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(Lipo)polysaccharide interactions of antimicrobial peptides

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ABSTRACT

Due to rapidly increasing resistance development against conventional antibiotics, as well as problems associated with diseases either triggered or deteriorated by infection, antimicrobial and anti-inflammatory peptides have attracted considerable interest during the last few years. While there is an emerging understanding of the direct antimicrobial function of such peptides through bacterial membrane destabilization, the mechanisms of their anti-inflammatory function are less clear. We here summarize some recent results obtained from our own research on anti-inflammatory peptides, with focus on peptide-(lipo)polysaccharide interactions.

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1. Introduction

Infections remain a leading cause of mortality, both in indications directly associated with a pathogen (e.g., pneumonia or sepsis), and in diseases where microbes may cause or deteriorate inflammation (e.g., chronic obstructive pulmonary disease) [1]. Particularly relevant to the present discussion, sepsis remains the leading cause of death in intensive care units, with 30–40% overall mortality (≈70% for elderly and chronically ill patients) [2], and no efficient and safe drugs on the market. Considering this, as well as their attenuated susceptibility to resistance development, antimicrobial peptides (AMPs; also referred to as host defense peptides) have attracted considerable interest as potential therapeutics against both infections and resulting inflammation [3–5].

2. Bacterial membranes as AMP targets

AMPs generally have distinct amphiphilic characteristics with a sizeable fraction of hydrophobic residues. They are also frequently rich in arginine and lysine residues, and thus carry a net positive charge. Electrostatic interactions therefore facilitate peptide binding to anionic bacteria membranes. In addition, presence of hydrophobic residues is important for the ability of many AMPs to disrupt membrane bilayers, particularly at high ionic strength, in the presence of serum, and for low-charged pathogens [6]. Key for AMP functionality is membrane selectivity, so that bacteria and other microbes are efficiently killed, while human cells are left intact. The basis for such selectivity is the different composition of human and bacterial membranes. For example, human cell membranes are rich in cholesterol (up to ≈50 mol%), whereas fungal membranes contain ergosterol, and bacteria membrane no sterol at all [7]. Also the phospholipid composition differs between these membranes. For example, the outer leaflet of erythrocyte membranes is dominated by zwitterionic lipids, such as phosphatidylcholine and sphingomyelin, thus being essentially uncharged [8]. In contrast, bacterial membranes are rich in anionic lipids. In addition, the outer membrane in Gram-negative bacteria is rich (>70% [9]) in highly anionic lipopolysaccharide (LPS) (Fig. 1), while Gram-positive bacteria contain lipoteichoic acid (LTA). Together, these differences in membrane composition provide opportunities for reaching AMP selectivity.

Several mechanisms have been proposed for AMP-induced membrane disruption, including pore formation and membrane disruption by detergent-like effects [6]. For pore formation, the peptide initially adsorbs at the membrane surface, where it subsequently inserts and induces a positive curvature strain. Complete membrane disruption and micellization may ultimately result at sufficiently high peptide densities. Membrane destabilization may also occur through chemical potential gradients, which drive translocation of peptides initially adsorbed to the outer membrane leaflet. In addition, peptide binding to the polar headgroup region of membranes causes lateral expansion, which allows alkyl chain relaxation and results in membrane thinning. Also peptide-induced phase transitions and lipid segregation may cause membrane rupture.

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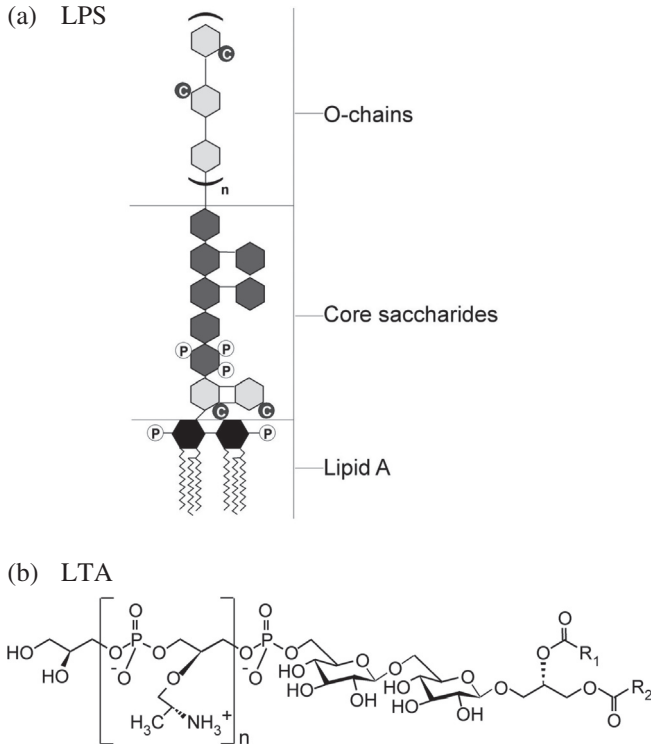


Fig. 1. Structure of LPS and LTA in Gram-negative and Gram-positive bacteria, respectively.

