Lipopolysaccharide Neutralization by Antimicrobial Peptides: A Gambit in the Innate Host Defense Strategy

David Pulido\textsuperscript{a}    M. Victòria Nogués\textsuperscript{a}    Ester Boix\textsuperscript{a}    Marc Torrent\textsuperscript{a, b}

\textsuperscript{a}Department of Biochemistry and Molecular Biology, Faculty of Biosciences, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, and \textsuperscript{b}Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Biomedical Research Park of Barcelona, Barcelona, Spain

Key Words
Peptide design \slash Peptide structure-activity relationship \slash Antibacterial peptides \slash Antimicrobial peptides \slash Endotoxin \slash Host defense \slash Immune response \slash Pathogen-associated molecular patterns \slash Pattern recognition receptors \slash Sepsis \slash Septic shock \slash Toll-like receptor

Abstract
Antimicrobial peptides (AMPs) are nowadays understood as broad multifunctional tools of the innate immune system to fight microbial infections. In addition to its direct antimicrobial action, AMPs can modulate the host immune response by promoting or restraining the recruitment of cells and chemicals to the infection focus. Binding of AMPs to lipopolysaccharide is a critical step for both their antimicrobial action and their immunomodulatory properties. On the one hand, removal of Gram-negative bacteria by AMPs can be an effective strategy to prevent a worsened inflammatory response that may lead to septic shock. On the other hand, by neutralizing circulating endotoxins, AMPs can successfully reduce nitric oxide and tumor necrosis factor-\alpha production, hence preventing severe tissue damage. Furthermore, AMPs can also interfere with the Toll-like receptor 4 recognition system, suppressing cytokine production and contributing to modulate the inflammatory response. Here, we review the immune system strategies devised by AMPs to avoid an exacerbated inflammatory response and thus prevent a fatal end to the host.

Introduction

The innate immune system is the first line of defense against invasive organisms. It comprises humoral and cellular components endowed with different protective functions together with anatomical barriers against pathogens, like the skin and the internal epithelial tissues [1].

The innate immune response begins with the host recognition of highly conserved molecular structures in pathogens, known as pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors. Bacterial lipopolysaccharide (LPS) is one of the most active PAMPs and can promote a resilient induction of the innate immune system [2]. Actually, LPS has a predominant role in septic shock syndrome caused by Gram-negative bacteria [3]. Other PAMPs such as Gram-positive lipoteichoic acid or peptidoglycan (PGN) are also recog-
nized by the host pattern recognition receptors and stimulate proinflammatory responses, although much less exacerbated than LPS [3]. Additionally, compounds known as ‘endogenous danger signals’, including uric acid and extracellular heat shock proteins [4], can activate proinflammatory responses in the host.

LPS is an amphiphilic molecule composed by lipid A and a polysaccharide moiety which comprises the polysaccharide core and the antigenic ‘O’ region, conformed by several repeats of variable oligosaccharide units (fig. 1a). LPS forms supramolecular aggregates in aqueous environments above the critical micellar concentration [5]. Typical critical micellar concentration values for lipid A or deep rough mutant LPS Re are comprised between $10^{-7}$ and $10^{-8}$ M [6]. In fact, LPS aggregates are the biologically active units recognized by the host immune system [7].

Due to the central role of LPS in triggering septic shock, new molecules that prevent such an exacerbated immunity response are actively searched. Noteworthy, the development of new drugs based on LPS-binding molecules could contribute to fighting a life-threatening condition that leads, worldwide, to $>$500,000 deaths per year [8]. Among the host molecules that can interact and neutralize LPS and prevent sepsis, antimicrobial proteins and peptides (AMPs) are especially attractive because of their high affinity and modularity.

