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Peptides with dual mode of action: Killing bacteria and preventing endotoxin-induced sepsis☆

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ABSTRACT

Bacterial infections, with the most severe form being sepsis, can often not be treated adequately leading to high morbidity and lethality of infected patients in critical care units. In particular, the increase in resistant bacterial strains and the lack of new antibiotics are main reasons for the worsening of the current situation, As a new approach, the use of antimicrobial peptides (AMPs) seems to be promising, combining the ability of broad-spectrum bactericidal activity and low potential of induction of resistance. Peptides based on natural defense proteins or polypeptides such as lactoferrin, *Limulus* anti-lipopolysaccharide factor (LALF), cathelicidins, and granulysins are candidates due to their high affinity to bacteria and to their pathogenicity factors, in first line lipopolysaccharide (LPS, endotoxin) of Gram-negative origin.
In this review, we discuss literature with the focus on the use of AMPs from natural sources and their variants as antibacterial as well as anti-endotoxin (anti-inflammatory) drugs. Considerable progress has been made by the design of new AMPs for acting efficiently against the LPS-induced inflammation reaction in vitro as well as in vivo (mouse) models of sepsis. Furthermore, the data indicate that efficient antibacterial compounds are not necessarily equally efficient as anti-endotoxin drugs and vice versa. The most important reason for this may be the different molecular geometry of LPS in bacteria and in free form. This article is part of a Special Issue entitled: Antimicrobial peptides edited by Karl Lohner and Kai Hilpert.

1. Introduction

Bacterial infections still represent a severe threat to human health worldwide. This is fundamentally different to the view up to approximately 1990, where it was general consensus that antibiotics (AB) would be able to combat most infectious diseases caused by bacteria. A reason for this paradigm change is, in particular, the dramatic increase in bacterial resistance, connected with a lack of newly approved antibiotic drugs [1–2]. At the same time, the demographic change with increasingly elder people worsens the situation. One alternative strategy to improve the antibacterial fight is the use of antimicrobial peptides (AMPs). These polypeptides, such as cathelicidines, defensins, and dermicines, are endogenous (body-own) compounds, which play an important role as efficacious mucosal defense factors [3]. They are expressed in compartments of the body, with the main role of exerting defense, for example in the oral and respiratory tract [4], and in the human skin [5]. In the case of severe infections, which in the final form leads to sepsis (blood poisoning), this health state is characterized by an inflammatory state affecting the patient's whole body, i.e., a systemic inflammation with high morbidity and lethality [6–7]. The naturally occurring AMPs, however, are never exposed to the systemic environment, rendering them inherently toxic at therapeutically necessary concentrations.

One of the main problems in the fight against sepsis is the fact that an efficient drug must be able to kill the bacteria without releasing the inflammation-inducing toxins, i.e., lipopolysaccharides (LPS, endotoxin) in the case of Gram-negative and lipoproteins (LPs) for Gram-positive bacteria. Former data showing a significant contribution of lipoteichoic acids or peptidoglycans could not be confirmed recently [8]. It has been frequently found in critical care units that the situation for patients treated with AB is worsened due to the release of endotoxins from the bacteria [6,9]. Since existing therapies aim at killing the bacteria or modulating the immune response, such treatments do not address the major underlying cause of sepsis, i.e. the release of toxins by the bacteria. Thus, no satisfying therapeutic option exists to date.

Abbreviations: AMP, antimicrobial peptide; LPS, lipopolysaccharide; SALPs, synthetic anti-LPS peptides; AA, amino acid; LAL, *Limulus* amebocyte lysate; LF, lactoferrin; LP, lipoprotein; LALF, *Limulus* anti-LPS factor; ENP, endotoxin neutralizing protein; LBP, lipopolysaccharide-binding protein; BPI, bactericidal permeability-increasing protein; SAXS, small-angle X-ray scattering; ITC, isothermal titration calorimetry; AFM, atomic force microscopy; RAW, monocytic cell line; MNCs, mononuclear cells.

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For the development of AMPs as drugs against bacteria and their toxins three strategies seem to be promising: (i) amino acid (AA) sequence variations of the endogenous AMPs mentioned above to reduce their inherent toxicity, but to maintain their antibacterial actions, (ii) analysis of the AA sequences of LPS-binding domains from natural defense proteins and subsequent synthesis of these protein-derived polypeptides, and (iii) development of polypeptides which are not antibacterial but modulate the immune system for a better fight against the infections.

In this review, option (ii) will be discussed more profoundly, because it seems to have the most promising perspectives for the development of an effective antibacterial drug. Details of option (iii) are described in [10–12].

An overview of various classes of AMP, i.e. derived from CAP37 (cationic antimicrobial protein from human neutrophil leukocytes), LBP (lipopolysaccharide-binding protein), BPI (bactericidal-permeability increasing protein), LALF (*Limulus* anti-LPS factor from *Limulus polyphemus*), CAP18 (18 kDa cationic antimicrobial protein from rabbit granulocytes), and lactoferrin (iron-binding glycoprotein of mammals), is given by Pristovsek and Kidric [13]. Furthermore, Martin et al. [14] recently published a short review about the role of AMPs in human sepsis. Furthermore, it should be stated here that from the multitude of papers, only selected reports are discussed which may give closer insights into the mechanisms of action, i.e., how AMPs kill bacteria and neutralize LPS. Furthermore, reports dealing with pore-forming AMPs, which belong to a completely different class of drugs are not subject of this overview. These aspects are discussed in detail in a number of reviews, see e.g. [15].

The main findings for the different AMPs discussed in the following are summarized in Table 1.

