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Peptide interactions with bacterial lipopolysaccharides

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Abstract

Peptide and protein interactions with (lipo)polysaccharides are important in various biological contexts, including lipoprotein deposition at proteoglycan-covered endothelial surfaces in atherosclerosis, lectin functionality, and the interaction of antimicrobial and anti-inflammatory peptides and proteins with (lipo)polysaccharides. The latter of these areas, which is the topic of this review, has attracted considerable interest during the last few years, since antimicrobial peptides may offer novel therapeutic opportunities in an era of growing problems with antibiotics resistance, and persisting problems with both acute and chronic inflammation. In the present overview, physicochemical factors affecting peptide interactions with bacterial (lipo)polysaccharides are discussed, both in solution and at membrane interfaces. In doing so, an attempt is made to illustrate how physicochemical factors affect antimicrobial and anti-endotoxic functionality of such peptides, and how knowledge on this can be translated into therapeutic opportunities, e.g., in sepsis.

Introduction

Infectious diseases cause millions of deaths each year and result in tremendous socioeconomic costs. The disease spectrum is broad and includes acute indications, e.g., sepsis and pneumonia, directly associated to a pathogen, but also various chronic diseases, e.g., chronic obstructive pulmonary disease (COPD), where microbes may cause, or deteriorate, a long-standing inflammatory state. Due to increasing antibiotic resistance, effective therapeutic agents are no longer available for an increasing number of infections. Methicillin- and vancomycin-resistant *Staphylococcus aureus* are well-known examples of this, but also numerous other bacterial strains display extensive antibiotics resistance (1).

Despite progress in the understanding of the underlying causes of sepsis during the last decades, the mortality rate has not decreased. Instead, sepsis remains the leading cause of death in intensive care units (with 30–40% overall mortality, and $\approx 70\%$ for chronically ill and elderly patients), accounting for 40% of total ICU expenditures (2). Despite massive efforts from pharmaceutical industry, there are currently no safe and efficient drugs on the market. The main reason for this is that sepsis is a complex syndrome, in which an exacerbated systemic inflammatory host-defense response to local tissue damage or microbial infection is complicated by a compensatory antagonistic response to the systemic inflammation. This results in the simultaneous occurrence of pro- and anti-inflammatory effects, alternatively predominating over time. Furthermore, the exacerbated systemic inflammation leads to activation of the coagulation and complement systems, which can lead to deposition of microvascular thrombi in various organs. This, in turn, may contribute to the pathogenesis of hemodynamic and metabolic derangement that ultimately can result in septic shock and multiple organ failure.

Given the above, antimicrobial peptides (AMPs), also referred to as host defense peptides, have attracted considerable interest in both infections and resulting inflammation, including sepsis. AMPs are key components of the innate immune system, where they constitute a first line of defense against invading pathogen (3-6). Such peptides provide direct broad-spectrum antimicrobial effects, but also a range of additional functionalities, including anti-inflammatory and immune modulating effects, as well as effects on angiogenesis and chemotaxis, the mechanisms of which are only recently becoming clarified.

Membrane disruption by antimicrobial peptides

Most AMPs carry a net positive charge, but simultaneously contain a relatively high fraction ($\approx 20-50\%$) of hydrophobic residues, frequently appearing in patterns of 1-2 for every 3-4 residues. As a result of this, many AMPs form ordered secondary structure, notably α -helices, which occur in about 30% of the AMPs (7). Among the amino acids, cationic lysine and arginine are abundant, while anionic aspartic and glutamic acid are relatively rare. Another frequently occurring amino acid is tryptophan, which is 50% more common in AMPs than its general occurrence. Thus, by electrostatic attraction, AMPs are able to reach lytic concentration at anionic bacteria membranes, both adsorption and membrane rupture being further facilitated by peptide amphiphilicity.

For Gram-positive bacteria, the bacterial wall consists of a single lipid membrane surrounded by a thick peptidoglycan layer. The wall of Gram-negative bacteria, on the other hand, contains two lipid membranes with a thinner peptidoglycan layer between them, and with the outer membrane

rich in bacterial lipopolysaccharide (**Figure 1**). Several mechanisms have been observed for peptide-induced bacterial membrane rupture, including packing disruption by a detergency-like mechanism, or through formation of membrane pores or defects of barrel-stave or toroidal type (**Figure 2**) (3,8). Since barrel-stave pores can only be formed by oligomers of α -helical peptides, in which hydrophobic and charged residues are precisely spaced to reduce free energy, thus placing quite severe constraints on both peptide and membrane, such pores have only been experimentally demonstrated for a couple of peptides. Toroidal pores, on the other hand, place fewer constraints on peptide properties, and can be formed by a wider range of peptides, as does the “carpet mechanism”, in which peptide adsorption results in localized packing disruptions (4). Irrespective of defect formation mechanism, peptide adsorption initially occurs parallel to the membrane surface (9). On reaching a critical adsorption density, the peptide either inserts into the membrane, or induces a positive curvature strain, resulting in the formation of a toroidal pore. Higher peptide densities at the membrane surface may subsequently cause membrane disruption and micellization (10). Alternatively, the chemical potential gradient across the bilayer may result in peptide translocation across the membrane to the inner membrane leaflet, in turn resulting in transient defects (11). In addition, peptide localization to the polar headgroup region causes lateral expansion of the lipid membrane, allowing relaxation of the alkyl chains and resulting in membrane thinning, further facilitating membrane rupture (12). Depending on membrane composition, also peptide-induced phase transitions and/or lipid segregation may cause membrane rupture (13).

Membrane selectivity is critical to AMP functionality, so that bacteria and other microbes are efficiently killed, while human cells are left intact. The basis for this, in turn, is the different

composition of human and bacterial membranes. Thus, cholesterol is abundant in human cell membranes ($\approx 20\text{-}50$ mol%), but replaced by ergosterol in fungal membranes, and absent in bacteria. There are also considerable differences in phospholipid composition. For example, the outer leaflet of erythrocyte membranes is dominated by zwitterionic phosphatidylcholine and sphingomyelin, rendering the outer part of the membrane essentially uncharged. On the contrary, the outer membrane of bacteria is rich in anionic lipids. In addition, both Gram-positive and Gram-negative bacteria contain peptidoglycan, while Gram-negative bacteria contain also negatively charged LPS (up to 70% of the outer membrane), and Gram-positive bacteria contain lipoteichoic acid. Based on these differences, considerable efforts have been directed to identifying selective AMPs through screening for AMPs from various species, through use of combinatorial libraries and quantitative structure-activity relationships (QSAR), through identification of AMPs generated through proteolytic degradation of endogenous proteins during bacterial infection, or through directed peptide modifications (notably aromatic amino acid substitutions) (3-6,8).