3. AMP binding to LPS

As discussed in greater detail previously [10], there are an increasing number of studies addressing peptide interactions with bacterial lipopolysaccharides [10]. For example, investigating the role of electrostatics for AMP–LPS interactions, Singh et al. studied a series of peptides derived from S1 peptidases, and demonstrated that while phospholipid membrane binding was largely driven by conformation-dependent amphiphilicity of these peptides, LPS binding depends on peptide net charge (Fig. 2a), as well as hydrophobicity [11]. In a subsequent study, the same authors found pronounced effects of peptide linear amphiphilicity on LPS and LTA binding of GKY25 peptide variants. Specifically, both the native thrombin-derived peptide GKY25 (GKYGFYTHVFRLKKWIQK-VIDQFGE) and its D-amino acid variant GKY25d adsorbed much less at both LPS and LTA than did WFF25 (WFFFYY-LIIGGGVVTHQQRKKKKDE), with identical composition but with amino acids sorted according to hydrophobicity, thus displaying pronounced linear amphiphilicity, i.e., a gradient in hydrophobicity when going from one end of the peptide to the other (Fig. 2b) [12]. Similarly, Andrä et al. reported on electrostatically driven LPS binding of NK-2, but also that hydrophobic interactions are important for LPS neutralization in this system [13]. In line with the latter, Rosenfeldt et al. found adsorption to LPS-containing liposomes to increase with increasing length of the hydrophobic conjugation of K/L peptides [14].

Investigating the relative affinity of AMP binding, Sing et al. studied binding of thrombin-derived peptides to phospholipid membranes of different composition, to LPS, and to its lipid A

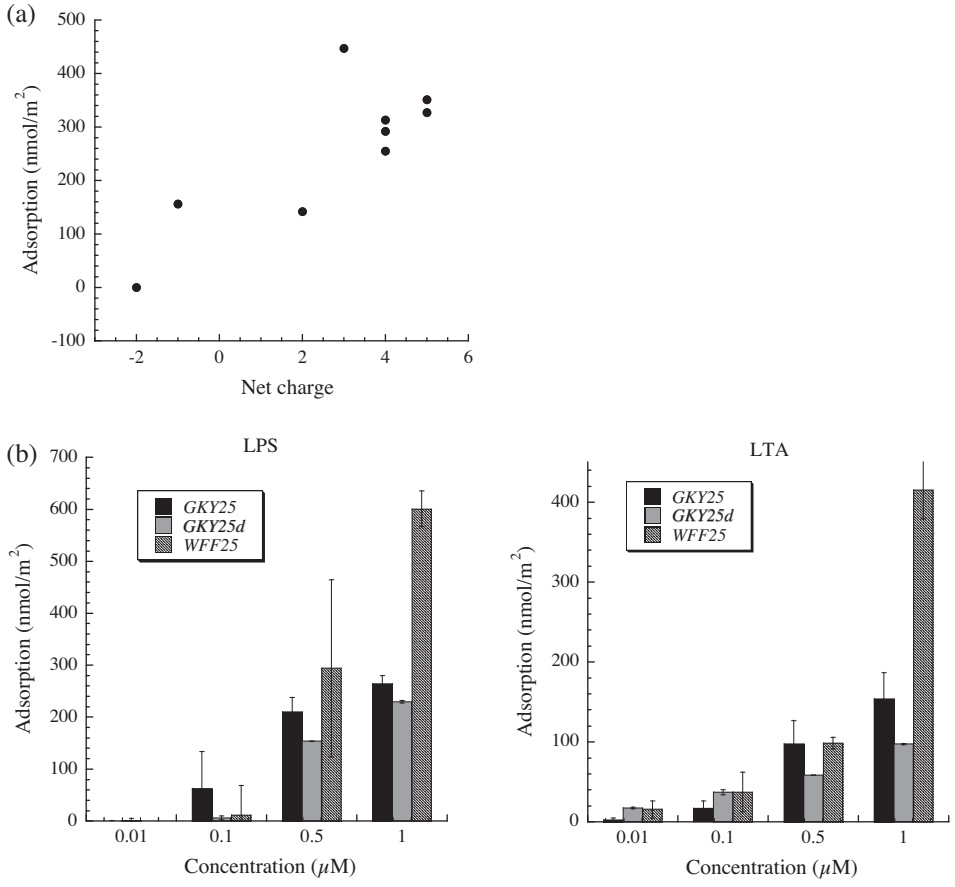


Fig. 2. (a) Correlation between adsorption to *E. coli* LPS and peptide charge density (11). (b) Peptide adsorption to LPS (left) and LTA (right) from 10 mM Tris, pH 7.4, with additional 150 mM NaCl [12].