AMPs generally display a high content of cationic and hydrophobic amino acids. It is widely believed that they act through nonspecific binding to biological membranes, even though the exact nature of these interactions is currently unclear [9, 10]. Although AMPs differ widely in sequence and source, several designs in their 3-dimensional topology are recursive. Hence, AMPs have been categorized accordingly into four main groups: (1) α-helices, (2) β-sheet structures, stabilized by two or more disulfide bonds, (3) extended helices (polypeptide helices), with a predominance of one or more amino acids, and (4) loop or disordered structures [11]. However, in proteins, a combination of such structures is often found. Additionally, some modified constructions exist, i.e. cyclic peptides such as θ-defensins or lipid-derived peptides such as polymyxin. Nonetheless, all of them are folded to exhibit an amphipathic structure displaying a hydrophobic surface that comprises non-polar amino acids and a hydrophilic face containing most polar and positively charged residues [9]. Despite this amphipathic nature, polypeptides may differ widely in length and amino acid composition.

In addition to direct bacteria killing, AMPs also display essential immunomodulatory functions that may be involved in the clearance of microorganisms, including: (1) the ability to act as chemokine and/or induce chemokine production, (2) the capacity to inhibit LPS-induced proinflammatory cytokine production, and (3) the capability to modulate the response of dendritic cells and adaptive immune system cells [12].

**Molecular Basis of AMP Interaction with LPS**

Commonly, LPS-binding AMPs are mainly unstructured in solution but gain defined conformation (typically α-helical) upon interaction with LPS micelles. This is the case, for example, of fowlcidin [13], SMAP29 [14], pardaxin [15], temporins [16] and magainin analogues [17]. As stated before, all AMPs display an amphipathic structure, a critical trait required not only for LPS binding but also for their antimicrobial activity. In AMPs, this kind of structure confers (1) the ability to perform strong electrostatic interactions with the negatively charged phospholipids in the membrane bilayer of microbes, (2) the ability to partially or entirely insert into the lipid bilayer, promoting membrane disruption and, additionally, (3) a stability gain when bound to the lipid bilayer as the unfolded-to-folded transition stabilizes the membrane-bound state (e.g., in amphipathic α-helices, the folded conformation stabilizes the membrane-bound state by approximately 0.4 kcal/mol) [18, 19]. However, not all amphipathic designs can efficiently neutralize LPS. In fact, in all the nuclear magnetic resonance-solved structures of AMPs bound to LPS, the hydrophobic portion of the peptide appears segregated from the cationic part. As reference examples, we have compiled the structures of lactoferrin, pardaxin, Y112WF and MSI954 (a magainin analogue) when bound to LPS in figure 1b. Peptides with analogous structures but devoid of antimicrobial activity, such as the HIV-1 gp120 viral protein V3 loop, have also been described to bind LPS [20].

On the one hand, the reason for this segregation could be explained by the high rigidity and low permeability of the LPS molecule compared with phospholipids; thus, LPS molecules are unable to reorient in response to AMP action [21]. On the other hand, a high cationic content is critically required in LPS-binding peptides to enable the replacement of divalent cations that tighten the structure and neutralize the LPS-negative charge [14, 22, 23]. In summary, the highly charged portion of the peptide would direct the binding of AMPs to LPS, immediately substituting the divalent cations and directing the peptide to the interphase of the structure [24–28]. There, the hydropho-
Fig. 1. LPS recognition by AMPs. a Chemical structure of LPS (left). The chemical structure of LPS is divided into the O-antigen, the polysaccharide core and the lipid A moiety. Molecular structure of LPS based on the LPS 3-dimensional structure (right). LPS structure (without O-antigen) was determined in complex with protein FhuA by X-ray diffraction (1FI1.pdb) [64]. b Molecular structure of LPS-binding AMPs MSI594, pardaxin, lactoferrin and YI12WF. Cationic residues are depicted in blue, polar residues in green, hydrophobic residues in yellow and aromatic residues in red. Side chains for aromatic and cationic residues are depicted in the cartoon model to show their location. Surface models are colored by electrostatic potential, from blue (positive charge) to red (negative charge). c Rational design of new LPS-binding peptides. The scheme shows the steps to design new LPS-binding peptides based on the analysis of detailed molecular information on LPS-polypeptide complexes. The steps include: (1) identification of the pharmacophore, (2) rational design of new peptides that resemble the pharmacophore, and (3) peptide refinement. All molecules were drawn using Pymol (DeLano Scientific).
bic moiety of the peptide would insert into the lipophilic phase promoting the disturbance of LPS. For example, in boomerang peptides, the hydrophobic regions of the peptide are particularly involved in the disorganization of LPS [29]. Consistently, the deletion of hydrophobic residues in these peptides promotes a dramatic reduction in LPS-neutralizing and antimicrobial activities [29].