Effects of peptide physicochemical properties on LPS binding

Although LPS covers $>70\%$ of the outer leaflet of Gram-negative bacteria (14), constituting an important barrier for antimicrobial peptides as well as triggering inflammatory effects, the interaction between AMPs and LPS is only recently becoming increasingly understood. Thus, while membrane disruption by AMPs is by now relatively well understood, as is its connection to the antimicrobial action of these compounds, there is considerable uncertainty regarding the mechanisms of peptide/LPS interactions, and on their relationship to anti-endotoxic effects displayed by some AMPs. As discussed below, it has been widely noted in literature that many

structural characteristics are similar for AMPs and anti-endotoxic peptides, e.g., positive charge and amphiphilicity, and many anti-endotoxic peptides are indeed also AMPs. This was illustrated, e.g., by Tack et al. who investigated variants of the antimicrobial peptide SMAP-29, and found a good correlation between minimal affective concentrations (MECs) for bacteria killing, on one hand, and LPS binding affinity, on the other (**Figure 3**) (15). Although many anti-endotoxic peptides are also AMPs, the reverse does not necessarily hold, suggesting that the membrane binding characteristics of AMPs, or underlying characteristics resulting in membrane binding and rupture, constitute a necessary, but not sufficient, requirement for anti-endotoxic effects. Addressing this issue, Singh et al. investigated a series of peptides derived from S1 peptidases, and demonstrated that while phospholipid membrane binding was largely driven by hydrophobic interactions and the (conformation-dependent) amphiphilicity of these peptides, LPS binding correlates primarily with the net charge of the latter (**Figure 4**), although hydrophobic interactions influence LPS binding as well (16). In analogy, Andrä et al. found that LPS carbohydrate chains provide electrostatically driven binding of the antimicrobial peptide NK-2, but also that hydrophobic interactions are necessary for efficient LPS neutralization (17). The same authors found C12 modification of LF11, a lactoferricin-derived peptide, to result in more potent inhibition of LPS-induced cytokine generation (18). Similarly, Rosenfeldt et al. investigated effects of fatty acid conjugation of K/L peptides, and found peptide adsorption to LPS-containing liposomes to increase with increasing length of the hydrophobic conjugation, mirrored by a suppression of inflammatory TNF- α generation (**Figure 5**) (19). The simultaneous importance of hydrophobic and electrostatic effects on LF11 binding to LPS was reported also by Japelj et al. (20). Furthermore, Rosenfeldt et al. investigated the binding and anti-endotoxic effects of LL-37, magainin, and a 15-mer all-L synthetic K/L peptide and its D,L-counterpart

(21). These peptides were found to bind to LPS, and to disintegrate LPS aggregates, the latter suggested to play a central role in the anti-endotoxemic effects of these peptides. Importantly, however, LPS binding in itself was not found to be sufficient for anti-endotoxic effect of these peptides. Later on, Rosenfeld et al. investigated a set of K₆L₉ peptides with regard to peptide charge distribution and conformation (the latter controlled through selected D-amino acid substitutions) (22). While the size of the K and L blocks was found to influence LPS binding only modestly, as were effects of partial D-substitutions in these peptides, the latter caused significant reduction in TNF- α generation. Thus, the anti-endotoxic effect of these peptides seems to be related to the helix induction in the peptides (cf, discussion below). In line with this, peptide truncation has been found to result in a decrease in both helix formation, peptide binding to LPS, and anti-endotoxic effects (23,24), the latter analogous to membrane binding and direct antimicrobial effects of such peptides (8,25).

Peptide binding to LPS, its polysaccharide moieties, and lipid A

In order to understand the observations outlined above, it is instructive to look into the composition of LPS, and peptide interactions of its different moieties. As illustrated in Figure 1a, LPS is a major component of the outer membrane in Gram-negative bacteria. It is anchored to the outer leaflet through its hydrophobic lipid moiety (lipid A), while a short oligosaccharide moiety (R-core) and an outer polymeric carbohydrate (O-antigen) region extend into the surrounding aqueous solution (**Figure 1a**). Through its carboxyl and phosphate groups, LPS is negatively charged, and readily binds cationic and amphiphilic AMPs through a combination of electrostatic and hydrophobic interactions, as outlined above. The question remains, however, what is the relative affinity of AMP binding to the phospholipid membrane and LPS, and to the

polysaccharide and the lipid A regions within LPS. Addressing this issue, Sing et al., investigated binding of thrombin-derived peptides to lipid membranes of different composition, to LPS, and to lipid A (23). It was found that saturation binding of these peptides was comparable for DOPE/DOPG bilayers, LPS, and lipid A, indicating that the peptides are capable of binding to either of these bacterial membrane components. However, from reduced peptide-induced liposome leakage observed in the presence of LPS, it was concluded that these peptides bind preferentially to LPS over anionic phospholipid membranes. Similar results were obtained by the same authors for a series of S1 peptidase-derived peptides (16), and for a series of peptides derived from heparin cofactor II (26). Moreover, since LPS binding to negatively charged lipid membranes is driven by lipid A incorporation, preferential peptide binding to lipid A over the carbohydrate region in LPS was demonstrated by the strongly reduced LPS binding to such membranes after LPS complexation with these peptides (**Figure 6a**) (23). In parallel to a reduced membrane binding, destabilization, and direct antimicrobial effect, truncation may result in reduced binding to LPS (although depending on composition). Truncation may also, however, provide further insight into the driving forces for binding to polysaccharide and lipid A domains of LPS. Demonstrating this for the thrombin-derived peptide GKY25, Singh et al. found the truncated peptide VFR12 to display higher adsorption than GKY25 to lipid A (**Figure 6b**) (23). Since GKY25 is less hydrophobic than VFR12 (mean hydrophobicity on the Kyte-Doolittle scale -0.52 and + 0.28, respectively), but carries a higher net charge (+3 as compared to +2), these results demonstrate that electrostatics dominate peptide binding to the polysaccharide moiety of LPS, whereas hydrophobic interactions dominate peptide binding to lipid A.

Comparing peptide adsorption to LPS either adsorbed to a solid methylated silica surface or

incorporated in preformed supported phospholipid bilayers, Singh et al. were able to draw further information on mechanisms involved in peptide binding to the different LPS moieties. At the former of these surfaces, which are strongly hydrophobic but also negatively charged, LPS binds through lipid A, in analogy to other hydrophobically modified polysaccharides, e.g., peptidoglycans (27). Although partly screened by the densely packed LPS carbohydrate chains, lipid A will not be immersed in a separate phase as in the case of membrane-anchored LPS, and therefore at least partially accessible to peptide binding. When LPS is anchored to a lipid membrane, on the other hand, peptide interaction with lipid A can only occur if the peptide is either incorporated in the lipid bilayer, or disrupts the latter to expose lipid A. However, GK Y25 binds to almost the same extent to DOPE/DOPG bilayers and to such bilayers containing LPS at a comparable LPS density, and also disorder such membranes similarly (23). At first sight, these results may be taken to indicate preferential peptide binding to the DOPE/DOPG bilayer. However, the reduction in peptide-induced liposome leakage in the presence of LPS shows this not to be the case. Furthermore, since LPS adsorbs to preformed DOPE/DOPG bilayers, while peptide/LPS aggregates do not (**Figure 6a**), the combined binding and leakage and adsorption experiments demonstrate that the binding affinity of GK Y25 is highest for lipid A. Together with the finding that GK Y25 causes similar membrane destabilization, irrespectively of the presence of LPS, these results show that GK Y25 binds preferentially to lipid A also when the latter is incorporated into a (bacteria-mimicking) DOPE/DOPG bilayer, and that this occurs partially by membrane disruption and partly by peptide incorporation in the hydrophobic part of the lipid membrane.