moiety [15]. While saturation binding of these peptides was found to be comparable for anionic DOPE/DOPG bilayers, LPS, and lipid A, reduced liposome leakage in the presence of LPS showed that these peptides bind preferentially to LPS over anionic phospholipid membranes (Fig. 3a). Similar results were obtained for S1 peptidase-derived peptides [11] and peptides derived from heparin cofactor II [16]. Furthermore, since binding of anionic LPS to anionic lipid membranes is driven exclusively by lipid A, preferential peptide binding to lipid A over the carbohydrate region in LPS was demonstrated by the strongly reduced LPS binding to such membranes after peptide complexation (Fig. 3b). Using selective truncation, electrostatics was demonstrated to dominate peptide binding to the polysaccharide moiety of LPS, whereas hydrophobic interactions dominate peptide binding to lipid A [15].

Similarly, Yang et al. found a higher binding affinity for lipid A of rALF-Pm3 [17], while Brandenburg et al. demonstrated preferential lactoferrin binding to the phosphate groups of lipid A [18]. This preference seems to be system dependent, however, as reversed binding preference has also been reported. For example, Junkes et al. reported that binding of cyclic R/W-rich peptides decreases on removal of the O-antigen and outer polysaccharides from LPS, demonstrating that these moieties contribute to peptide binding [19]. It should here be noted that cationic peptide binding to the anionic LPS polysaccharide domain is expected to lead to osmotic deswelling [20,21]. Despite this, small cationic peptides are able to penetrate LPS layers to the lipid A moiety. Through this, lipid A disordering may occur, as reported, e.g., for Pep19 variants [22] and polymyxin B [23].

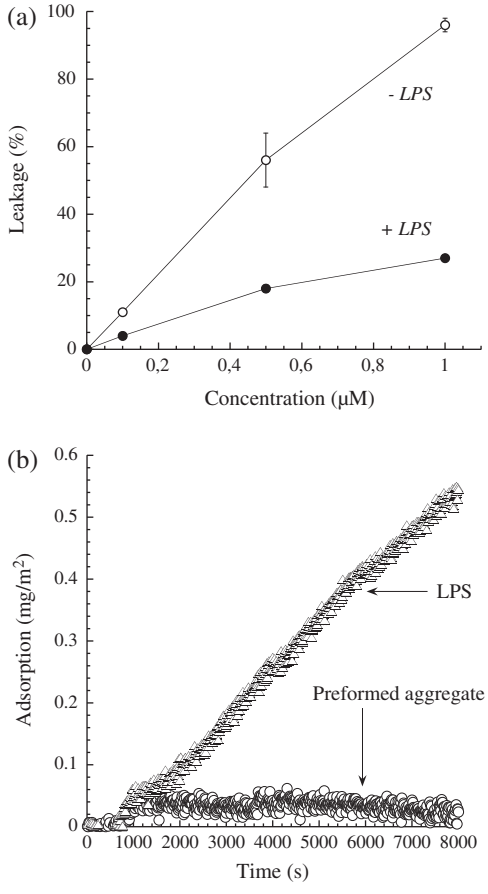


Fig. 3. (a) GKY25-induced leakage of DOPE/DOPG (75/25 mol/mol) liposomes in the absence (open) and presence (filled) of 0.02 mg/ml *E. coli* LPS. Measurements were performed in 10 mM Tris, pH 7.4. (b) Comparison of adsorption of LPS and preformed LPS–peptide aggregates (0.02 mg/ml LPS and 1 μM peptide) to DOPE/DOPG bilayer in 10 mM Tris, pH 7.4. For the pre-formed aggregates, LPS and peptide were mixed together 1 h before addition [15].