A defined distance between the charged residues has also been proved to be essential. Actually, the positively charged amino groups of Lys and Arg in LPS-binding peptides show a typical distance range of 12–15 Å, in good accordance with the interphosphate distance of the lipid A moiety of LPS [15, 16]. Thus, the charged residues in the peptide will interact with the negatively charged phosphate groups of the lipid A portion of LPS, whereas the hydrophobic residues will be embedded in the lipophilic core region.

Last but not least, aromatic residues are essential to pack the peptide structure, by stabilizing a compact structure. Hence, the packing of aromatic side chains can have a remarkable impact in the LPS-binding affinity [29]. For example, in MSI594, Phe 5 to Ala mutation generates a relaxed structure where the distance between the charged clusters exceeds the mean interphosphate distance. Hence, the cationic clusters are no longer in appropriate vicinity [30]. Additionally, the compact structure could also be important for antimicrobial activity, by helping with the translocation across the LPS bilayer in Gram-negative bacteria and allowing the peptide to reach the lipid membrane. Similar results are observed for the recently reported β-boomerang peptides, designed using a rational structure-guided approach based on the crystal structure of protein FhuA in complex with LPS (fig. 1c) [29]. The authors show that the long-range aromatic stacking (from residues i to i+5) is extremely important for the endotoxins and antimicrobial properties of boomerang peptides. It appears that the aromatic packing promotes, in fact, the specific boomerang structure [29]. The replacement of those residues by Ala or Leu in peptides, though retaining amphipathicity, generates a relaxed structure with impaired neutralization activity. Similar results are found in temporins where intimate packing of aromatic residues sustains the dimeric structure of temporin TL [16]. Interestingly, peptide oligomerization and the aggregation state have also been found critical for antimicrobial activity [31, 32]. Recent studies by Bhunia and coworkers [16] have shown that the oligomerization state after binding to LPS modulates antimicrobial activity. Hence, temporins TA and TB that form oligomeric structures in the presence of LPS exhibit low antimicrobial activity on Gram-negative species. In particular, TB undergoes oligomerization in complex with LPS by contacts among hydrophobic residues in the N and C termini of the molecule that hinder the antimicrobial action of the peptide [16]. Accordingly, temporin TL, which shows reduced oligomerization, also displays higher antimicrobial activity.

Changes in LPS Structure upon Interaction with AMPs

It has been shown that LPS micelles undergo a certain rearrangement when bound to AMPs, altering their aggregation levels. Thus, some peptides (e.g. temporins and pardaxins) can disturb LPS aggregates producing singular structures with smaller size [15, 16]. These smaller particles reduce the availability of LPS to bind LPS-binding protein (LBP), ensuing a decrease in the level of tumor necrosis factor (TNF)-α secretion by macrophages. However, other peptides have been described to increase the size of LPS aggregates. In these cases, like the Limulus anti-LPS factor, the decrease in LPS bioactivity can be explained by the fact that binding sites for LPS-binding proteins are hidden in multilamellar aggregates (in contrast to cubic aggregates in the absence of AMPs). Thus, the change of LPS from a cubic to multilamellar structure would be a prerequisite for LPS inactivation [33].

Despite the fact that peptide affinity can be modulated by LPS sugar moiety, it seems not to be involved in the final structure defined by the peptide once bound to LPS. For example, in polymyxin M, the presence of a Thr residue in position 7 favors the interaction with the KDO (2-keto-3-deoxyoctonic acid) molecules of LPS, whereas in polymyxins B and E the presence of a Leu residue contributes to strengthen the hydrophobic contacts with the lipid moiety. In fact, the backbone region of polymyxin would act as a scaffold for the appropriate orientation of the side chains rather than being primarily involved in LPS binding [34, 35].