In agreement with these results, Neville et al. previously studied lipid A monolayers, and found that the antimicrobial peptide LL-37 is able to incorporate into such monolayers in a

concentration-dependent manner, also at high lateral pressure (**Figure 7**) (28). Furthermore, Yang et al. investigated rALF-Pm3 binding to LPS and lipid A, and found a higher binding affinity to lipid A, based on peptide attachment and detachment rates (29). Analogously, Brandenburg et al. demonstrated preferential binding of lactoferrin to the phosphate groups of lipid A (30), as did Fukuoka et al. for peptides derived from magainin 2 (31). This preference, however, seems to be relatively marginal, as other peptides have been reported to display a reversed binding preference. For example, Junkes et al. found LPS binding of cyclic R/W-rich peptides to decrease on removal of the O-antigen and outer polysaccharides from LPS, demonstrating that these moieties contribute to peptide binding as well (**Figure 8**) (32). As for other anionic polyelectrolytes (33,34), binding of cationic peptides (and similarly so with multivalent cations) to the LPS polysaccharide domain is expected to lead to osmotic deswelling. For example, Schneck et al. investigated adsorbed *P. aeruginosa* LPS with specular X-ray reflectivity, and found Ca^{2+} to induce a collapse of the negatively charged O-chains (35), analogous to results reported for other membrane-bound polysaccharides (27). Despite this deswelling, however, small cationic peptides (30) are able to penetrate deeply into LPS layers, all the way to the lipid A moiety, and to interact with its negatively charged phosphate groups. Through this, disordering of lipid A may occur, as reported, e.g., for Pep19 peptide variants (36), NK-lysine peptide variants (37), and polymyxin B (38). From the above, it is thus clear that peptide binding to LPS can be controlled with a range of peptide properties. As discussed further below, however, peptide binding to LPS seems to be a necessary, but not sufficient, condition for such anti-endotoxic effects, other factors contributing as well.

Peptide-LPS aggregate structure

In the classic view, LPS inflammation triggering occurs through LPS binding to lipopolysaccharide-binding protein (LBP) through its lipid A-recognizing epitope at macrophage surfaces (39). Subsequently, CD14 and Toll-like receptor 4 (TLR4) recognizes the LPS/LBP complex through the MD2 protein (40), resulting in an up-regulation of NF- κ B and proinflammatory cytokine production (**Figure 9**). Besides this mechanism based on individual LPS molecules, the state of LPS aggregation has also been found to be important for inflammation triggering. For example, Mueller et al. investigated lipid A variants and found that compositions favoring aggregate formation displayed enhanced endotoxic effects (41). They also found that monomeric LPS, prepared through dialysis, was largely inactive, while LPS aggregates displayed pronounced endotoxic effects (**Figure 10**). Since findings of this type identifies LPS aggregates as responsible for the endotoxic effect of LPS, efforts have been placed on investigating how peptides affect LPS aggregate structure, and on whether peptide-induced LPS aggregate disruption can be coupled to anti-endotoxic effect of peptides. For example, Rosenfeld et al. demonstrated that LL-37, as well as a 15-mer all-L synthetic K/L peptide and its D,L-counterpart, were all able to disintegrate LPS aggregates (21). Similar findings were reported by Bhunia et al. for fowlicidin-1 peptide fragments (42), and by Mangoni et al. (43,44) and Shrivastava et al. (45) for temporin variants. However, the situation is complex, illustrated, e.g., by Kaconis et al., who demonstrated that presence of anti-endotoxic Pep 19 peptide variants resulted in an *increased* aggregate size, related to a structural change, from cubic to multilamellar (36). The correlation between the lipid A ordering transition and the anti-endotoxic effect of peptides has also been reported by Brandenburg et al. (**Figure 11**) (37), Fukuoka et al. (31), and Chen et al. (46). In this context, it is also interesting to note that pronounced helix formation has been reported for a range of peptides in LPS complexes, fowlicidin-1 fragments (42), melittin

(47), K_mL_n (49), as well as CAP18 (48) and SAMP-29 (15) cathelicidins being some examples. Furthermore, Rosenfeldt et al. investigated a series of K_6L_9 peptides with regard to charge distribution and D-substitutions, and found that while the effect of partial D-substitutions in these peptides on LPS binding and LPS aggregate disintegration was relatively minor, it caused significant reduction in TNF- α generation (22). Since the helical and the disordered peptide variants displayed comparable LPS binding, the anti-endotoxic effect of these peptides seems to be related to the helix induction in the peptides. Similarly, Singh et al. investigated secondary structure transitions in S1-peptidase peptides on interaction with phospholipid membranes and LPS. The extent of helix induction in the presence of DOPE/DOPG liposomes was found to correlate to liposome leakage induction and bacterial killing, demonstrating the importance of conformationally induced amphiphilicity for peptide-induced membrane rupture and direct antimicrobial effect. Similarly, a dramatic helix induction was observed for all peptides investigated displaying anti-endotoxic effects, suggesting a potential functional role of such secondary structure formation (16). Analogous results were reported for a series of thrombin-based peptides (23). Thus, while the anti-endotoxic peptide GKY25 displayed significant binding to both LPS and lipid A, so did two control peptides with either selected D-amino acid substitutions or with maintained composition but scrambled sequence, both displaying strongly attenuated anti-endotoxic effects, but retained or even increased LPS and lipid A binding, compared to the endogenous GKY25 peptide. Hence, the extent of LPS or lipid A binding is clearly not the sole discriminant for the anti-endotoxic effect of these peptides. In contrast, helix formation in peptide/LPS complexes correlates to the anti-endotoxic effect of these peptides, and is potentially linked to this functionality. Dynamic light scattering furthermore demonstrated that helix formation was correlated to LPS micelle disintegration and densification, thus pointing to

the relation between secondary structure formation in the peptide/LPS complexes and overall packing transitions.

In a follow-up study, Singh et al. investigated membrane and LPS interactions for a series of peptides derived from human heparin cofactor II (26). Antimicrobial effects of these peptides were compared to their ability to disorder bacterial lipid membranes, while their capacity to block endotoxic effects of LPS was correlated to the binding of these peptides to LPS and its lipid A moiety, and to charge, secondary structure, and morphology of peptide/LPS complexes (**Figure 12**). While the peptide KYE28 displayed potent antimicrobial and anti-endotoxic effects, its truncated variants KYE21 and NLF20 displayed partially retained and substantially reduced anti-endotoxic effects, respectively, hence locating the anti-endotoxic effects of KYE28 to its C-terminus. While the anti-endotoxic effects decrease in the order KYE28>KYE21>NLF20, these peptides bind to a similar extent to both LPS and lipid A. In contrast, ordered secondary structure formation was found to correlate to the anti-endotoxic effects of these peptides. Thus, CD results demonstrate that the peptides are largely disordered in buffer, with low (<15%) helix content. In the presence of LPS, there is a pronounced helix induction for KYE28, but considerably less so for the less anti-endotoxic KYE21 and NLF20. Parallel experiments with cryoTEM show the peptides to have effects on LPS aggregate structure, which correlate to the anti-inflammatory effects of the peptides. Thus, in the absence of peptide, LPS aggregates are elongated and interconnected due to poor packing efficiency caused by electrostatic repulsion between the negatively charged carbohydrate domains of LPS, and the bulky and partially charged nature of lipid A. On addition of KYE28, LPS aggregates initially disintegrate to short linear fragments, and subsequently reorganize to form dense spherical particles at higher peptide concentration

(Figure 12). This fragmentation and densification is most likely due to a reduction of the electrostatic repulsion between negatively charged LPS chains through cationic peptide binding, and similarly so in the vicinity of lipid A phosphate groups, both facilitating denser packing. In comparison, KYE21 is less efficient in fragmenting LPS aggregates, and NLF20 the least efficient of the peptides investigated. Correlating this to the secondary structure of peptide/LPS aggregates, it seems like helix formation facilitates increased packing density of the composite aggregates, and that this in turn is correlated to the anti-endotoxic effect of these peptides (KYE28>KYE21>NLF20). To further characterize LPS aggregate disruption, 8-anilino-1-naphthalenesulfonic acid (ANS) fluorescence was monitored, reporting on lipid A packing. On initial peptide addition, there is a shift to higher wavelengths in ANS fluorescence spectrum, demonstrating that these peptides to all localize to the lipid A moiety. At higher peptide concentration, ANS fluorescence intensity reports on a dramatic transition in the lipid A moiety of the LPS aggregates, mirroring the overall morphological change observed with cryoTEM, as well as the anti-endotoxic effects of these peptides. Thus, and as discussed further below, peptide-induced LPS aggregate disruption seems to correlate to the anti-endotoxic effects. This capacity, in turn, may be tuned by peptide-LPS/lipid A binding, as well as packing constraints controlled by secondary structure in peptide/LPS complexes.