3.1. Peptide effects on LPS aggregate structure

In the major pathway, triggering of the inflammation cascade occurs through lipid A-mediated binding to lipopolysaccharide-binding protein (LBP) at macrophage surfaces [24]. Recognition of the LPS/LBP complex by CD14 and Toll-like receptor 4 (TLR4) [25] subsequently results in an up-regulation of NF-κB and in the generation of proinflammatory cytokines. In addition, however, LPS aggregation has been found to be important for inflammation triggering [26]. Hence, peptide-induced LPS aggregate disruption can potentially be coupled to anti-inflammatory effects. Along this line, Rosenfeld et al. demonstrated LPS aggregate disintegration by anti-inflammatory peptide LL-37, as well as two synthetic 15-mer K/L peptides [27]. Similar findings were obtained by Bhunia et al. for fowlicidin-1 fragments [28] and by Mangoni et al. for temporin variants [29]. Here, it should also be noted that helix formation (reporting on structural transition) has been reported for a range peptide–LPS complexes, including fowlicidin-1 fragments [28], melittin [30], and S1-peptidase peptides [11]. Addressing such peptide-induced LPS aggregate disruption, Singh et al. investigated membrane and LPS interactions for a series of peptides derived from human heparin cofactor II [16]. While the peptide KYE28 (KYEITTIHNLFRKLT HRLFRRNFGYTLR) displayed potent anti-inflammatory effects, its truncated variants KYE21 (KYEITTIHNLFRKLTHRLFRR) and NLF20 (NLFRKLTHRLFRRNFGYTLR) displayed partially retained and substantially reduced anti-endotoxic effects, respectively. While the anti-inflammatory effect decreases in the order KYE28 > KYE21 > NLF20, these peptides bind to a similar extent to both LPS and lipid A (Fig. 4). In contrast, cryoTEM showed peptide effects on LPS aggregate structure to correlate with their anti-inflammatory potency. Thus, KYE28 causes LPS aggregates to disintegrate, initially to short linear fragments, and subsequently to dense spherical particles at higher peptide concentration. KYE21 is less efficient in fragmenting LPS aggregates, and NLF20 the least efficient of the peptides investigated. Analogously, 8-anilino-1-naphthalenesulfonic acid (ANS) fluorescence showed that these peptides bind to the lipid A moiety. At higher peptide concentration, a transition in the lipid A moiety of the LPS aggregates is observed, mirroring the overall morphological change observed with cryoTEM, as well as the anti-inflammatory effects of these peptides. The ability of AMPs to disrupt LPS aggregates thus seems to be of importance for the anti-inflammatory effect of these peptides.

3.2. Mechanisms of peptide anti-inflammatory effects

As discussed in greater detail previously, numerous studies have reported that LPS binding to be a necessary, but not sufficient, criterium for anti-inflammatory effects of AMPs [10]. Similarly, several studies have demonstrated that lipid A binding is insufficient for AMPs to be anti-inflammatory, thus arguing against blocking of lipid A-mediated LBP binding as the only anti-inflammatory mechanism of AMPs. Interesting in this context is a study by Chen et al. who found the presence of granulysin-derived peptides to facilitate membrane binding of LPS, thereby scavenging the alternative (inflammation–triggering) LBP binding [31]. Since membranes of human cells are rich in zwitterionic lipids and therefore essentially uncharged, incorporation of cationic peptides may induce a net positive charge, which may facilitate electrostatically driven binding of anionic LPS to these membranes. As this offers an alternative to TLR4 binding and NF-κB activation, such localized LPS scavenging provides a potential anti-inflammatory mechanism for AMPs. Indeed, for the heparin cofactor II peptides discussed above, some correlation was observed between