The binding affinity of AMPs to LPS is similar at different temperatures, 298 K (below the phase transition temperature of LPS, gel phase) and 313 K (above the phase transition temperature of LPS, liquid crystalline phase). However, the thermodynamics of the process are quite different [15]. For AMPs (e.g. pardaxins and temporins), peptide binding to the gel phase of LPS is an endothermic process, primarily driven by a positive change in entropy, and hence, determined by hydrophobic interactions [15, 36]. In contrast, in the liquid crystalline phase, the reaction is exothermic and becomes enthalpically driven though retaining a positive change of entropy. The ele-
trostatic attraction between the positive charges of AMPs and the negative charges of LPS results in an exothermic process which is superimposed by the interaction with ordered water and counterion layers and results in an endothermic reaction. Whereas in the gel phase, the endothermic enthalpy change in the LPS backbone exceeds the exothermic change of the charge attraction, in the liquid gel phase, the opposite process is observed [37]. In fact, the enthalpy increase in the interaction between AMPs and LPS has been correlated with the antimicrobial activity in Gram-negative species [38].

How AMPs Block LPS-Mediated Inflammation

As multifunctional peptides, AMPs can regulate LPS action at several levels, from recognition to downstream signaling. Here, we describe, at the molecular level, the LPS transduction pathway and the diverse effects of AMPs in the system.

Recognition and Binding of Circulating LPS Molecules

During bacterial death or division, endotoxins are released into the blood stream. These travelling molecules are recognized by LBP, which is responsible for stimulating mononuclear cells, even in the presence of small LPS amounts. At these low concentrations, LBP binds LPS and transfers it to CD14 (fig. 2), promoting the activation of mononuclear cells [39]. Other properties of LBP comprise the ability to transfer phospholipids to LPS aggregates and LPS to phospholipid membranes (fig. 2) [40]. Therefore, LBP can be considered as a lipid transfer protein. In fact, LBP is able to transfer LPS to high-density (HDL) and to low-density lipoprotein, attenuating the activity in Gram-negative species.

Transfer of LPS Molecules from LBP to mCD14

CD14 is an important LPS-binding molecule that can be present in serum as a soluble form (sCD14) and as a membrane form (mCD14) anchored by glycosylphosphatidylinositol on the surface of proinflammatory cells (fig. 2) [43]. The structure is based on a leucine-rich repeat folded into a horseshoe-like shape with a deep hydrophobic pocket where it is assumed that the lipid A portion of LPS is buried, in contrast with the carbohydrate chain that sticks out of the pocket [44] (online suppl. fig. S1; for all online suppl. material, see www.karger.com/doi/10.1159/000336713). Binding of LPS to CD14 does not induce a global structural change but local changes in two surrounding α helices. The hydrophobic N-terminal pocket of CD14 is not the only region involved in the binding of hydrophilic molecules such as PGN, though a partial overlap has been described. Instead, the binding of PGN to CD14 could be shifted to the C-terminal region of the protein [44]. The interaction between LPS and CD14 is required for the activation of human monocytes and murine macrophages. CD14-negative endothelial and epithelial cells are also activated by sCD14 in conjunction with LBP-LPS complexes [43, 45]. sCD14 can mediate the transport of cell-bound LPS to plasma lipoproteins thereby tempering the cellular response to LPS. In fact, CD14-deficient mice are highly resistant to septic shock induced by injection of either LPS or live bacteria [46].