Relation between peptide-LPS binding and anti-endotoxic effects

As discussed above, there are numerous studies in literature by now, in which it has been found that LPS binding is a necessary, but not sufficient, criterium for anti-endotoxic effects. While studies on peptide-lipid A binding is fewer than those with LPS, and while there are studies suggesting a correlation between peptide anti-inflammatory effects and binding to LPS and lipid

A (50,51), there are numerous studies demonstrating that lipid A binding is insufficient for anti-endotoxic effect, thereby arguing against blocking of LBP binding through the lipid A epitope after peptide binding as the only anti-endotoxic mechanism of AMPs. Apart from the studies discussed in some detail above, one can here note a study by Chen et al., who investigated LPS interactions of granulysin-derived peptides, as well as its consequences for LBP binding (46), and demonstrated a characteristic change in the aggregate structure of LPS into multilamellar stacks in the presence of these peptides. LPS neutralization was found not to be due to scavenging in solution, but rather to proceed after incorporation into target membranes, suggesting a requisite membrane-bound step. Specifically, it was found that presence of the peptide facilitated membrane binding of LPS, causing a reduction in the inflammation-triggering LBP binding (**Figure 13**). Also in the absence of LPS/LBP complex formation, however, one could imagine membrane-localized, rather than bulk-localized, LPS scavenging. Thus, as human cells (e.g., monocytes and macrophages) have membranes rich in zwitterionic lipids, and therefore carry a low negative charge, incorporation of cationic peptides into such membranes may induce a net positive charge, which may facilitate electrostatically driven LPS binding to these membranes. Investigating such events for peptides derived from heparin cofactor II, Singh et al. found that such peptides induced a positive electrostatic potential on these zwitterionic membranes, which in turn facilitated electrostatic binding of LPS to such peptide-loaded membranes (26). As this offers an alternative to CD14 binding and NF- κ B activation, such localized LPS scavenging provides an anti-endotoxic mechanism for such peptides. Indeed, for the heparin cofactor II peptides investigated, some correlation was observed between peptide-induced potential build-up on the membranes, subsequent LPS binding, and anti-endotoxic effects displayed by these peptides. Similarly, Kalle et al. found that GKY25 interacts with monocytes and macrophages *in*

vitro, *ex vivo*, and *in vivo*, reducing TLR4- and TLR2-induced NF- κ B activation, and inhibiting LPS-induced TLR4/MD2 dimerization, whereas the control peptide WFF25 (of identical composition but scrambled sequence) showed much less membrane binding and resulting anti-endotoxic effects (52). Thus, cell-binding of GKY25 seems to mediate inhibition of TLR4-dimerization and subsequent reduction of NF- κ B activity and pro-inflammatory cytokine production in monocytes and macrophages. Although the above studies indicate a role of non-specific membrane-bound events in the anti-endotoxic effects of these peptides, further work is needed to more clearly demonstrate the relative importance of peptide-induced LPS scavenging localized at eukaryotic cell membranes.

As discussed above, numerous studies indicate a correlation between structural changes in LPS/peptide complexes and the anti-endotoxic effects of such peptides. Both fragmentation and densification of LPS aggregates, as well as ordering transitions in lipid A domains, have been found to correlate to anti-endotoxic effect of peptides, and to depend on the secondary structure in the peptide/LPS aggregates. What is not entirely clear, however, is why such fragmentation, packing, and ordering transitions should provide anti-endotoxic properties. As discussed above, such ordering transitions may indeed be related to localized membrane scavenging, by which LPS membrane binding is facilitated, correspondingly decreasing the relative importance of inflammation-triggering LBP/CD14 activation of NF- κ B. One could, however, imagine also other mechanisms. Specifically, LPS fragmentation, as well as reduction of the negative charge of LPS, may both contribute to facilitating phagocytosis, in analogy to size and charge dependence of phagocytosis of other types of nanoparticulate systems. Through this, an alternative pathway to LPS-LBP/CD14 binding/activation is provided, resulting in attenuation, or

even blocking, of the inflammatory activation occurring in the absence of peptide. Indeed, such phagocytosis-related scavenging as anti-inflammatory response has been previously observed, although for inflammation caused by amyloid A β rather than by LPS. Thus, Richman et al. investigated protein-microspheres with an A β -recognizing peptide, and were able to correlate anti-inflammatory effects of the latter with triggering of A β phagocytosis, thereby avoiding the alternative triggering pathway (53). Along the same line, the cationic peptide LL-37 has been demonstrated to transfer complexed negatively charged molecules into cells, which has been used, e.g., to transfect eukaryotic cells (54). The further consequences of this for inflammatory cytokine generation, however, remains unclear, as inhibition of TLR responses and of the generation of inflammatory cytokines has been observed for myeloid cells (55), whereas inflammatory activation was observed following peptide-induced LPS cell internalization in the case of lung epithelial cells (56). Thus, additional work with peptide/LPS is needed. Here, much can potentially be learned from comparisons with the area of particle phagocytosis, including effects of particle size, shape, and charge (57,58).

A vast majority of studies on anti-inflammatory peptides has focused on their effects on LPS from Gram-negative bacteria. While Gram-negative bacteria are certainly dominating in many indications, inflammation may be caused or deteriorated also by other pathogens. For example, inflammatory effects in Gram-positive bacteria are caused by other components, specifically by lipoteichoic acid (LTA), while zymosan is central in this context for fungi. LPS and LTA are structurally related, as they both contain hydrophobic hydrocarbon chains, forming a hydrophobic anchor moiety, as well as a saccharide-containing anionic polyelectrolyte moiety, again rich in phosphate charges. Zymosan, on the other hand, contains no hydrophobic moieties,

and only carries a low negative charge to its polysaccharide chains. From a biophysical perspective, one could thus expect peptide interactions to be comparable for LPS and LTA, whereas zymosan should differ from the other two in this respect. It is therefore interesting to note, that a couple of studies have compared peptide antagonistic effects against inflammation triggered by LPS, LTA, and zymosan. For example, Kasetty et al. investigated a series of truncations of the thrombin-derived peptide GKLY25, e.g., regarding inflammation triggered by LPS, LTA, and zymosan. In all cases, truncations from either the C- or N-terminal, or both, resulted in deterioration of the anti-inflammatory effects of this peptide. Strikingly, little difference was observed between LPS, LTA, and zymosan (24). Similarly, Kasetty et al. investigated anti-inflammatory effects of a series of peptides derived from S1-proteases, and found similar effects for inflammation triggered by LPS and zymosan (59). Given the structural differences between LPS and LTA, on one hand, and zymosan on the other, this illustrates the complexity of the anti-inflammatory effects of these peptides. While so far not investigated in literature, the absence of hydrophobic domains in zymosan, as well as its lower charge density compared to LPS and LTA, most likely results in different peptide binding to LPS, LTA, and zymosan, but also different effects on LPS/LTA/zymosan self-assembly, phagocytosis, and membrane-localized scavenging. Conversely, comparative biophysical studies of the type discussed above may help discriminating between possible action mechanisms for different peptides.

