1 Endotoxin effects and interaction with the immune system

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Sepsis is defined as “a life-threatening organ dysfunction caused by a dysregulated host response to infection.”[1] Therefore, the interaction of sepsis with the immune system is implied by its very definition. The term “dysregulated” can be conceptualized as a maladaptive response that is responsible for organ dysfunction and death. The immune imbalance resulting from this altered response is characterized by multiple concomitant pro-inflammatory and immunosuppressive aberrations involving both the innate and the adaptive immune systems.[2] The loss of the normal homeostasis between the pro- and anti-inflammatory systems results in uncontrolled activation of the inflammatory response, leading to the development of progressive physiological dysfunction in ≥2 organs.[3] This uncontrolled activation of the inflammatory response and the inability of the host to limit inflammation at the injury site lead to systemic inflammation and a widespread insult.[4] Consequently, regardless of the nature of the initial trigger, a progressive functional failure of organ systems due to an uncontrolled systemic inflammatory response may lead to a multiple organ failure (MOF) syndrome.[5]

Septic shock due to gram-negative bacterial infection is a leading cause of mortality in critically ill patients, and it causes a huge burden on our health care systems. Due to the widespread nature of antimicrobial resistance and co-infections, the incidence of gram-negative bacterial infection is continuously increasing.[6, 7] Endotoxin, also known as lipopolysaccharide (LPS), is an antigen localized in the outer membrane of gram-negative bacteria.[8] Detection of LPS by the host represents a signal of microbial invasion and triggers a potent anti-inflammatory response.[9] The consequent activation of the immune system leads to the release of several cytokines and chemokines, which orchestrate the antimicrobial and inflammatory response, although they may cause MOF.[10] Consequently, it is important to highlight that this “dysregulated

host response" to LPS, rather than the intrinsic properties of LPS itself, is responsible for the potentially lethal consequence of gram-negative bacterial infection.[11] Due to the high incidence in critical care patients and its fatal consequences, a deep knowledge of pathophysiology of endotoxin effects and interaction with the immune system is necessary to understand clinical manifestations and the role of endotoxin in multiple organ dysfunction.

In this chapter, we provide an overview of the current knowledge regarding the possible interaction between endotoxin and the immune system and endotoxin organ-specific mechanisms of dysfunction.

Endotoxin structure

Endotoxin is a constitutive component of the gram-negative bacterial wall and acts as a barrier, protecting the bacteria from external insults.[8, 12] LPS is an amphiphilic molecule consisting of a hydrophilic polysaccharide part and a covalently bound hydrophobic lipid component, called lipid A.[8]

The polysaccharide can be divided into two subdomains: the core region and the O-specific chain. The core region structure, between the O-chain-proximal outer core and the lipid-A-proximal inner core, is less variable. The O-specific chain consists of up to 50 repeating units, each composed of 2-8 sugar monomers. It is unique for a given endotoxin and is distinctive for each bacterium.

Lipid A is composed of a phosphorylated 1,6-linked D-glucosamine disaccharide that carries up to six acyl residues. In particular, the lipid A component is responsible for the toxic properties of the entire LPS structure. This has been underlined by Huang *et al*.[13] They showed that exposure of human dendritic cells to whole *Escherichia coli* upregulated 466 genes, and 88% of the gene transcript profile was replicated upon exposure to purified LPS alone. The general structure of endotoxin is shown in Figure 1.1.

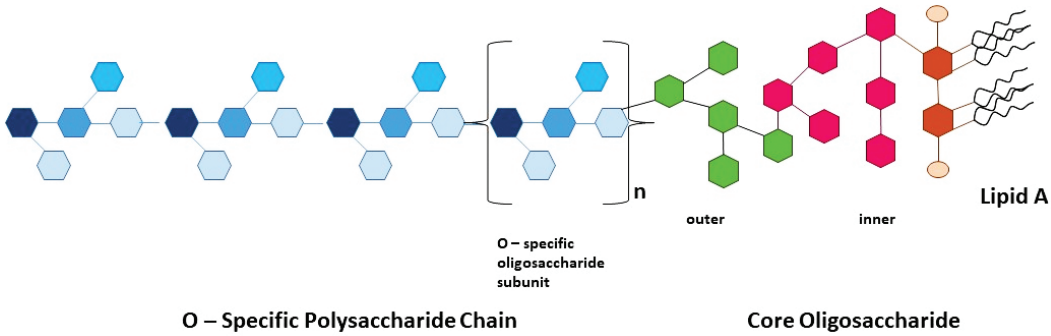

Figure 1.1. Structure of bacterial endotoxin.