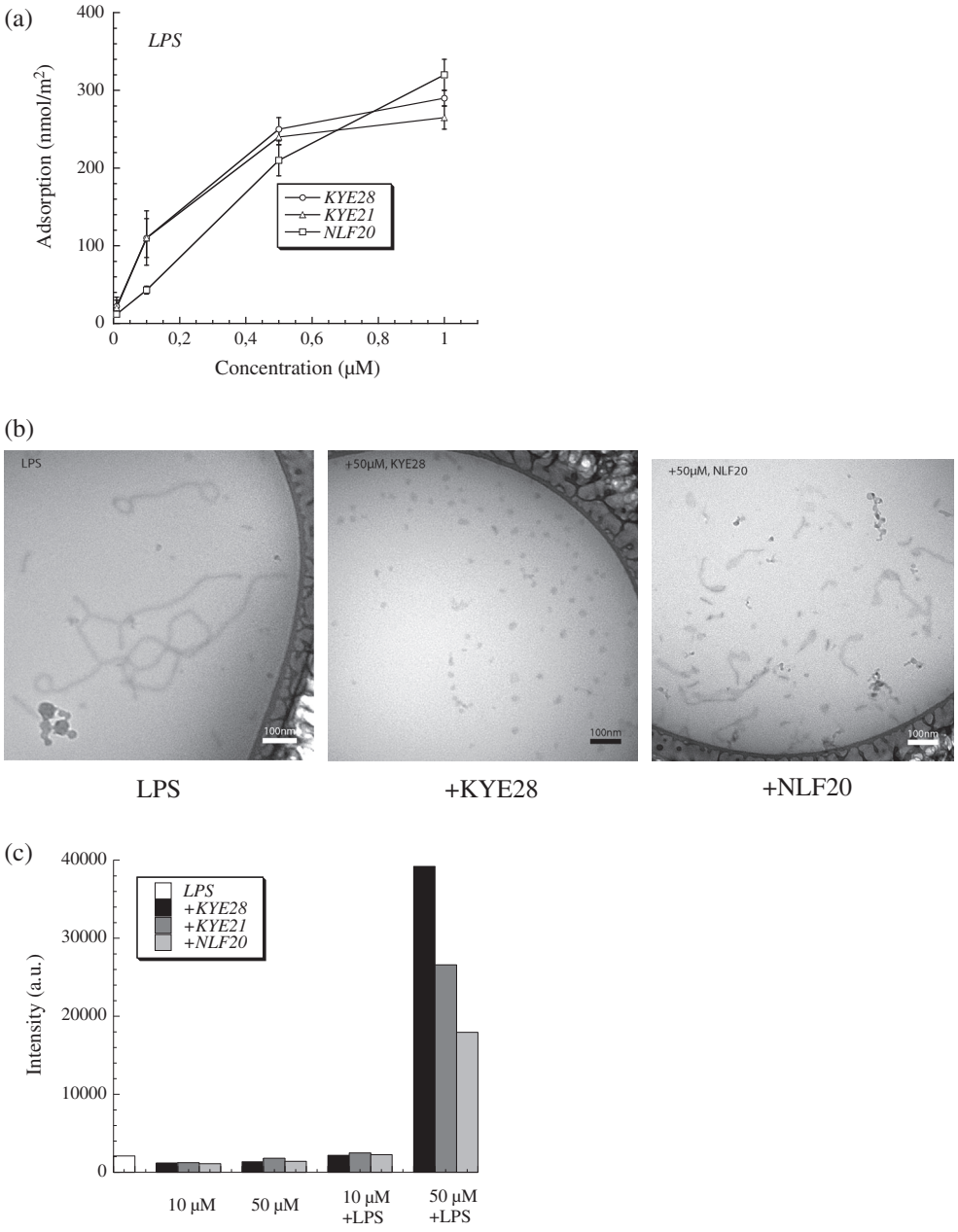


Fig. 4. (a) Peptide binding to preadsorbed *E. coli* LPS. (b) Representative cryoTEM images of LPS (0.2 mg/ml in 10 mM Tris, pH 7.4) in the absence and presence of KYE28 and NLF20 at a peptide concentration of 50 μM. (c) Maximum ANS fluorescence intensity after addition of 10 or 50 μM peptide to 10 μM ANS and 0.2 mg/ml LPS in 10 mM Tris, pH 7.4 [16].

peptide-induced potential build-up on the membranes, subsequent LPS binding, and anti-inflammatory effects displayed by these peptides (Fig. 5) [16]. Further work is, however, needed to more clearly demonstrate the relative importance of peptide-induced LPS scavenging localized at eukaryotic cell membranes.

In addition, both peptide-induced fragmentation of LPS aggregates and reduction of LPS negative charge due to peptide binding may facilitate phagocytosis [32]. Through this, an alternative pathway to CD14 binding is provided, thus suppressing the inflammatory activation occurring in the absence of peptide. Indeed, such phagocytosis-related scavenging was previously observed by Richman et al. who were able to correlate anti-inflammatory effects of an Aβ-recognizing peptide with its effect on Aβ phagocytosis [33]. Analogously, the AMP LL-37 has been reported to be able to transfer other anionic polyelectrolytes (notably DNA) into eukaryotic cells after complexation [34].