While there are an increasing number of biophysical studies on peptide-LPS interactions, and its consequences for cytokine generation, relatively few investigations span the entire range from detailed biophysical investigations to broader biological evaluation and effect studies, the latter

of course critical in the evaluation and development of potential peptide therapeutics against inflammatory indications. It could here be noted, however, that the thrombin-derived peptide GKY25, as well as truncations and other versions of this peptide, have been evaluated also from a broader therapeutic perspective (60,61). Thus, apart from reducing a range of pro-inflammatory cytokines, including IL-6, TNF- α , INF- γ , and MCP-1, and transiently increasing anti-inflammatory IL-10, GKY25 resulted in dramatically increased survival in mice after inflammation caused by either LPS or Gram-negative bacteria. While mice challenged with inflammatory shock displayed massive platelet infiltration and fibrin deposition into lungs, those surviving after peptide treatment displayed baseline normality for the healthy animal (**Figure 14**), as well as modulated coagulation. Correspondingly, truncation of the peptide diminished anti-inflammatory cytokine levels and attenuated survival effects, as did scrambling of the sequence to form WFF25, unable to form helices and small aggregates together with LPS. Analogous effects were subsequently found also for the heparin cofactor II-derived peptide KYE28 and its truncations KYE21 and NLF20 (62). Although lacking detailed biophysical back-up data to support such broad comparisons, such multimodality function of selected AMPs, including attenuation of pro-inflammatory cytokines, promotion of anti-inflammatory cytokine levels, and induction of chemokine expression, have been observed also for other peptides, including LL-37, β -defensins, and IDR (63-65).

As clearly demonstrated by a range of clinical trials on sepsis, suppression of specific inflammatory cytokines are unlikely to be the entire effect needed of potent anti-inflammatory peptides for them to be successfully developed to therapeutics. Thus, a number of approaches for targeting specific pro-inflammatory responses have been attempted, including antibodies against

TNF- α , interleukin-1 receptor antagonists, interleukin-6 antagonists, and anti-endotoxin antibodies, all failing to show clinical efficiency, clearly showing sepsis not to depend on a single factor, but rather being multifunctional (66,67). There is thus a need for new therapeutic approaches, multifunctional peptides offering interesting opportunities in this context. Apart from providing direct antimicrobial activity from rapid and broad-spectrum response towards both Gram-negative and Gram-positive bacteria, as well as fungi, such peptides may also activate the complement system to combat and clear bacteria, as demonstrated, e.g., for peptides derived from the tissue factor protein inhibitor (TFPI) (68). In addition, and as exemplified above, some antimicrobial peptides mediate diverse immunomodulatory roles, reducing a wider range of anti-inflammatory cytokines, and increasing anti-inflammatory cytokines (61). They may also interfere with coagulation by modulating contact activation and tissue factor-mediated clotting. Thus, in response to LPS challenge, the coagulation cascade is activated, leading to excessive activation of the coagulation system, followed by consumption of coagulation factors in the blood, resulting in prolonged clotting times. In addition, coagulation activation, fibrinolysis inhibition, and consumption of coagulation inhibitors lead to a procoagulant state and fibrin deposition in the microvasculature (61). As a consequence, microvascular thrombosis contributes to promotion of organ dysfunction. In parallel, excessive contact activation leads to the release of the pro-inflammatory peptide bradykinin and a subsequent induction of inflammatory reactions, which contribute to serious complications such as hypotension and vascular leakage. As demonstrated, e.g., for the thrombin-derived peptide GK25, such direct and indirect LPS activation of the coagulation system can be reduced by some peptides (61). Similar effects were observed for EDC34, a peptide derived from TFPI-2, which was found to abolish fibrin-deposition irrespective of bacterial load and antibiotic usage in the animal models,

thus linking the anti-coagulative effects *in vitro*, including blocking of bacteria-induced kallikrein activation and bradykinin release, to those observed *in vivo* (69). In essence, therefore, multi-modality is ideally provided by such peptides, addressing both the infection as such and its detrimental consequences for inflammation and coagulation.

Summary and outlook

As summarized, e.g., by Pasupuleti et al. (70), there are currently no AMPs on the market. There are, however, a number of peptides currently undergoing various stages of clinical trials. Key factors in the development of these towards successful antimicrobial therapeutics are factors relating to cost-of-goods and peptide stability, translating into needs for reducing the number amino acids in the peptide to increase the chemical and proteolytic stability of the latter, both in formulation and after administration, yet maintaining efficacy and limited toxicity. Drug delivery systems may facilitate this, and may also provide additional advantages, e.g., relating to drug release rate, triggerability, and improved efficacy/safety ratio (71). Given current regulatory frame works, AMPs furthermore need to outperform existing antibiotics on the market to reach introduction, in turn driving development in the direction of activity against multi-resistant bacteria and additional host defense functions. It is in this latter context that AMPs displaying also anti-inflammatory effects offer interesting opportunities. It is clear, however, that anti-endotoxic effects of some AMPs and related peptides are quite complex processes, with multiple molecular mechanisms, including direct prevention of LPS binding to LPB through its lipid A epitope, membrane-localized LPS scavenging, and peptide-triggered phagocytosis. Despite an emerging understanding of these processes, considerable uncertainty remains as to the relative importance of these effects, and how this may be tailored in peptide design for optimizing

biological effects. The comparable effects observed for the structurally quite diverse LPS, LTA, and zymosan indicate that hitherto undisclosed processes may also be involved. From a biological and therapeutic perspective, a holistic approach is most likely needed, covering not only effects on cytokines, but also less understood effects on coagulation and complement activation. As both various scavenging processes, phagocytosis, cytokine generation, complement activation, coagulation activation, and consumption of various coagulation factors, are dynamic and transient processes, depending also on the pharmacokinetics and biodistribution of the peptide after administration, more detailed bioanalysis of these processes and of peptide localization and consumption is therefore needed for optimal control of these transient effects. Additional work is furthermore needed to clarify possibilities to selectively control the kinetics of these events by various drug delivery systems.

Acknowledgement

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Figure captions

Figure 1. Schematic illustration of the cell walls of Gram-negative (a) and Gram-positive (b) bacteria. In (a), a schematic illustration of the structure of LPS is shown as well.

Figure 2. Schematic illustration of different modes of AMP interaction with lipid membranes.

Figure 3. Correlation between LPS binding affinity and antimicrobial potency (expressed as MEC, Minimum Effective Concentration) of CAP18 peptides against different *E. coli* strains (Redrawn from (15).)

Figure 4. (a) Correlation between DOPE/DOPG (75/25 mol/mol) liposome leakage induction and peptide adsorption density (both at a peptide concentration of 1 μ M) at the corresponding supported bilayers in 10 mM Tris, pH 7.4. Also indicated is the fractional helix content of the peptides in the presence of DOPE/DOPG liposomes (X_{helix}). (b) Correlation between peptide net charge and adsorption density at preadsorbed *E. coli* LPS. (Redrawn from (16).)

Figure 5. (a) Effect of acyl length of fatty acid-conjugated K7L5 peptides on (a) LPS binding, and (b) TNF- α generation. (Redrawn from (19).)

Figure 6. (a) Comparison of adsorption of LPS and preformed LPS-peptide aggregates (0.02 mg/ml and 1 μ M WFF25) to DOPE/DOPG bilayer in 10 mM Tris, pH 7.4. For the pre-formed aggregates, LPS and peptide were mixed together 1hour before addition. (b) Comparison of the

adsorption of GKY25 and its truncated version VFR12 at LPS (left) and lipid A (right). (Redrawn from (23).)

Figure 7. Insertion of LL-37 into lipid A monolayers as a function of surface pressure and peptide concentration. (Redrawn from (28).)

Figure 8. Binding of c-WFW peptides to POPC liposomes containing lipid A (i.e., no polysaccharide chain), LPS with short polysaccharide chain (rLPS), and LPS with long polysaccharide chain (sLPS). (Redrawn from (32).)