Endotoxin release and effects on the immune system

LPS is the most potent bacterial mediator involved in gram-negative bacterial infection: It is viewed by the host as a signal of microbial invasion and triggers a potent anti-inflammatory response.[9] Moreover, endotoxin is released into the circulation after the disruption of intact bacteria due to cell lysis. Several antibiotics may play an important role in the induction and the release of endotoxin.[14]

The innate immune response is the first line of defense against external pathogens and is based on recognition of pathogens structures, called pathogen-associated molecular patterns (PAMPs).[15] Immune cells carry surface pattern recognition receptors (PRRs) on their surfaces; these receptors recognize PAMPs. Circulating necrotic cell fragments can initiate a similar response via damage-associated molecular pattern (DAMP) receptors. PAMPs and DAMPs can trigger leukocyte activation and transmigration.

The linkage between PAMPs and PRRs triggers cellular pathways responsible for production of inflammatory mediators. LPS is a PAMP; in other words, it alerts the eukaryotic organism to the presence of a bacterial invader within its internal milieu. Since its original description by Pfeiffer and Koch in 1892, endotoxin has been recognized as a key trigger of sepsis and the inflammatory cascade.[16]

Toll-like receptors (TLRs) are a family of PRRs that play crucial roles in the innate immune system by recognizing PAMPs derived from various microbes.[17] In particular, TLR4, which recognizes lipid A, is the PRR expressed on cells surface involved in the innate immune response after exposure to LPS.[18] LPS-binding protein (LPB) and CD14 are necessary for the interaction between TLR4 and LPS. LPB is required to transfer LPS to the TLR4/MD2 complex on the cell surface.[19] MD-2 is a molecule associated with TLR4 on the cell surface. The presence of MD-2 is required for LPS recognition.[20]

The process starts when LPB binds LPS and then recruits CD14; next, the complex links to MD-2. As a consequence, two different pathways of cellular activation may be triggered: MyD88-dependent or MyD88-independent pathways (Figure 1.2).[21] The intracellular signal transduction cascade is activated by different kinases (*e.g.*, interleukin-receptor-associated kinases [IRAKs] and mitogen-activated protein kinases [MAPKs]), leading to the activation of a transcriptional program, which includes the nuclear transcription factor nuclear factor kappa B (NF-κB), and the subsequent production and release of inflammation factors (*e.g.*, tumor necrosis factor alpha [TNF-α], interleukin 1 [IL-1], IL-1B, and IL-6).[22, 23] The MyD88-independent pathway is characterized by activation of TIR-domain-containing adaptor protein (TRIF) that, in turn, activates interferon regulatory factor 3 (IRF3) and interferon beta (IFN-β).[24] This pathway is involved in the late phase of transcriptional activation (*i.e.*, IL-10 and transforming growth factor beta [TGF-β]).[25]

The release of cytokines and chemokines triggers the classical manifestations of sepsis, characterized by leukocyte activation and transmigration, increased capillary

permeability, and endothelial damage. The consequent exposure of tissue factor (TF) to circulating coagulation factor represents an important linkage between the inflammatory and coagulation systems. The cross-talk and interplay between the endothelium, vascular smooth muscle, inflammation, and coagulation may initiate a procoagulant response that results in thrombin generation and increased fibrin formation.[26, 27] Fibrinolysis may also be impaired. Specifically, the stimulation of endothelial cells with TLR agonists, such as LPS, upregulates several adhesion molecules (E-selectin, P-selectin, intercellular adhesion molecule-1, etc.) as well as cytokine (IFN-α, INF-γ, and IL-6) and chemokine (CCL2, CCL3, and CCL5) production.[28] Consequently, the rolling, adherence, and migration of leukocytes into infected tissues is enhanced exponentially, and endothelial cells also shift the hemostatic balance from an anticoagulant to a procoagulant state through decreased expression of thrombomodulin, tissue-type plasminogen activator, and heparin, as well as increased expression of TF and plasminogen activator inhibitor 1 (PAI-1).[29] The hemostatic balance is further altered by LPS-induced apoptosis of endothelial cells, which exposes prothrombotic subendothelial proteins to clotting factors in the blood. Moreover, apoptosis leads to cytoskeletal alterations that decrease endothelial barrier function, so intravascular fluids leak into the extravascular space.[30] Although the phenotypic changes in the activated endothelium may limit the spread of regional infection, during severe systemic inflammation, upregulation of endothelial adhesion molecules, disruption of the endothelial barrier, and loss of the anticoagulant properties of the endothelium can worsen organ injury and mortality.[30] Consequently, microcirculation flow and distribution can be widely altered, with subsequent changes in oxygen circulation, oxygen distribution, and organ perfusion. All the aforementioned mechanisms are responsible for the development of MOF.[31] In 2004, the MEDIC study was the first large observational cohort study to correlate endotoxin and patient outcome. Marshall *et al*.[32] enrolled 857 patients admitted to the intensive care unit (ICU) and used the Endotoxin Activity Assay (EAA) to measure endotoxin on the first day of admission. The rates