3.3. Other microbial (lipo)polysaccharides

Although Gram-negative bacteria and LPS are responsible in many indications, inflammation may be caused or deteriorated also by other pathogens. For example, inflammatory response to Gram-positive bacteria is caused primarily by LTA, while zymosan is central for fungi. LPS and LTA both contain hydrocarbon chains and an anionic polysaccharide moiety rich in phosphate charges. Zymosan, on the other hand, carries only a low negative charge, and no hydrophobic moieties. From a biophysical perspective, one could thus expect peptide interactions to be comparable for LPS and LTA, as indeed found for GKY25 variants (Fig. 2b) [12], whereas zymosan should differ from the other two. As found by Kasetty et al. however, both a series of thrombin-derived peptides [35] and peptides derived from S1-proteases [36] showed similar effects on inflammation triggered by LPS, LTA, and zymosan. While

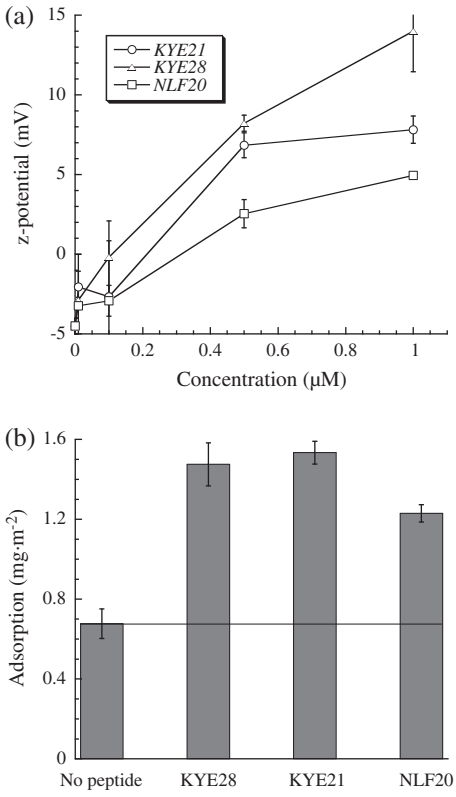


Fig. 5. (a) Effect of peptide adsorption on z-potential of DOPC/cholesterol (60/40 mol/mol) liposomes. (b) LPS adsorption at DOPC/cholesterol (60/40 mol/mol) supported bilayers with and without peptide preadsorption (peptide concentration 1 μM). Measurements were made in 10 mM Tris, pH 7.4 [16].

so far not investigated, the lower charge density and absence of hydrophobic domains in zymosan most likely results in different peptide binding, as well as different effects on self-assembly, membrane-localized scavenging, and phagocytosis. Therefore, comparative biophysical studies between LPS/LTA and zymosan may help discriminating between possible action mechanisms for different peptides.

3.4. Biological effect spectrum of anti-inflammatory AMPs

Despite a growing number of studies on peptide–LPS interactions and their consequences for cytokine generation, few investigations cover the range from detailed biophysical investigations to broader biological effect studies. Exemplifying such studies, GKY25, discussed above regarding biophysical aspects, has been evaluated also from a broader therapeutic perspective [37,38]. After both intraperitoneal and subcutaneous injection, this peptide was found to reduce a number of pro-inflammatory cytokines, including TNF-α, IL-6, INF-γ, and MCP-1, and transiently increasing the anti-inflammatory cytokine IL-10. Furthermore, GKY25 resulted in dramatically reduced platelet infiltration and fibrin deposition into lungs, and increased survival in mice after inflammation caused by either Gram-negative bacteria or LPS. In addition, it decreased histone 3, histone 4, and MPO levels in the pancreas, abolished neutrophil expression of Mac-1, and inhibited CXCL2-induced chemotaxis of isolated neutrophils [39]. Truncation of GKY25 diminished anti-inflammatory cytokines and attenuated survival, as did scrambling of the sequence to form WFF25, unable to disrupt LPS aggregates. A similar effect spectrum was subsequently found for the heparin cofactor II-derived peptide KYE28, discussed above from a biophysical perspective [40]. Thus, for mice challenged with *Escerichia coli* LPS, 98% of the animals survived after KYE28 treatment, whereas all control animals had to be sacrificed within the first 24 h. In parallel, pro-inflammatory cytokines