A basic strategy used by the host immune system to regain homeostasis in response to infection is based on the detoxification and neutralization of endotoxins by LBP and CD14. Among others, one mechanism lies in the ability of AMPs to neutralize endotoxin molecules, covering the recognition sites of LPS for CD14 and LBP and therefore precluding the expression of Toll-like receptor 4 (TLR4) proinflammatory genes [47]. Hence, several AMPs act as scavengers of the innate immune system. For example, cathelicidins are able to: (1) interact and neutralize LPS, blocking its binding to CD14, (2) disaggregate LPS, reducing the binding affinity to LBP, and (3) clear LPS from cell surfaces in monocyte- or macrophage-like cells, inhibiting the production of proinflammatory cytokines [48]. Similarly, some defensins have been shown to interact with LPS in vivo, inhibiting the acute cell response in mice [49]. More interestingly, bactericidal permeability-increasing protein (BPI) displays a multifunctional modular structure based on an N-terminal antimicrobial and LPS-neutralizing domain and a C-terminal domain that binds and transfers LPS to proteins located in the surface of host immune cells and LBP [50]. Thus,
Fig. 2. Recognition and neutralization of LPS in the human body. LPS molecules can be recognized and detoxified by diverse elements in the human body such as LBP, CD14 and AMPs. Moreover, LBP can also transfer LPS to sCD14 or mCD14 that can be recognized by TLR4, initiating the immune response by releasing different mediators by diverse cell types. Protein structures were obtained from the protein data bank (www.pdb.org). When human structures were not available (LBP), a homology model was constructed using Swiss Model (www.swissmodel.expasy.org).
BPI can inhibit LPS bioactivity by four known mechanisms: (1) by direct interaction and neutralization of LPS, (2) by transferring LPS to lipoproteins, (3) by forming LPS-LBP aggregates that are internalized, and (4) by interfering with the transfer of LPS bound to CD14 and TLR4 [40].

Activation of TLR4 and Other Signaling Molecules by the CD14-LPS Complex

Since CD14 does not have an intracellular signaling domain, transfer of the LPS molecule to other molecules is required for downstream signaling. The receptor responsible for coupling the LPS recognition and transduction systems is the TLR4. The TLRs are members of the Toll/interleukin-1 receptor superfamily that display a common and highly conserved Toll/interleukin receptor homology domain [51]. The Toll/interleukin receptor domain is the central element that triggers the proinflammatory signal from the cell membrane to subsequent intracellular domains by interaction with adaptor molecules resulting, at the end of the signaling cascade, in the expression of several proinflammatory genes (online suppl. fig. S1) [52].

Another way of cellular activation beyond the receptor-mediated pathway is the insertion of endotoxin molecules into the lipid matrix of the host cells [40]. There are two ways to accomplish the intercalation of endotoxin in host cell membranes: via a direct interaction with LPS aggregates or via interaction with LBP, sCD14 or plasma proteins that promote host cell activation [53, 54].

AMPs can also intervene in this stage by disturbing the local membrane environment of the receptor modifying its activation state. As a matter of example, cathelicidins can abolish TLR4 induction during dendritic cell maturation by inhibiting the upregulation mechanisms of other stimulatory molecules such as CD40, CD80 and CD86 and impeding cytokine release by altering the cell membrane structure. Altogether, cathelicidins cause a loss of responsiveness to LPS in dendritic cells [55]. Besides, Carratelli et al. [56] studied the TLR4-mediated induction of β-defensin 2 and suggested that TLRs could not only recognize their natural ligands but also induced AMPs.

Signal Downstream Transduction

LPS-TLR4 signaling falls into two different pathways: the myeloid differentiation factor 88-dependent and -independent pathways, both ending with the expression of proinflammatory effectors that start the acute phase of the inflammatory response (online suppl. fig. S2).

Genomic approaches suggest that AMPs also display a direct regulatory control over proinflammatory gene expression. In fact, LL-37 was found to significantly inhibit the expression of specific proinflammatory genes in the presence of LPS, by constraining nuclear translocation of the nuclear factor (NF)-κB p50 and p65 subunits, promoting a drastic decrease in the expression of NF-κB1 (p105/p50) and TNF-α-induced protein 2 genes and subsequently hindering the expression of TNF-α and interleukin-6. However, LL-37 did not significantly inhibit LPS-induced genes that antagonize inflammation, such as TNF-α-induced protein 3, the NF-κB inhibitor NF-κBI, or certain chemokine genes that are classically considered proinflammatory. Those results suggest that human host defense peptide LL-37 plays an important role in balancing the host innate immunity in response to infection [57].

Design of New Anti-LPS Peptides Based on Peptide and Protein Structures

As many AMPs show strong affinity for LPS and are able to neutralize TNF-α production after incubation with LPS, they are good candidates for the development of new drugs to neutralize the endotoxic activity of LPS. Thus, the design of peptide analogues from antimicrobial proteins and peptides can be an important pharmaceutical advance.