Figure 9. Schematic illustration of LPS inflammation triggering, initiated by LPS binding to lipopolysaccharide-binding protein (LBP) through its lipid A-recognizing epitope at macrophage surfaces. Subsequently, CD14 and Toll-like receptor 4 (TLR4) recognizes the LPS/LBP complex through the MD2 protein, resulting in an up-regulation of NF- κ B and proinflammatory cytokine production.

Figure 10. Endotoxic activity of lipid A and LPS aggregates (dark) and monomers (white) at the same concentration (10^{-9} M) in *Limulus* amoebocyte lysate assay. (Redrawn from (41).)

Figure 11. Effect of NK-2-derived peptides on the structure of LPS. The logarithm of the SAXS scattering intensity ($\log I$) against the scattering vector s ($=1/d$, where d is the reflection spacing). As demonstrated, these peptides induce an ordering transition to lamellar structure. (Redrawn from (37).)

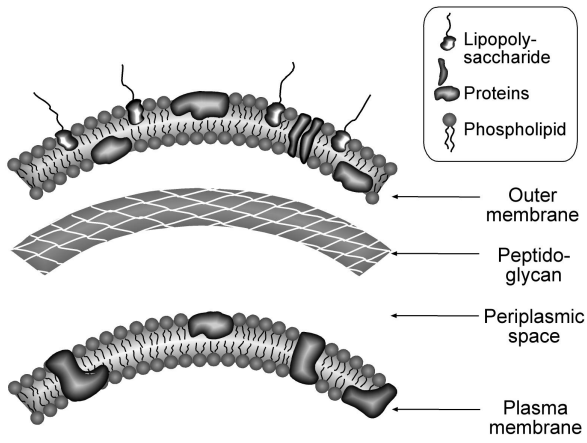
Figure 12. (a) Effects of the indicated peptides on NO production by macrophages. RAW264.7 mouse macrophages were incubated with LPS from *E. coli* in presence of peptides at the indicated concentration. Shown also are results on peptide binding to preadsorbed *E. coli* LPS (b), CD spectra for the indicated peptides in the presence of *E. coli* LPS (c), and representative cryoTEM images of LPS (0.2 mg/ml in 10 mM Tris, pH 7.4) in the absence and presence of KYE28 and NLF20 at a peptide concentration of 50 μ M. (Redrawn from (26).)

Figure 13. Effect of the G12.21 peptide on LPS binding to phosphatidylserine liposomes, as well as its effect on LBP binding. As can be seen, presence of the peptide enhances LPS binding to the lipid membrane, and causes a corresponding decrease in the competing LPS/LBP binding. (Redrawn from (46).)

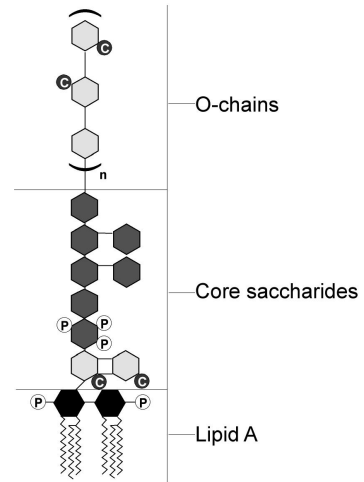
Figure 14. The thrombin-derived peptide GKY25 shows potent and multi-mode anti-endotoxic effects, including suppression of pro-inflammatory cytokines (a, right) and reduction of fibrin deposition and platelet infiltration into lungs (b), resulting in drastically improved survival in mice challenged by LPS chock (a, left). (Redrawn from (60).)

Figure 1.

(a) Gram-negative



LPS



(b) Gram-positive

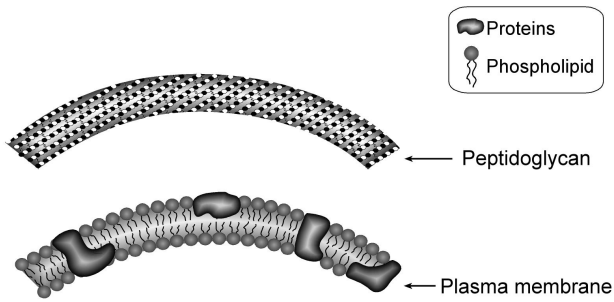


Figure 2.

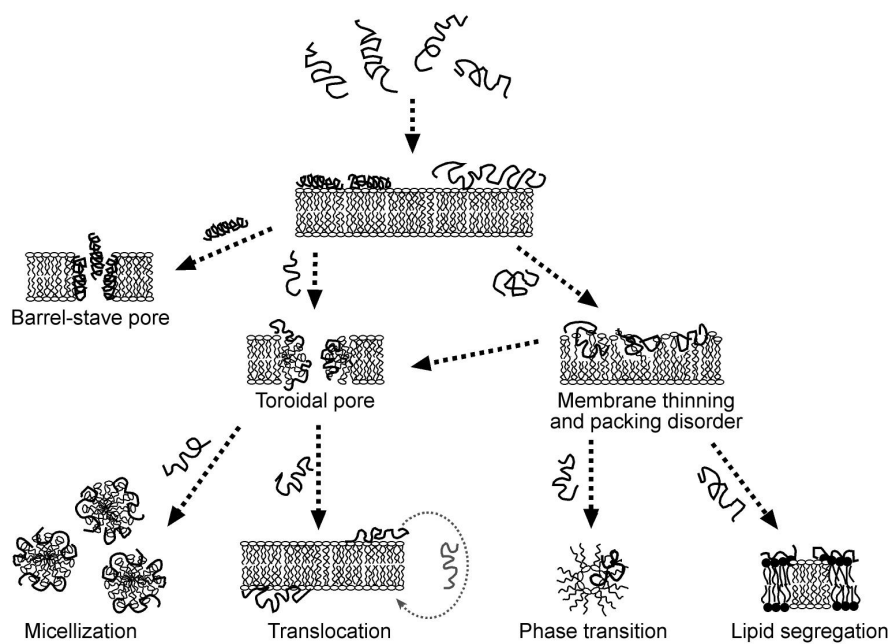


Figure 3.

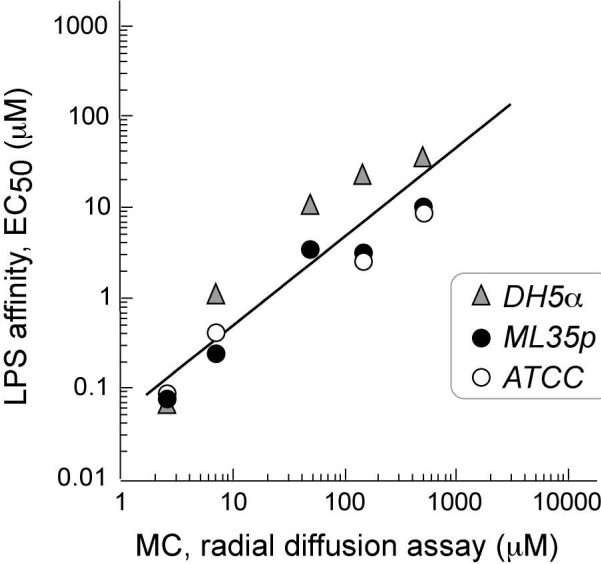
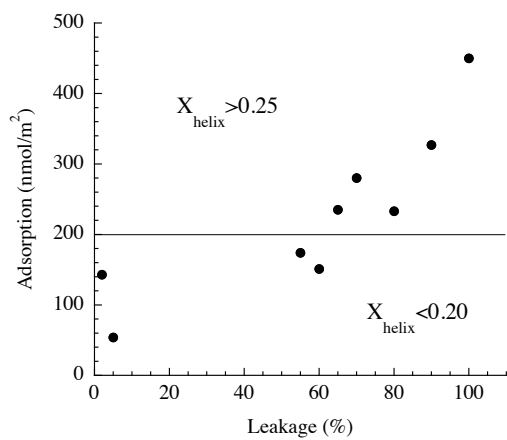


Figure 4.