2. Peptides based on lactoferrin

Lactoferrin (LF) is an iron-binding glycoprotein, a major component of secondary granules of neutrophils, present in high levels in milk, pancreatic juice, and secretions like saliva and tears and is released from neutrophil granules during inflammation [16]. It was reported that in excess concentration of LF, the LPS-induced cytokine (tumor-necrosis-factorα, TNFα) secretion of human mononuclear cells is considerably reduced [17]. Interestingly, this was not reflected in the *Limulus* assay (*Limulus* amebocyte lysate, LAL), in which increasing LF concentration yielded even an increase in the *Limulus* reaction (endotoxin units/ml, 1 ng/ml correponding to approximately 10 EU/ml). This can be explained by the fact that the recognition structures in the *Limulus* test are in the lipid A-part of LPS, an acylated (3 to 7 acyl chains) diglucosamine backbone including the 4′-phosphate, whereas for the induction of cytokines relevant for inflammation a fully acylated (6 or 7) lipid A backbone including the 1-phosphate is necessary as outlined in [18]. One important point with respect to the prerequisite for LPS inactivation was the observation that the non-lamellar cubic aggregate structure of lipid A was converted into a multilamellar structure as demonstrated by small-angle X-ray scattering (SAXS) using synchrotron radiation. A high lamellar repeat distance of 9.95 nm apparently results from the voluminous LF protein bridging neighboring lipid A bilayers, since normal lipid A bilayers have periodicities in the range 4.9 to 5.3 nm [19]. A further interesting observation was made by isothermal titration calorimetry (ITC); the LPS–LF interaction, measured at 37 °C, is clearly exothermic, while the lipid A–LF interaction showed only an endothermic reaction, but both saturated at 0.2 to 0.3 M ratio [LF]:[lipid]. This surprising behavior could be understood only on the basis of a later ITC study, in which the temperature dependence of the binding of the model peptide polymyxin B with various endotoxins including free lipid A was investigated [20]. It could be clearly demonstrated that endothermic as well as exothermic processes occur. Below the gel to liquid–crystalline phase transition temperature (T_m) at 30 or 36 °C for LPS and 45 °C for lipid A, endothermic reactions are observed and above T_m exothermic reactions are predominant. The former reactions can be understood by an entropic reaction of the peptide with

Table 1
Peptides, their AA sequence, and mode of action against bacteria and LPS; anti-Mic and anti-LPS mean antimicrobial activity and anti-inflammatory action, respectively.

Compound	Amino acid sequence	Antibacterial activity/mode of action	Reference
Lactoferricin-derivatives	4–16 AA from sequence FKCRRWQWRMKKLGAPSITCVRRAF	Permeabilizing agents for antibiotics	Farnaud et al. [22]
Lactoferricin derivatives	FKCRRWQWRMKKLGA W substitution by A	Decrease of anti-Mic by A	Haug et al. [23]
LF11 and lauryl-LF11	LF11: FQWQRNIRKVR	Enhancement of anti-Mic and anti-LPS by lauryl group	Andrä et al. [24]
Acylated and non-acylated 8-mers from LF	FWRIRIRR, FWRRFWRR, and octanoyl derivatives	Anti-LPS, LPS aggregation into multilamellae	Brandenburg et al. [25]
LALF/LBP/BPI hybrid peptides	LL-10-H-14: KPTFRRLKWKCRWKVRKSFFKLQC	Survival benefit in mice model of endotoxemia	Dankesreiter et al. [27]
LALF-peptide #28–54		Bactericidal activity, no survival benefit for mice	Weiss et al. [28]
LALF-peptide LPS-binding domain	DHEGHYRIKPTFRRLKWKYKGKFWCPS	Linear form better in anti-Mic and anti-LPS than cyclic form	Leslie et al. [29]
LALF variant CLP-19	CRKPTFRRLKWKIKFKFKC	Anti-LPS action, survival benefit for mice	Ren et al. [30]
Cyclic LALF variants	HYRIKPTFRRLKWKYKGKFWCG and shorter	Inhibition of LPS-induced inflammation	Andrä et al. [31–32]
SALP	Lead structure: GCKKYRRFRWKYKGKFWWG	High binding affinity to LPS, survival benefit for mice in endotoxemia	Gutsmann et al. [33], Heinbockel et al. [34]
SALP	GCKKYRRFRWKYKGKFWWG	Survival benefit in cecal ligation and puncture	Schuerholz et al. [39]
SALP	GCKKYRRFRWKYKGKFWWG	Inhibition of lung inflammation	Heinbockel et al. [34]
Granulysin-derived peptides	G12.34: rrvsrrfmrryrsrrirrlv G12.21 rrvsrrpmrryrsrrprrlv (Peptides in D-configuration)	Inhibition of LPS-induced inflammation, anti-Mic	Chen et al. [43]
NK-2 from NK-lysin	KILRGVCKKIMRTFLRRISKDILTGKK	Anti-Mic and anti-LPS best with rough mutants	Andrä et al. [44]
Variant NK11 from NK-2	KISKRILTGKK	No anti-Mic and anti-LPS activities	Hammer et al. [45]
Cecropin-derived peptides	Gm1: ENFFKEIERA GQRIRDAIIS AAPA VETLAQAQKIIKGGD ΔGm1: ENFFKEKERK GQRIRDAIIS RRPRVETLAQAQKIIKGGD	Gm1 stronger anti-Mic	Correa et al. [46–47]
CM4, cecropin derivative		Anti-LPS activity, blocking of cytokine secretion	Lin et al. [48]
LL-37	LL-37: LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTESAnti-Mic, anti-LPS, cytotoxicSigurdardottir et al. [50]LL-37 derived peptide, GKEGKEFKRIVQRIKDFLRNLVPRAnti-Mic, less hemolytic than LL-37Sigurdardottir et al. [50]11-Mer peptides$K_6L_2W_3$Efficient against MRSA, anti-LPSPark et al. [51]FowlicidinsFowl-1: RVKRVWPLVIRTVIAGYNLYRAIKKK Fowl-3: KRFWPLVPVAINTTVAAGINLYKAIRRKAnti-Mic, but cytotoxicBommineni et al. [52]CAP18 part structures $LL37_{15–32}$1) KEFKRIVQRIKDFLRNLV 2) KLFKRIVQRILDFLRNLV 3) KLFKRIVKRILKFLRKLVStrongly varying anti-Mic, best for 3)Nagaoka et al. [53]Abbreviations: LF lactoferrin; LALF *Limulus* anti-LPS factor, SALPs synthetic anti-LPS peptides, MRSA methycillin-resistant *S. aureus*.		