There are few cases in which the structure of a protein or peptide bound to LPS is known. In these cases, a rational approach to identify the pharmacophore can be pursued. Boomerang peptides embody a noteworthy example of this case as they were developed based on the crystal structure of protein FhuA bound to LPS [29]. A careful analysis of the contacts between the protein and the ligand reveals two characteristic regions: a cationic one that interacts with the KDO moiety of LPS (making electrostatic contacts with the phosphate groups) and a hydrophobic area (making contacts with the lipid portion of LPS) (online suppl. fig. S1). With all this information, a pharmacophore can be defined to guide the design of new peptides that retain a significant LPS-binding activity. Once template peptides are synthesized and assessed for LPS binding and neutralization, their structure in the presence of LPS can be solved and compared again with the protein to improve the design (fig. 1c).

If the structure of the protein bound to LPS is not available, one can resort to docking simulations. Though not as reliable as nuclear magnetic resonance or X-ray dif-
fraction, in silico studies can efficiently identify the main LPS-binding regions and thus guide the pharmacophore identification process [58, 59].

In this line, Bahl and coworkers [60] efficiently managed to dissect the LPS-binding epitopes of hemoglobin by in silico methods. By using a computational algorithm to assess phospho-group binding propensity in proteins together with docking simulations, they found evolutionary conserved cationic clusters in hemoglobin that are implied in LPS-binding activity. Afterwards, the authors synthesized a battery of peptides that mimic hemoglobin LPS-binding regions and have shown that they efficiently bind to LPS and neutralize endotoxic activity in vitro.

When the polypeptide structure is unknown but the size is small, other approaches can be used to guide the design. In the case of enhancing the activity of short peptides, the systematic substitution of peptide residues by Ala provides an assessment of the discrete role of individual side chain functional groups at specific positions and enables us to correlate it with biological activities, like LPS neutralization or TNF-α production. The alanine scanning technique has been successfully applied to AMPs to identify the key residues involved in the antimicrobial activity, but it has also been applied to assess the LPS-neutralizing properties of these peptides. For example, alanine scanning has been applied to an endotoxin-neutralizing peptide derived from the BPI [61]. In this case, the authors conclude that substitution of Lys residues by Ala abrogates the antimicrobial activity of the peptide. However, the replacement of hydrophobic residues also promotes a decrease in the LPS-neutralizing activity, ratifying that both the electrostatic interaction and hydrophobic contacts are indispensable for effective LPS neutralization. Other less global approaches can also be performed, e.g. the replacement of Lys by nor-Leu, which removes the charge of the residue while the hydrophobic properties remain [61].

Additionally, to improve the biological activity of LPS-neutralizing peptides, some synthetic strategies can be applied such as dendrimeric peptide synthesis or lipid anchorage. Commonly, dendrimers display increased activities compared with monomeric peptides, probably related to the fact that a higher local concentration of the molecule is reached. However, other factors are also important like higher stability to proteases, probably due to high steric hindrances caused by the branched core. Thus, better pharmacokinetic properties can be achieved [62]. The introduction of a lipid acyl chain in peptides can also be a useful strategy to drive the molecules to hydrophobic regions, such as membrane-like structure or LPS micelles [63].

In conclusion, all the hallmarks reviewed here show that AMPs are not only intended to kill pathogens through their antimicrobial activity but display a central role in regulating and balancing the inflammatory response of the innate immune system. The ability to reproduce the immunomodulatory properties of AMPs in synthetic molecules would be determinant for a better understanding of our innate immune system, contributing to the design of promising drugs against infection and inflammation processes.

Acknowledgements

The work was supported by the Ministerio de Educación y Cultura and FEDER funds (grant number BFU2009-09371) and by the Generalitat de Catalunya (2009 SGR 795). D.P. is a recipient of a UAB predoctoral fellowship, and M.T. is a recipient of an Alianza Cuatro Universidades postdoctoral fellowship.

References

Antimicrobial LPS-Neutralizing Peptides


58. Torrent M, Nogues MV, Boix E: Eosinophil cationic protein (ECP) can bind heparin and other glycosaminoglycans through its RNase active site. J Mol Recognit 2011;24:90–100.