(a)



(b)

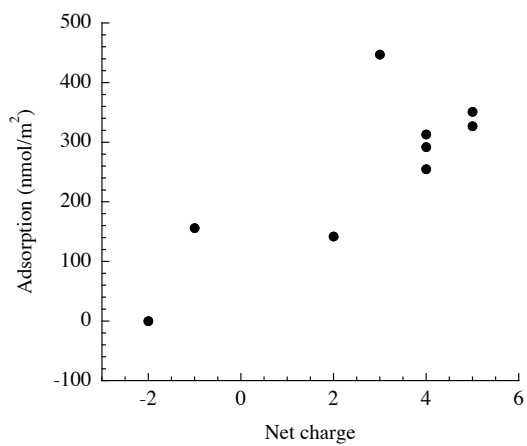
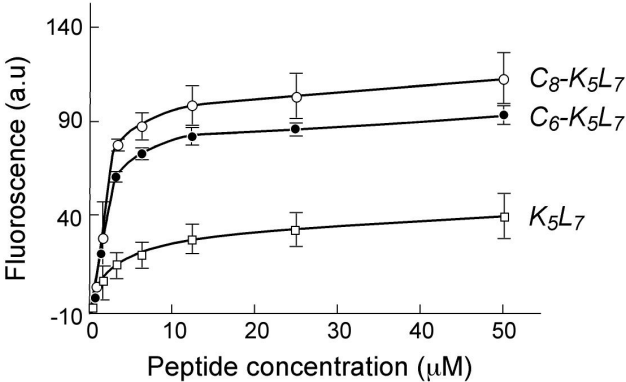


Figure 5.

(a)



(b)

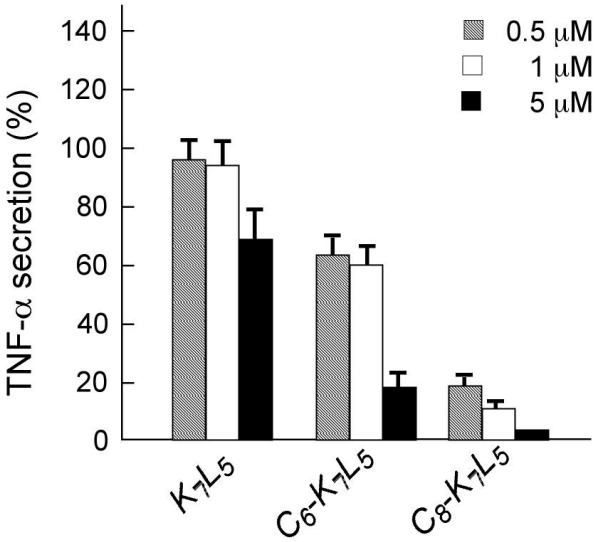
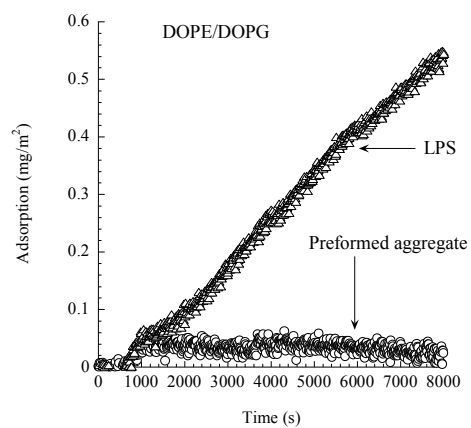


Figure 6.

(a)



(b)

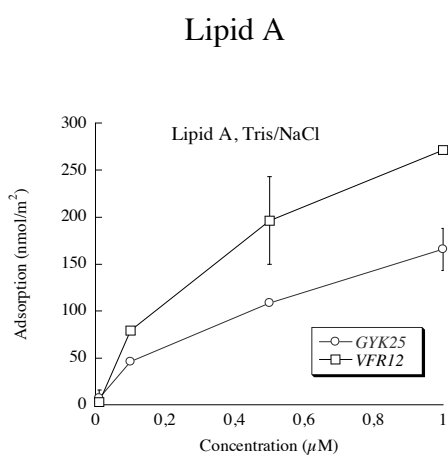
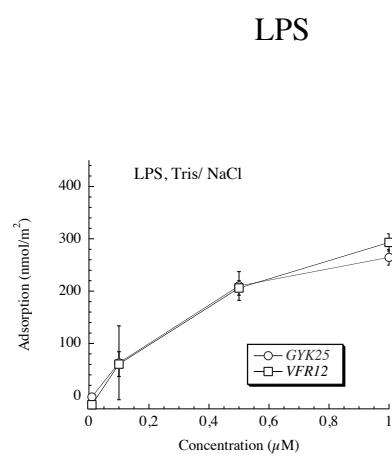


Figure 7.

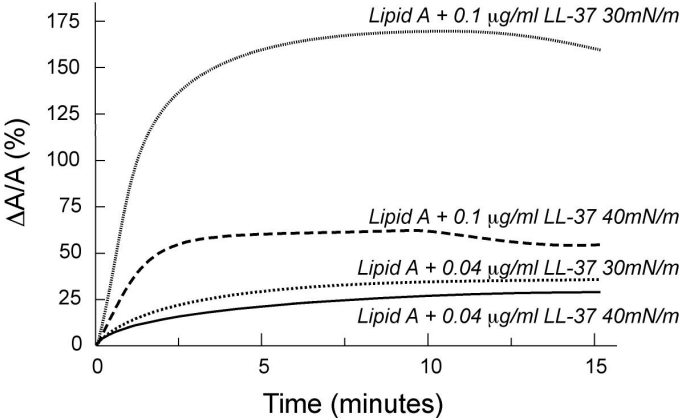


Figure 8.

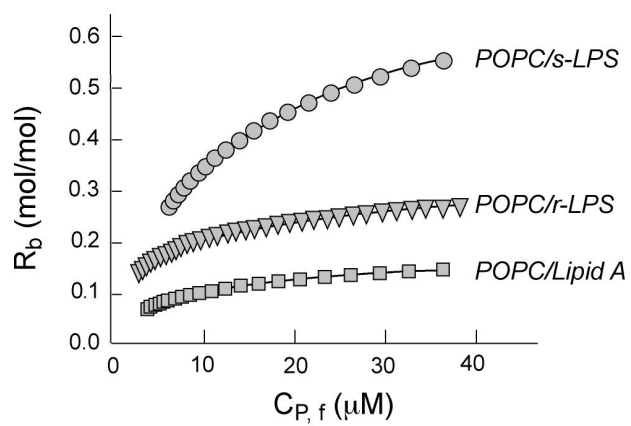


Figure 9.

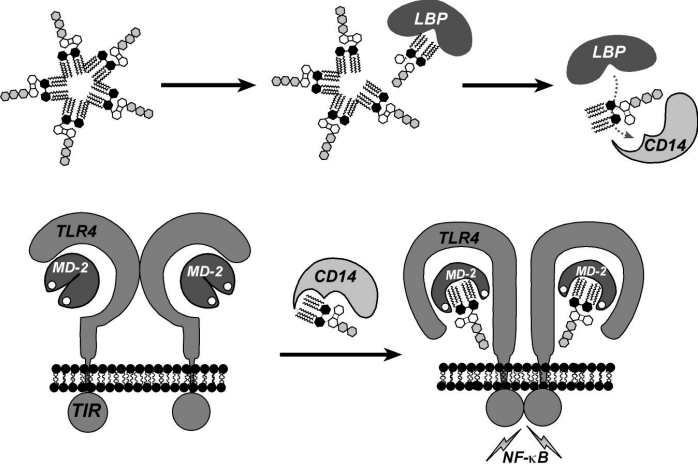


Figure 10.