the ordered water layers in the endotoxin aggregates, which are decomposed, whereas the exothermic interactions result from the Coulomb binding of the positive peptide charges with the negative groups of the endotoxin (phosphates, carboxylates). The latter interaction in each case is the driving force of the peptide–endotoxin binding, which was confirmed by LPS–PMB binding studies [21].

Farnaud et al. analyzed the interaction of lactoferricin-derived cationic peptides with structurally different LPSs [22]. The LF derivatives were selected from different AA sequence ranges of the lactoferricin peptide, which is released after cleavage of the N-terminal fragment by pepsin. The lengths varied between 4 and 16 AA. They found only restricted antimicrobial activity (only the peptides with tryptophan and net positive charges of at least 6 exhibited some activity against *Escherichia coli* K12 mutants) and hence postulated that 'cationic peptides should not be taken as antimicrobial agents, but probably more as disorganizing or permeabilizing agents that could increase susceptibility to more efficient antibiotics'. A more profound review of the action of short lactoferricin peptides against a variety of bacteria was published by Haug et al. [23]. They identified important structural parameters in alanine scans that influence the antibacterial activity of the peptides. They found in a 15-mer (FKCRRWQWRMKKLGA) that a substitution of the tryptophan (W) residues by alanine resulted in a considerable decrease of activity. A more detailed analysis of the hydrophobic property of W by substitution with other hydrophobic moieties, for example phenylalanine (F), led to lower activity against *aureus*, but higher activity against *E. coli*. The authors discussed a variety of variants leading to different folding of the peptides accompanied with variations in antimicrobial and hemolytic activities. The authors concluded that it was not the specific AA sequence but rather the AA content of the peptides that are highly significant for biological activity. Furthermore, electrostatic interactions are not only important for the binding to the bacterial membranes, but also vital in ensuring selectivity against bacteria versus eukaryotic cells.

The lactoferricin peptide, LF11 (AA 21–32) was modified by coupling of a C12-alkyl group (lauryl-LF11) at the N-terminus. This led to an enhancement of the anti-endotoxin as well as antibacterial action as compared to the non-acylated LF11 [24]. The authors found that a compensation of the surface charges of LPS was a prerequisite for action, as seen by Zeta potential measurements. For an effective LPS neutralization, however, even an overcompensation of the negative surface charge was observed. An extensive study of the interaction of lactoferricin-derived peptides TS140–15 (FWRIRIRR), TS140–27 (FWRRFWRR) and lipopeptides VSI-55 (octanoyl-FWRIRIRR) and VSI-60 (octanoyl-FWRRFWRR) with LPS was performed, i.e., their antibacterial activity and their ability to inhibit the LPS-induced inflammation in vitro (cytokine secretion of human mononuclear cells, MNCs) and in vivo (mouse model of endotoxemia) [25]. The compounds exhibit a significant effect on the gel to liquid–crystalline phase transition, as measured with Fourier-transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC). The FTIR data are plotted in Fig. 1, in which the symmetric stretching vibrational band at 2850 to 2853 cm^{-1} is plotted versus temperature. The figure shows a fluidization of the LPS (deep rough mutant from *Salmonella minnesota* strain R595) acyl chains by VS1–60 in both phases as evidenced by the decrease in wavenumbers, but the temperature of the phase transition remains nearly constant. In addition, it was found that the aggregate structure of LPS is converted into a multilamellar one. As example for this, the electron density of LPS is plotted versus temperature, and the structural parameters d, d_W, and d_B are presented showing only a slight dependence on temperature (Fig. 2). Further, it was found that both non-acylated compounds had a better antimicrobial activity against *E. coli* ATCC 25922, particular strains from *Pseudomonas aeruginosa*, *Bordetella bronchiseptica*, *Brucella abortus*, and *S. aureus*, but were weaker in inhibiting the LPS-induced inflammatory response in human MNCs than the acylated compounds. The difference in antimicrobial activity was in part explained by a reduced effective concentration of the acylated peptides at the cytoplasmic membrane, where the killing event takes place, due to their higher affinity to LPS [26]. These interpretations are indicative that the mechanisms of action in the interaction of the peptides with LPS in bacteria as planar monolayer and in free form as aggregate may be completely different.