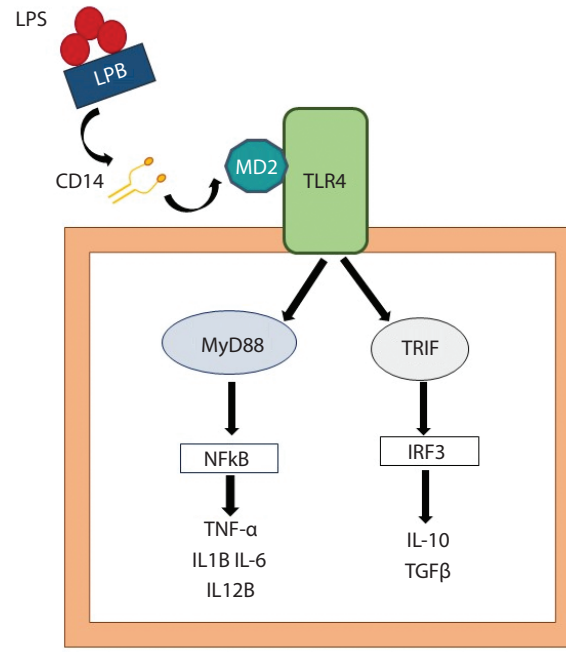

Figure 1.2. The MyD88-dependent and MyD88-independent pathways.

of severe sepsis were 4.9%, 9.2%, and 13.2%, and the ICU mortality rate was 10.9%, 13.2%, and 16.8% for patients with low, intermediate and high endotoxin activity (EA), respectively. These data indicate that in septic patients, increasing EA is associated with increased hospital mortality.[32] Since then, several studies have highlighted the possible role of endotoxemia on progression and outcome of septic patients.

Host exposure to large amounts of LPS is deleterious, because it triggers a potentially lethal massive release of pro-inflammatory mediators and pro-coagulant factors; however, a phenomenon known as endotoxin tolerance has also been observed and extensively studied in *in vitro* and *in vivo* models. Endotoxin tolerance consists of desensitization to endotoxin after exposure to a sublethal dose of endotoxin.[9] Nevertheless, lower doses may prime the immune system and, consequently, produce a more potent inflammatory response.[33] The mechanisms of endotoxin tolerance are still poorly understood. Endotoxin-tolerant macrophages and monocytes produce lower levels of pro-inflammatory cytokines (TNF-α and IL-6) and more anti-inflammatory molecules (IL-10 and TGF-β). Functionally, endotoxin-tolerant monocytes exhibit an increased phagocytic ability coupled with a conserved capacity to kill internalized pathogens, albeit with an impaired antigen presentation capacity.[34] Monocytes and macrophages as well as all other immune cells, including dendritic cells, neutrophils, T cells, and natural killer (NK) cells develop tolerance to secondary TLR stimuli.[2, 35]

Endotoxic tolerance is also characterized by cyclooxygenase-2 activation, inhibition of MAPKs, and reduced NF-κB translocation.[36] This shift from a pro-inflammatory to an anti-inflammatory profile may be explained by sepsis-induced epigenetic reprogramming, involving altered nuclear translocation of transduction molecules, decreased stability of messenger RNA for cytokine genes,[9] and upregulation of micro-RNAs (*e.g.*, miR-132, miR-146, and miR-155).[37, 38] Moreover, the ability of monocytes to present antigens is highly impaired due to reduced expression of major histocompatibility class II (MHC II) molecules (*e.g.*, HLA-DR).[2, 35] Pathophysiological adaptations to regulate overexuberant inflammation serve as an important mechanism for host protection against endotoxic shock. Considering the *in vivo* relevance of the above phenotype, a poor inflammatory capacity coupled with upregulation of anti-inflammatory cytokines would contribute to protect against septic shock, and increased phagocytosis would allow efficient bacterial clearance. In contrast, impaired antigen presentation would possibly either inhibit or alter the development of an adaptive response.[35] Although endotoxin tolerance has been thought of as a protective mechanism against septic shock and ischemia, its incidence is associated with a high risk of secondary infections.[39]