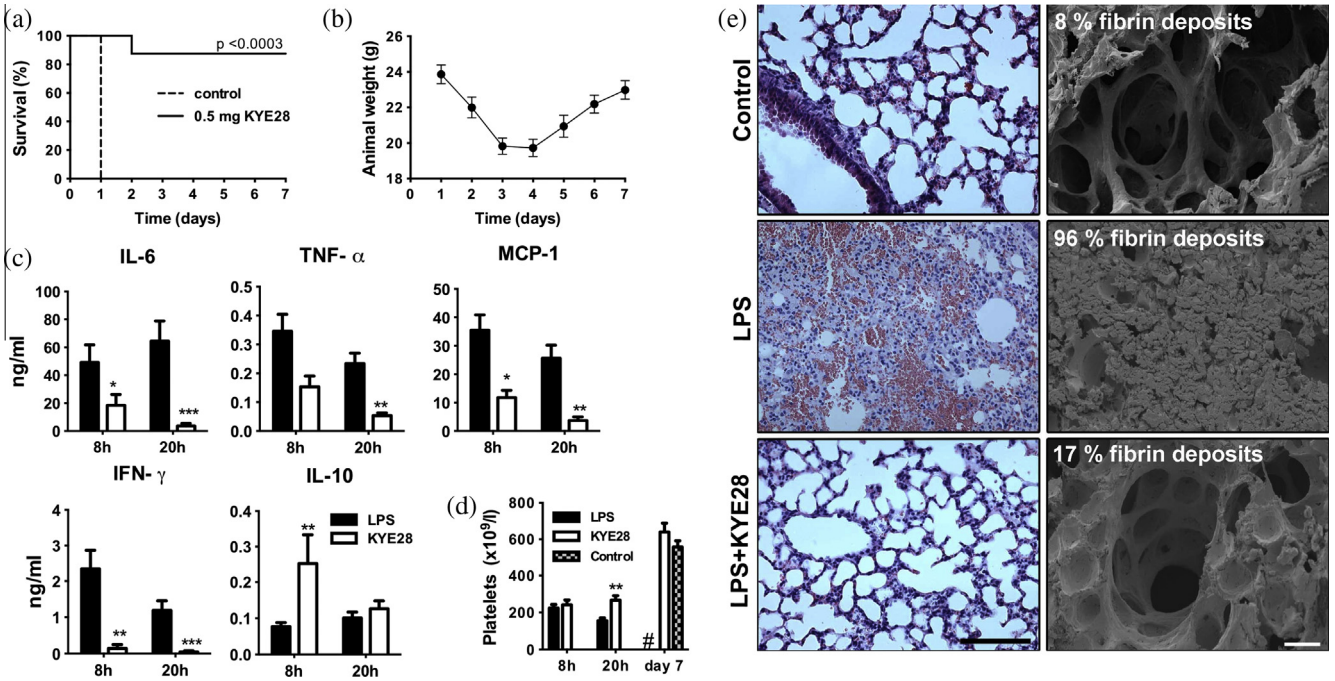


Fig. 6. Effects of KYE28 against LPS *in vivo*. Septic shock in C57BL/6 mice was induced by intraperitoneal (i.p.) injection of *E. coli* LPS (18 mg/kg) followed by i.p. injection of 0.5 mg KYE28 or buffer 30 min later. (a) Survival of the animals challenged with LPS. (b) Weight development during the experiment in (a) for KYE28-treated mice. (c) Cytokine levels 8 and 20 h after LPS injection in mouse plasma. (d) Number of platelets determined 8 and 20 h after LPS, as well as in survivors at day 7. (e) Lung sections of healthy (Control), LPS-treated, and LPS + KYE28-treated mice analyzed 20 h after LPS injection [39].

IL-6, IFN-γ, TNF-α, and MCP-1 were reduced in KYE28-treated mice, whereas the anti-inflammatory IL-10 was transiently increased. Furthermore, platelet counts of recovered KYE28-treated mice were similar to those of healthy controls, while histological and scanning electron microscopy analyses of lung infiltration showed red blood cells and fibrin deposition to be notably suppressed in KYE28-treated animals (Fig. 6).

3.5. PEGylation for enhanced AMP performance

Poly(ethylene)glycol (PEG) conjugation has been extensively investigated as a tool to increase performance for a range of protein and peptide therapeutics [41]. Such 'PEGylation' offers a series of potential advantages, including increased bloodstream circulation time and reduced uptake in tissues related to the reticuloendothelial system. Furthermore, PEGylated proteins/peptides frequently display increased resistance to proteolytic degradation, as well as reduced aggregation, toxicity, and immune responses. Also for AMPs, PEGylation may result in reduced proteolytic susceptibility and toxicity, but generally at a cost of reduced antimicrobial effect [42,43]. For example, Singh et al. investigated conjugation of KYE28 with PEGs of different length and conjugation site [44]. It was found that PEGylation results in a suppressed KYE28 antimicrobial potency in an Mw-dependent manner, but also in a strongly reduced toxicity and in improved selectivity. After PEGylation, conditions could be found, at which the PEGylated peptide was able to effectively kill bacteria added to blood, but at the same time causing no measurable hemolysis. Importantly, while PEGylation reduced peptide binding to both LPS and lipid A, peptide-induced LPS aggregate disruption was less affected by PEGylation, and anti-inflammatory properties largely retained (Fig. 7). Consequently, PEGylation seems to offer an interesting approach for optimizing the performance of anti-inflammatory peptides, e.g., in terms of reduced toxicity and increased proteolytic stability.