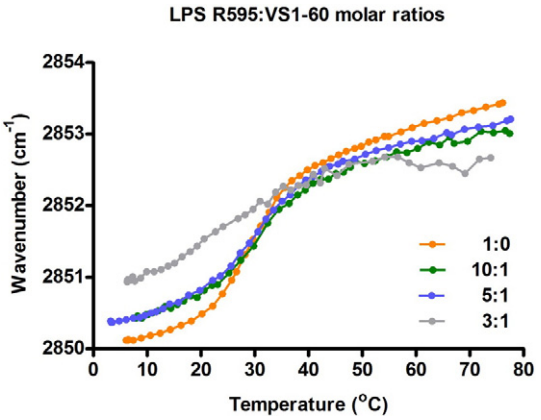

Fig. 1. Gel to liquid–crystalline phase transition of the hydrocarbon chains of LPS R595 in the presence of varying amounts of peptide VS1–60 (molar ratios indicated in the panel). The peak position of the symmetric stretching vibration of the methylene groups of the lipid A moiety is plotted versus temperature. In the gel phase, the peak position lies around 2850, in the liquid–crystalline phase around 2852.5 to 2853 cm^{-1}. (From Brandenburg et al. [25], Anti-infective Agents in Medicinal Chemistry, 2010, 9, 9–22).

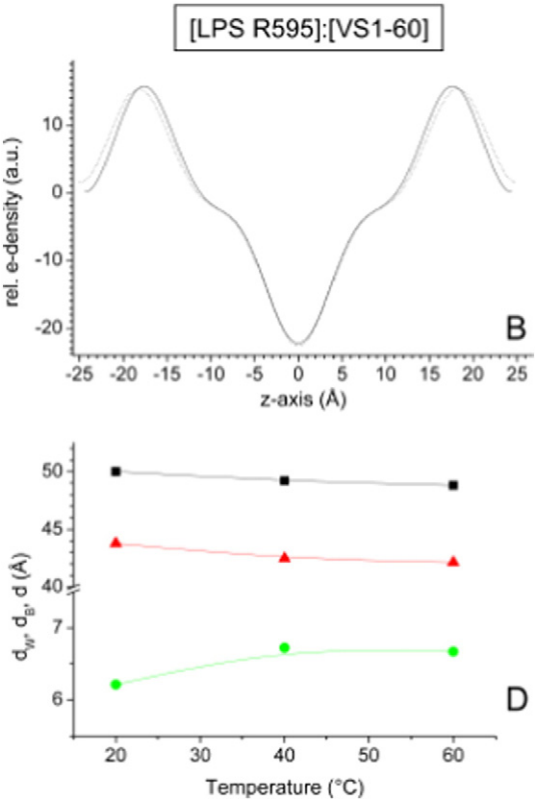

Fig. 2. Electron density maps of the L_β (dotted line) and L_α (solid line) phases, (top) and the structural parameters (bottom), lattice spacing, d (black line), the bilayer thickness, d_B (red line) and the free water layer thickness, d_W (gren line), where $d = d_B + d_W$. (From Brandenburg et al. [25], Anti-infective Agents in Medicinal Chemistry, 2010, 9, 9–22).

3. Peptides based on *Limulus* anti-LPS factor

The *Limulus* anti-LPS factor (LALF), called in its recombinant form endotoxin-neutralizing protein (ENP), is a potent neutralizer of LPS. A biophysical analysis of this protein showed a considerable inhibition of the LPS-induced cytokine (TNFα) response, when ENP was added to LPS at a high molar excess ([ENP]:[LPS] > 10 M/M). This inhibition was connected with a compensation of the LPS head group charges, a slight fluidization of the LPS acyl chains, and to a conversion of the cubic aggregate structure of lipid A into two multilamellar ones, one pure unperturbed lipid A and one lipid A:ENP complex.

Dankesreiter et al. [27] analyzed a variety of LALF-derived peptides in their ability to inhibit the LPS-induced release of tumor-necrosis-factor α in a murine macrophage cell line and in human PBMC, as well as in an in vivo mouse model. They found for the different peptides, including hybrid peptides with LBP- and BPI-LPS binding sections, good inhibition in the cytokine assay. The best compound, called LL-10-H-14, a dimeric peptide from linear LALF and cyclic hLBP, prevented lethality in a mouse model of endotoxemia at a 100-fold excess concentration (Pep:LPS).

The cyclic LALF–peptide (AA 28–54), consisting of the corresponding AA from the parent LALF protein, showed significant bactericidal and endotoxin-neutralizing activities, against bacteria and isolated LPS from *P. aeruginosa*, respectively [28]. The authors also investigated the survival of mice after challenge with LPS, but did not find a benefit for the animals in contrast to the behavior of the 'gold standard' PMB and the complete protein ENP. Only 10% survived, although the peptide was administered twice to the mice, one bolus directly before the LPS injection and one after 30 to 45 min. The authors explained these findings to be due to a possible very short half-life of the peptide. The complete LPS-binding domain of LALF, the 27-mer DHECHYRIKPTFRRLKWKYKGKFWCPS was investigated in the linear as well as in a cyclized form to neutralize LPS from *P. aeruginosa* in RAW264.7 cells, the fibroblast cell line WEHI 164, and in the LAL assay [29]. Cyclization was performed in this and in other investigations (see below) because it was thought that it would correspond to the constrained structure of the endotoxin-binding region of the complete LALF. The authors found acceptable LPS-neutralization of the linear peptide, which was, however, less active than the complete LALF and PMB, respectively. In particular, the cyclized peptide was much less active than the linear form with respect to bactericidal activity against bacteria. The authors concluded that cyclization of the linear peptide may not have created the domain outside the context of the full-length LALF protein.

Ren and co-workers [30] analyzed synthetic peptides based on LALF, one peptide called CLP-19 (sequence CRKPTFRRLKWKIKFKFKC) and another peptide corresponding to an essential part of the LPS-binding domain in the LALF, $LALF_{31-52}$, in their ability to neutralize LPS from *E. coli* O111:B4 in the *Limulus* assay, in an in vitro cytokine (TNFα) assay of RAW164.7 cells, and in an in vivo assay of mouse survival. Both peptides were able (i) to reduce the response in the LAL test, (ii) to inhibit the cytokine concentration, and (iii) to provide a survival benefit of the mice. At the same time, no signs of cytotoxic effects were observed in mouse RAW cells via the MTT test. Unfortunately, an assessment of these results in comparison with findings from other groups is not possible, since these authors did not use galactosamine-treated animals, which sensitizes the mice to the action of LPS.