Interestingly, gut microbiota can play a critical role in contributing to the release of LPS in the bloodstream.[40] Under normal physiological conditions, enteric bacteria live in symbiosis in the human gut, helping the immune system to develop tolerance and immune balance, and facilitating the destruction of invading organisms. In some pathological circumstances (*e.g.*, gut ischemia and low cardiac output states), the change in the gut barrier can facilitate bacterial or endotoxin translocation in the bloodstream,

leading to endotoxemia.[41] It is worth noting that the gut epithelium is an efficient barrier that prevents the absorption of LPS.[42] Structural changes to the intestinal epithelium in response to dietary alterations or low perfusion states allow LPS to enter the bloodstream, resulting in an increase in the plasma levels of LPS (termed metabolic endotoxemia).[43] LPS activates TLR4, leading to the production of numerous pro-inflammatory cytokines.

Role of endotoxin in organ dysfunction: organ-specific mechanisms of dysfunction

Endotoxin plays a pivotal role in the pathogenesis of multiple organ dysfunction in the setting of gram-negative sepsis. As previously described, the interaction between LPS and the immune system activates the inflammatory cascade, causing the dysregulated immune response typical of sepsis, with several degrees of severity. Endothelial dysfunction and the consequential barrier disruption underlie increased vascular permeability, which is critical to the pathogenesis of shock and MOF in endotoxemia.[44] LPS stimulates endothelial cells to produce cytokines, chemokines, and adhesion molecules (*e.g.*, E-selectin and P-selectin), and triggers apoptosis, destroying the endothelium. The effects of the inflammatory mediators clinically lead to cellular damage with the impairment of organ function.[45] Below, we summarize the organ-specific mechanisms of dysfunction mediated by endotoxin.

Effects on the heart

Septic cardiomyopathy is a well-known complication of septic shock; it is characterized by reversible cardiac dysfunction.[46] Cardiomyopathy may be due to dysfunction of the left or right ventricles, or both.[47] Several direct and indirect pathophysiological mechanisms are responsible for the cardiovascular complications during endotoxic septic shock (Table 1.I). Septic cardiomyopathy represents the consequence of a complex interaction between the guest, the host responses, and underlying cardiac condition.

Of note, TLR4 play a central role in the pathogenesis of cardiac dysfunction.[48, 49] Activation of TLR4 signaling pathways may induce the production of different mediators (*e.g.*, IL-6 and TNF-α) responsible for systemic inflammation and cardiac injury. Furthermore, TLR4 is expressed on the surface of myocardial cells; consequently, direct activation of TLR4 in the heart and/or the production of mediators, through indirect mechanisms, may lead to cardiac dysfunction.[50] Hence, immune cells, heart cells expressing TLR4, oxidative stress, circulating factors, and infiltrating blood leukocytes may alter myocyte function.

Regardless of the specific pathogenic mechanism, mitochondrial dysfunction, alterations in ion channels, changes in autonomic autoregulation, apoptosis, focal

necrosis, congestion, inflammatory infiltrates, and edema represent the possible final cardiac sequelae of endotoxic shock.[51] In the heart, endotoxin triggers multiple caspases and cytochrome *c* release from the mitochondria, causing end-stage apoptosis of myocardial cells. Additionally, caspase-3 activation may directly alter calcium myofilament response, troponin T cleavage, and sarcomere organization, without inducing myocardial cell death.[52] Alterations in calcium channels and a consequent reduction in intracellular calcium impair myocyte contractility.[53] Hence, myocyte contractility may be altered due to a direct effect of cytokines, but also due to structural myocyte alterations and to the presence of myocardial infiltrates (*e.g.*, polymorphonuclear leukocytes, macrophages, monocytes, and fibrin). Myocardial wall edema *per se*, mostly described after cardiac surgery and post-cardiopulmonary bypass—but also in endotoxin-challenged animals—can be an underestimated component of this reversible dysfunction by altering muscle compliance/elastance an exhibiting a "stunning"-like profile.[51, 54]

Table 1.I. Direct and indirect effects of endotoxin on the heart.