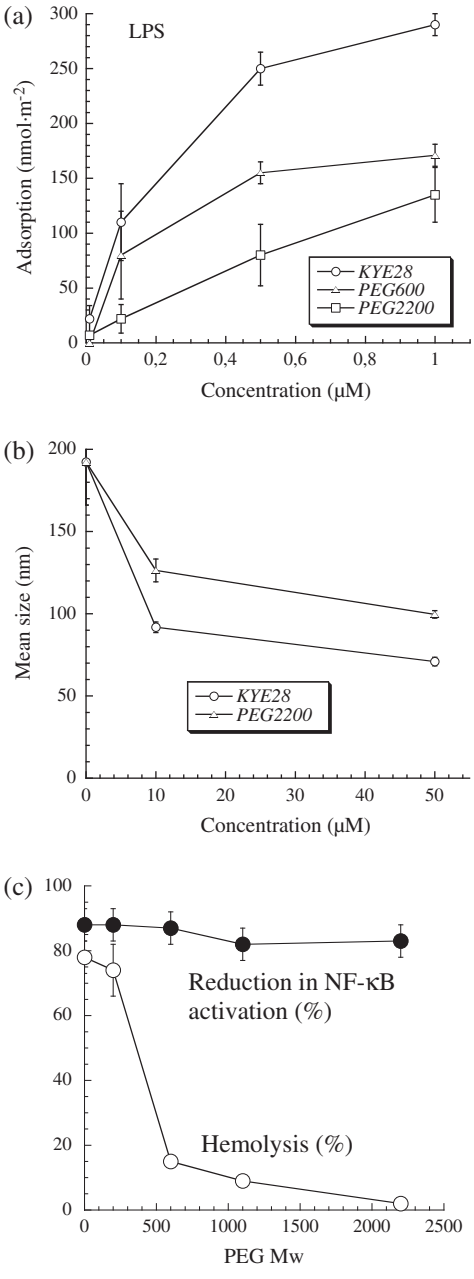


Fig. 7. (a) Peptide binding to *E. coli* LPS from10 mM Tris, pH 7.4. (b) Effect of KYE28 and KYE28PEG2200 (Mw PEG = 2200 Da) on the mean size of LPS aggregates in 10 mM Tris, pH 7.4. (c) Effects of PEG length on hemolysis and anti-inflammatory effect of PEGylated KYE28. For the latter, RAW-Blue macrophages were incubated with *E. coli* LPS in presence of peptides at the indicated concentration, followed by monitoring of NF-κB activation [43].

4. Summary and outlook

Although there are currently no AMPs on the market, a number of peptides are currently in various stages of clinical trials [3]. Such AMPs need to outperform existing antibiotics to reach the market, driving development in the direction of activity against multi-resistant bacteria and additional host defense functions. Considering this, and the considerable unmet medical needs in this area, AMPs displaying also anti-inflammatory effects offer interesting opportunities. The mode-of-action of such AMPs is, however, quite complex, with multiple molecular mechanisms. The most important of these seem to be:

(i) Prevention of LPS binding to LPB through peptide binding/blocking of the lipid A epitope of LPS, thereby avoiding triggering the cascade leading to NF-κB activation.
(ii) Positive charge build-up of macrophage and monocyte membranes through peptide adsorption, in turn causing LPS binding/scavenging.
(iii) Peptide-triggered phagocytosis through charge neutralization and LPS aggregate disruption.

At present, considerable uncertainty remains as to the relative importance of these mechanisms, and how knowledge on this may be used in peptide design for optimized biological effects. In particular, membrane-localized peptide-induced LPS scavenging needs to be further investigated in relation to factors determining peptide binding and positive potential build-up on various human cell types. Furthermore, trafficking of amphiphilic peptides in macrophages in particular needs to be clarified, together with the interplay between peptide properties and LPS complex formation and cell internalization through phagocytosis. Many parallels can here most likely be found with siRNA and DNA internalization through complex formation with cationic polymers and lipids. However, studies also need to pay attention to the similar effects of some AMPs against inflammation triggered by the structurally different LPS/LTA and zymosan, indicating that mechanisms may differ between systems, and also that additional processes may be involved. From a therapeutic perspective, an accentuated emphasis on broad biological effect spectra is required, as is work to clarify pharmacokinetics of AMPs, as well as possibilities to control this by drug delivery systems.

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