In the works of Andrä et al. [31–32] biophysical techniques were applied to understand the mechanisms of action of various cyclic LALF peptides ranging from a 9-mer to a 22-mer. The different compounds converted the non-lamellar structure of LPS into a multilamellar structure, with the amount depending on the affinity of the peptides to LPS. Interaction studies using ITC *exhibit, for all* peptides, saturation curves for LPS binding, with cLALF 22 ($LALF_{31-52}$: HYRIKPTFRRLKWKYKGKFWCG) having the highest affinity. Comparison of the LPS binding exotherms of the two peptides cLALF10 ($LALF_{38-45}$: TFRRLKWKCG) and cLALF22 is presented in Fig. 3, from which it can be deduced that the S-shaped saturation range lies around Pep:LPS 0.5 to 1 M ratio for the latter and 1 to 2.2 M ratio for the former explaining the much better inhibition of the cytokine production for cLALF22 (complete inhibition at [Pep]:[LPS] 30:1 as compared to cLALF10 [Pep]:[LPS] 300:1 M ratio). However, there is no conclusive interpretation regarding the phase transition behavior of LPS and correlation with the cytokine-inhibition efficiency. In most cases a fluidization of the acyl chains is observed, while in other cases only a very slight effect or even a rigidification is seen.

Newly designed linear peptides containing the 5-mer base sequence KGKFW from LALF were analyzed for their ability to act antiseptically as described by Gutsmann et al. [33] and Heinbockel et al. [34]. The design of these peptides, called synthetic anti-lipopolysaccharide peptides (SALPs) with the lead compound Aspidasept® (Pep19–2.5: GCKKYRRFRWKFKGKFWFWG), was directed in an optimization process to bind with high affinity to the lipid A part of LPS, its 'endotoxic principle' [35]. In this way, the multitude of Gram-negative bacteria should be neutralized by Pep19–2.5. As proof of this concept, ITC measurements of the binding of Pep19–2.5 to LPS Ra show a high binding constant $K_A = 3 \times 10^8$/mol [36]. LPS Ra (strain R60 from *S. minnesota*) was taken because it represents, within the heterogeneous wild-type LPS, the bioactive unit [37]. The binding is connected with a change of the aggregate structure of LPS into a multilamellar form, as seen in SAXS and freeze-fracture experiments [36], confirming the results mentioned above. Interestingly, the secondary structure of the SALP, taken from the peak position of the amide I at 1630 cm^{-1} vibrational band in FTIR experiments, exhibits a β-kind structure which does not change when LPS binds to the peptide. The binding leads simultaneously to a size increase of the LPS aggregates, which is a prerequisite that serum- or membrane proteins such as CD14 and TLR4 are no longer able to bind to the epitopes in the LPS backbone. The biological experiments are indicative of a considerable inhibition of the LPS-induced cytokine secretion (IL-6 and TNFα) in human mononuclear cells in vitro, and in in vivo mouse model of endotoxemia. In the latter model inhibition was observed even at very low peptide concentrations ([LPS]:[Pep19–2.5] 1:1 wt.%). Various in vitro data of the use of Pep19–2.5, in combination with classical antibiotics, are described elsewhere [38]. In another sepsis model of cecal ligation and puncture of mice, there was a significant survival benefit for the animals [39], and both strongly elevated IL-6, IL-10 and monocyte chemoattractant protein serum levels in septic animals were significantly reduced after Pep19–2.5 administration ($P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively). Similarly, Pep19–2.5 significantly reduced the sepsis-induced CD14 mRNA expression in the heart

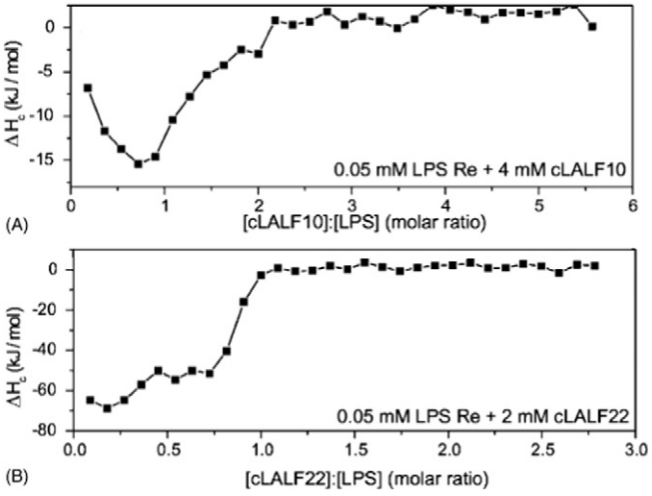

Fig. 3. Isothermal calorimetric titration of 0.05 mM LPS Ra with 4 mM cLALF10 (A) and 2 mM cLALF22 (B). Thirty titrations were performed by adding 3 μl peptide every 5 min. (From Andrä et al. [32].

(P = 0.003), lung (P = 0.008), and spleen tissue (P = 0.009). Interestingly, Pep19–2.5 outperformed the well-known anti-sepsis drug polymyxin B, which is the only approved drug against sepsis and is restrictedly used due to its inherent nephro- and neurotoxicity [40]. The data show the reduction of inflammation in mouse models of endotoxemia and bacteremia, in which an antibiotic kills the bacteria (*Salmonella*) and the peptide blocks the released LPS. This was also observed when the peptide addition was delayed until after the bacterial or endotoxin challenge [34]. The investigations were also applied to an ex vivo model of the human lung. In this model, LPS and MRSA were taken as stimulants. As shown in Fig. 4, the addition of Pep19–2.5 considerably reduced the inflammation signal in the lung [34]. These findings seem to be important since in approximately 40% of sepsis cases a severe lung inflammation takes place [41].