Direct miocardial effects	Indirect myocardial effects
Mitocondrial dysfunction	Activation of coagulation system
Adrenergic down-regulation	Microcirculatory dysfunction
Disturbed calcium trafficking	Autonomic dysregulation
Apoptosis	Circulation failure
Myocardial edema	Cytokine release

Hemodynamic alterations due to sepsis (*e.g.*, preload, afterload, and contractility) are involved in the pathogenesis of myocardial depression. Hypovolemia and reduced vascular tone can contribute differently to the severity of myocardial dysfunction, with consequent inadequate cardiac output and redistribution of regional blood flow.[55-57] The effect of endotoxin on coronary blood flow is controversial. However, it seems that, during endotoxic septic shock, coronary blood flow is preserved or in some case is increased, characterized by marked coronary vasodilatation.[58, 59] Direct and indirect effects of endotoxin on the heart are shown in Table 1.I.

Pulmonary effects

The effects of endotoxin on the lung are complex. Endotoxin interacts with the different cells of the lung, inducing immunomodulation and inflammatory responses, with possible disruption of alveolar and epithelial cells leading to acute respiratory distress syndrome (ARDS).[60] It is possible to recreate human acute lung injury (ALI) in different animal species by infusing LPS.[61] One hour after inhalation, intratracheal instillation, or intravenous infusion of endotoxin, there is considerable lung tissue injury characterized by neutrophil accumulation in the alveolar and interstitial space, alveolar wall thickening, and accumulation of proteinaceous edema and detritus in the alveolar space.[62] These alterations are mostly due to the presence of profound vascular leakage with not only

movement of fluid and macromolecules into the interstitium and airspace, but also transendothelial diapedesis of leukocytes into lung tissue, further contributing to vascular and alveolar dysfunction in ALI.[63]

In detail, endotoxin bound to the surface of endothelial cells activates endothelial signaling pathways and secretion of inflammatory mediators.[64, 65] LPS induces changes in extracellular matrix mechanics in lung tissue, increasing vascular permeability and causing pulmonary edema. Moreover, cytokine activation leads to increased adhesion of immune cells, causing acute neutrophil accumulation in lung tissue.[66, 67] Pro-inflammatory cytokines, TF, eicosanoids, and endothelin act in concert to alter the lung endothelial and epithelial barriers, changes that are characteristic of ALI.

In many animal models, LPS administration enhances lung microvascular pressure and permeability, thereby increasing the extravascular lung water content and worsening gas exchange. In addition, there is altered coagulation activity, resulting in the generation of microthrombi in lung capillaries.[68] Furthermore, LPS directly interacts with alveolar epithelial cell types I and II (ATI and ATII, respectively). LPS binding to the receptor of advanced glycation end products (RAGE) on the ATI cell surface activates NF-kB, determining the transcription of several inflammatory factors (*e.g.*, TNF-α and IL-1).[69] ATI and ATII cells constitutively express TRL4. ATII cells produce surfactant, which is required to maintain an adequate surface area at the end of expiration in the lungs.[70] Endotoxin may also interact directly with surfactant lipid film, causing fluidization and altering its properties, resulting in surfactant dysfunction.[71]

Increased apoptosis and autophagy have also been reported in several animal studies through mitochondrial signaling and activation of cell surface death receptors (Fas).[72] Moreover, LPS interacts with fibroblasts, inducing fibroblast proliferation and accelerating pulmonary fibrosis in early stages of lung injury.[73] The subsequent differentiation of fibroblasts into myofibroblasts, induced by inflammatory pathways, amplifies epithelial cell damage and apoptosis, creating a vicious circle of pro-fibrotic interactions.[74]

A recent study reported evidence of action potential duration (ADP) prolongation in human pluripotent stem cells treated with LPS. They authors hypothesized that this dysfunction partially explains the cardiac rhythm alteration observed in septic patients.[75]

Renal effects

The pathophysiology of acute kidney injury (AKI) during endotoxemia is not clearly understood. Moreover, it should not be viewed simplistically in the context of hypoperfusion and ischemia, but rather as the expression of a dysregulated host response to septic stimuli, through the recognition of PAMPs.[76] Surely, hypotension and hemodynamic instability represent important insults in critical care patients. However, several studies have observed a preserved or even elevated renal blood flow in septic patients with AKI.[77, 78] Hence, it seems that a major role is played by the direct pro-inflammatory state typical of septic shock, leading to microvascular shunting, cell stress, and direct injury of glomerular