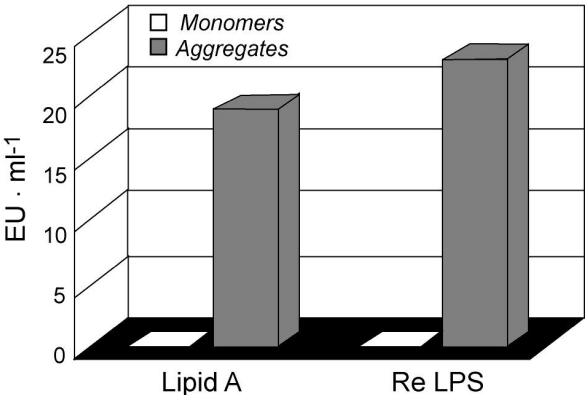


Figure 11.

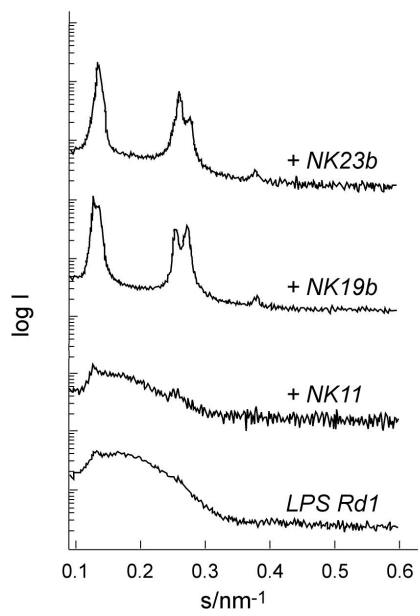
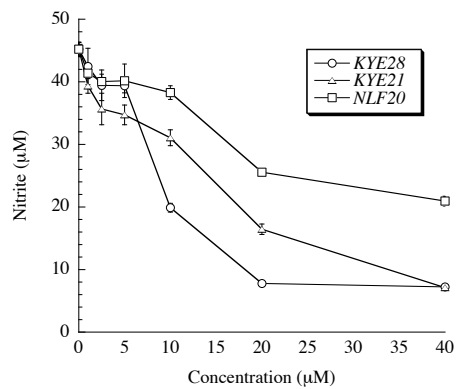
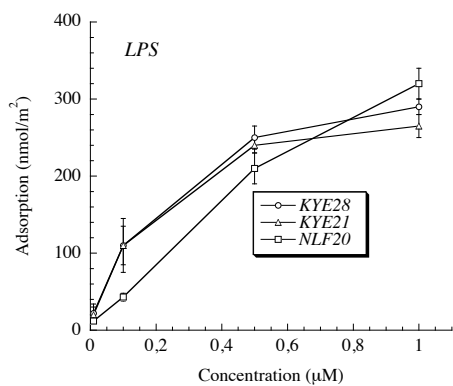


Figure 12.

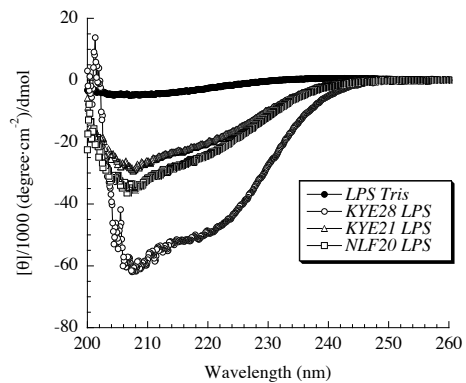
(a)



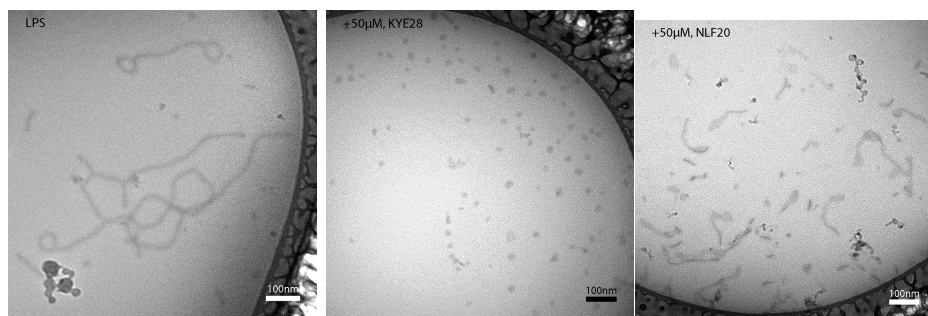
(b)



(c)



(d)



LPS

+KYE28

+NLF20

Figure 13.

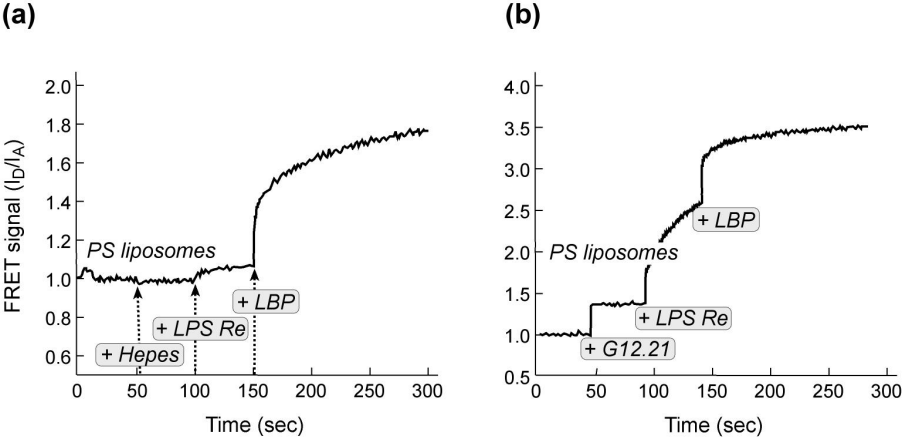
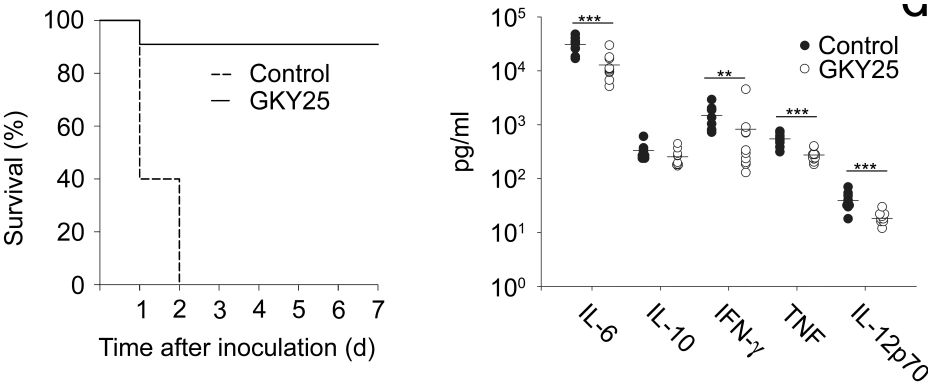


Figure 14.

(a)



(b)