4. Peptides based on other polypeptides

Granulysin, a cytolytic protein, is a mammalian defense protein. Synthetic peptides derived from granulysin were found to kill Gram-negative as well as Gram-positive bacteria [42]. The biophysical analysis of compounds G12.34 to G12.25, which were part structures in D-configuration, exhibited inhibition of LPS induced cytokine-induction already at 10:1 [peptide]:[LPS] wt.%, in particular at a LPS concentration of 10 ng/ml [43]. The gel to liquid–crystalline phase transition of the LPS acyl chains showed, if at all, only a slight rigidification, but the conversion of the LPS aggregates into a multilamellar form, which was also detected in freeze-fracture electron microscopy, was strongest for compounds with the highest inhibition efficiency. In addition, the ITC data are indicative of a nearly ideal S-shaped saturation curve.

NK-lysin is a granulysin-type defense protein from the pig. A potent AMP derived from this is compound NK-2, consisting of 27 AA (KILRGVCKKIMRTFLRRISKDILTGKK). The interaction of different *Salmonella enterica* strains (R595, R60, S-form) and the corresponding LPS with NK-2 showed a dependence on the length of the LPS sugar chains within the strains, since the bacteria with short sugar chains (R595) are more susceptible to the action of the peptide than the Ra- mutants or S-forms [44]. Similarly, the inhibition of the LPS-induced cytokine secretion by NK-2 exhibited a dependence in the sequence LPS R595 < LPS R60 < LPS S-form. Likewise, the increase of the Zeta potential concomitant with surface charge compensation was achieved at lower peptide concentrations for LPS R595 than for S-LPS. These data suggests that a hydrophobic interaction of AMPs with LPS is necessary for an efficient neutralization of the bioactivity of LPS, and that long sugar chains of LPS are also relevant regarding the protection of LPS in and outside the bacteria from binding to poly-cationic compounds.

The interaction of deep rough mutant bacterial strains from *E. coli* WBB01 and *Proteus mirabilis* R45 and the corresponding LPS with NK2

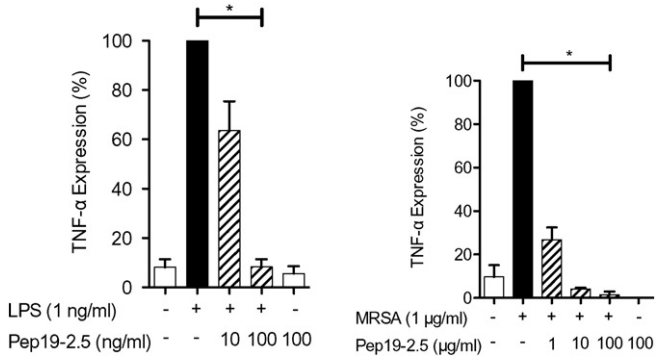

Fig. 4. Pep19–2.5 mediated protection against the LPS or MRSA-induced inflammation of human lung tissue: TNF-α expression levels from human lung tissue were determined after incubation with *S. enterica* LPS or heat-killed MRSA combined with different concentrations of Pep19–2.5. (From Heinbockel et al. [34], AAC 57(3) 2013).

and shortened analogs was studied by Hammer et al. [45]. Transmission EM and atomic force microscopy revealed that NK-2 mediated killing of these bacteria was corroborated by structural alterations of the outer and inner membranes of the bacteria, and fibrous structures within the bacteria. A shortened variant NK11 (KISKRILTGKK), unable to kill bacteria (MIC > 128 or 256 μg/ml), did not cause any morphological changes in the bacteria.

Correa and co-workers [46,47] have studied the interaction of particular AMPs from *Galleria mellonella*, a species which expresses an array of antimicrobials for its own protection. A native peptide Gm1 overall uncharged and a derivative thereof ΔGm1 with overall positive charges were investigated. These peptides, belonging to the class of cecropins, were analyzed with respect to their ability to interact with bacterial model membranes and with bacteria and endotoxin. FTIR was applied to analyze the phase transition behavior of DMPG, and LPS R60, showing a strong fluidization of the acyl chains of both lipids by Gm1, but a rigidification by ΔGm1. Binding constants measured by ITC for the peptide–LPS interaction, gave values $K_A = 1.2$ to 1.3×10^5/mol. These values are much lower than that found for the interaction of LPS with Pep19–2.5 mentioned above, which illustrates that the length (Gm1 and ΔGm1 are 39-mers) as well as the detailed sequence of the amino acids are of uttermost importance in the interaction process. This fact is also reflected in the efficiency of the LPS-induced cytokine secretion in human MNCs, which exhibited approximately 50% to 60% inhibition at [Pep]:[LPS] 100:1 wt.%, which is rather low as compared to findings with other AMPs described above. The authors used AFM for the study of morphological changes of bacteria by the attack of the peptides, and found more changes induced by the native Gm1, and also stronger action on bacteria R45 from *P. mirabilis*, a PMB-resistant strain, as compared to 'normal' *S. minnesota*.

