolved with the intent of finding an easily applicable scoring system to use in any clinical setting to predict the presence of shock, the risk of organ dysfunction, and the in-hospital mortality.

In case of a rapid identification of the source of infection, clinical investigations are individualized to the infected organ. On the contrary, in the absence of an apparent source, a time-sensitive search for infectious sources becomes a priority. Society guidelines endorse a routine collection of specimens from blood, sputum, urine, and any other wound for culture within the first hour of evaluation and before starting any antibiotic treatment [41].

Fundamental is the cardiovascular monitoring of patients with shock, who should be rapidly brought to a critical care area to assist, if necessary, the rapid resuscitation and optimal hemodynamic support. Continuous electrocardiographic monitoring and pulse oximetry are tools used in the management of critically ill patients. Monitoring venous oxygen saturation can give important information on the oxygen demand, especially in the early resuscitation phase of the shock therapy [42]. Indeed, a markedly low value of saturation indicates an imbalance in the oxygen supply/demand and likely indicates a need for augmenting global oxygen support.

Depending on the severity of endotoxin-induced shock, routine investigations can include the evaluation of indirect metabolic parameters to evaluate the extent of perfusion impairment and end-organ injury. The use of biomarkers is helpful for the diagnosis process. Among inflammatory biomarkers, there are procalcitonin, lactate, cytokines and chemokines, and C-reactive protein [43]. Lactate is currently the most commonly used metabolic parameter to monitor the effectiveness of resuscitation and cardiovascular support, since it can be indicative of tissue perfusion [42]. However, the other biomarkers are also essential to enhance lactate's effectiveness. Moreover, in a multi-marker panel, combinations of pro- and anti-inflammatory biomarkers may help to identify patients who are at major risk of developing severe shock and multi-organ dysfunction. However, one of the most significant direct parameters to assess the level of risk to develop a septic shock is related to the measurement of endotoxin activity assay. The Endotoxin Activity Assay (EAA) is a

useful test to risk stratify patients with severe sepsis and assess for Gram-negative infection evolution being assessed on a large multicenter study (Medic-study), demonstrating usefulness in flowing-up disease evolution in critically ill patients [44–46].

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