The AMP CM4, a 35-mer, belonging to the cecropin family, was tested for its activity to neutralize LPS O111:B4 [48]. The authors found LPS neutralization by CM4 in the LAL test in the range of 2 to 20 μM of CM4, lowering of LPS binding to RAW264.7 cells (using FITC-conjugated LPS) in the range of 1 to 10 μM, inhibition of the LPS-induced cytokine (TNFα) production in RAW cells, also in the range of 1 to 10 μM, and in a similar way blocking of the NO synthesis by LPS also in RAW cells. Regarding the binding data, it should be noted that the competitive binding of peptide and labeled LPS to the cells is of course only a relative measure, and does not allow to get quantitative statements on binding affinities.

The natural AMP human cathelicidin LL-37, released from activated neutrophil and endothelial cells, cannot be used as anti-sepsis drug since it is inherently cytotoxic. Nell et al. scanned the LL-37 sequence with a window size of 22- to 25-mers identifying a 24-mer (amino acid sequence 13–36) as the most promising segment in terms of antimicrobial activity and with similar efficacy as LL-37 in terms of LPS and LTA neutralization and lower proinflammatory activity [49]. This peptide was further modified to favor the formation of an amphipathic helix. The C-amidated and N-acetylated peptides (P60.4AC) were nontoxic and shown to be safe in animal studies and primary skin and eye irritation/corrosion study. In another approach the compound GKE (GKEFKRIVQRIKDFLRNLVPR) was shown to represent a promising template as antimicrobial agent and also inhibiting the LPS (O111:B4)-induced nitric oxide production of rat aorta [50]. Although the basic helical secondary structure of the compound did not change, the hemolytic activity was reduced considerably for the 21-mer GKE lying at >50 μM as compared to 6 μM for LL-37. These data may be useful in constructing further AMPs with even better anti-septic properties.

Park et al. [51] have designed tryptophan-rich 11-meric AMPs with the aim of targeting bacteria, but not eukaryotic cells, and acting anti-inflammatory. They have found optimized compounds, in particular $K_6L_2W_3$, showing also efficient action against multiresistant isolates such as MRSA and MDRPA (274.7 cells, multi-drug resistant *P. aeruginosa*), and inhibitory effects of LPS (O55:B5)-induced NO production in RAW, and low cytotoxicity against RAW cells. Also, the

binding of LPS monitored via the LAL test indicated the potential of such AMPs as new antimicrobial and anti-inflammatory agents.

Fowlicidins were identified as cathelicidins from chicken with antimicrobial and immune-modulating activities. Bommineni et al. [52] investigated the antimicrobial activities of Fowl-1 (RVKRVWPLVIRTVIAG YNLYRAIKKK) and Fowl-3 (KRFWPLVPVAINTVAAGINLYKAIRRK), which exhibited good antimicrobial activity against various Gram-negative and Gram-positive strains, with MICs lying in the range of 1 to 2 μM. However, the compounds were significantly cytotoxic, with LD_{50} of 1.5 μM for Fowl-1 and 9 μM for Fowl-3. Both AMPs were able to inhibit the LPS (O111:B4)-induced expression of interleukin-1β from RAW274.7 cells. The inhibition took place, however, at concentrations at which cytotoxic effects could be observed, from which it can be deduced that the 'therapeutic index' is low.

Antibacterial and LPS-neutralizing activities of partial structures of the cathelicidin peptide CAP11 were investigated by Nagaoka et al. [53]. The authors found largely varying MIC values from 0.2 μM for the 33-mer G1-E33 to 10–30 μM for the other AMPs against *E. coli*. In addition they recorded binding curves of the peptides to the system Alexa488-LPS-RAW264.7 ($CD14^+$) cells. The data showed saturation of binding at a peptide concentration of 0.1 μM for G1-E33, but much higher for the other. It should be mentioned again that these binding data are only a relative measure of the binding affinity.

5. Mode of action of endotoxin neutralization and of bacterial killing by AMPs

In the case of efficient neutralization of the LPS-induced inflammation reaction, the mode of action can be described as follows: First step in the interaction reaction is the Coulomb attraction between the poly-cationic charges of the AMPs and the negative head groups (phosphates, carboxylates) of LPS. This binding is followed in each case by a second step, consisting of a hydrophobic interaction of apolar regions in the AMPs with the acyl chains of the lipid A part of LPS. In order to become a very efficient AMP, this second step is absolutely necessary. There may be some endotoxin neutralization in the absence of the latter effect, but then the affinity is not high. A sketch of the neutralization of LPS by compound Aspidasept® is presented in Fig. 5. As shown in this figure, the neutralization of Gram-positive lipoproteins is obtained in a similar way by SALPs such as Aspidasept®, described by Martinez et al. [8]. Connected with LPS neutralization is the conversion of the aggregate structure of LPS, which may be cubic, unilamellar, or mixed cubic unilamellar, into a large multilamellar aggregate, this means that an increase in LPS aggregate size occurs. Concomitantly, the binding and morphology change are connected with a strong exothermic reaction with an S-shaped course characteristic for a chemical complex reaction. The details of these mechanisms of action are described in [32–34,36] (see Graphical abstract). Furthermore, Heinbockel and co-workers [54] compared the action of the SALP Pep19–2.5 with another peptide, Hbγ-35, which is a partial structure of the γ-chain of human hemoglobin and increases the bioactivity of LPS. High sensitivity 90°-light scattering showed a drastic increase of the light scattering signal of the LPS complex with the bioactivity-decreasing peptide, Pep19–2.5 and a considerable decrease with the bioactivity-increasing peptide, Hbγ-35. These observations clearly indicate that the size of LPS-aggregates, in the presence of bound peptides, which inhibit their activity, increases significantly.

Conversely to this interpretation, Rosenfeld and coworkers [55] reported for a series of poly-cationic AMPs that the binding complexes with LPS are smaller than pure LPS aggregates. This interpretation was taken from fluorescence spectroscopy and transmission electron microscopy of the binding complexes. The authors explained their

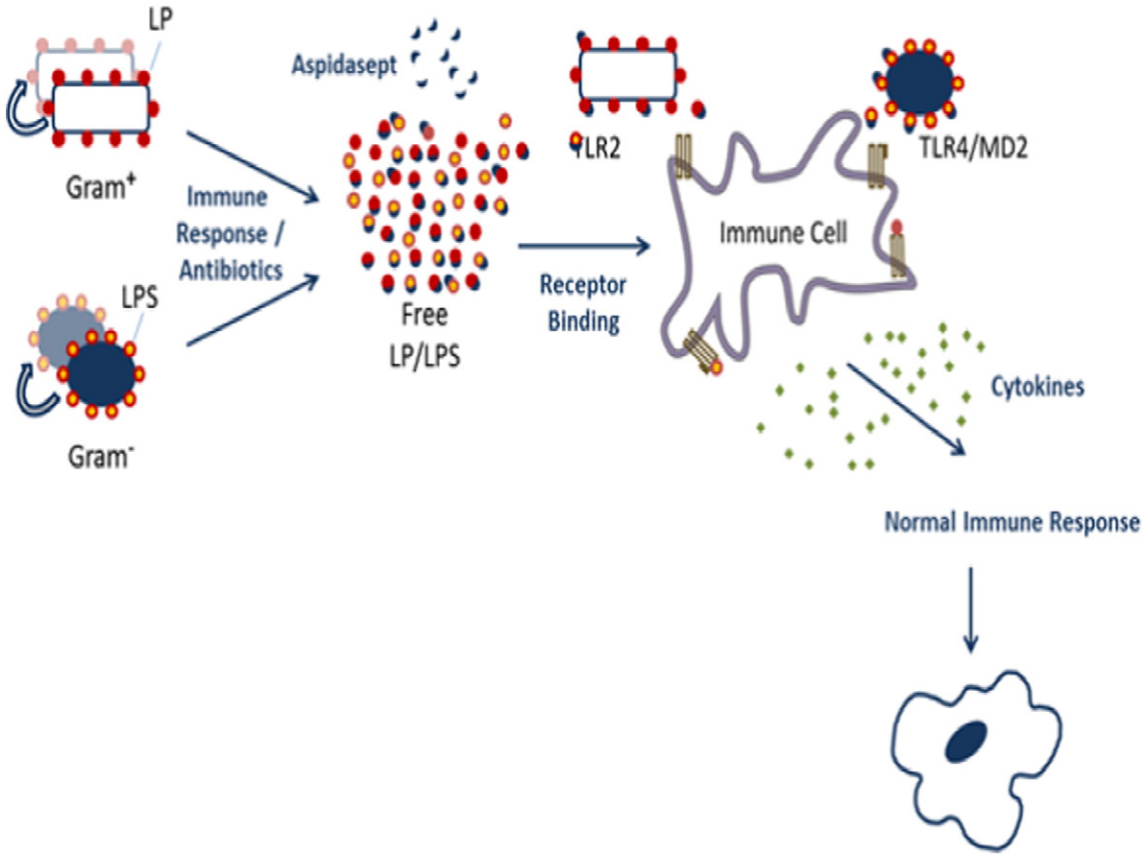

Fig. 5. Schematics of the inactivation of bacterial toxins LPS and lipoprotein (LP) by Aspidasept®. The peptide converts the LPS aggregate into an inactive multilamellar form, as described in the text [8,33,34,36], but in the same way also LP aggregates [8]. In this form, the toxins cannot interact with serum and cell receptors such as LBP, CD14, and the TLR4/MD-2 system.

deviating interpretation as resulting from the use of wild-type LPS rather than defined rough mutant LPS. This fact cannot be excluded, since S-form LPS is a heterogeneous mixture of various fractions as outlined above. Beyond this, however, the more direct techniques (for example, freeze fracture EM instead of transmission EM, saving the morphology of the complexes, SAXS) used in the studies cited above should be more adequate to solve this problem. Regarding cell signaling, it is known that this event starts by direct interaction of LPS with membrane- and serum proteins such as LBP, CD14 and the TLR4/MD2 system, probably after intercalation into the membrane of the immune cells [56,57]. The interaction of these proteins with epitopes within the LPS backbone may be of course more probable in small aggregates than in the large multilamellar aggregate, in which the epitopes are more or less hidden. In a recent paper, LPS aggregates from smooth form and various rough mutant LPSs were investigated in the absence and presence of the AMPs LL-37 and bLF (bovine lactoferricin) using small-angle neutron scattering (SANS) and cryo-TEM [58]. A lot of different LPS morphologies were observed: (i) elongated branched micelles of S-form LPS O111:B4 transformed to thinner, shorter, and less branched structures in the presence of the peptides, (ii) sheet-like structures of rough mutant LPS D21 changed upon peptide interaction into toroids, and (iii) undulated, irregular lamellae of rough mutant LPS E7 in the presence of Mg^{2+} converted into elongated particles and lamellae on peptide addition. Since these data strongly varied for the different LPS structures and as no biological data were presented, no general conclusions from the aggregation behavior in the absence and presence of antimicrobial peptides can be derived from these findings.

As is evident from several investigations, the antimicrobial action of AMPs does not necessarily correspond to their anti-endotoxin action. In particular, we have found a SALP Pep19-4LF with slight changes in the AA sequence which is a factor of 8 better in antimicrobial activity against Gram-negatives such as *E. coli*, but also Gram-positives such as *S. aureus*, but is worse in inhibiting the LPS-induced cytokine response in human MNCs (unpublished data). One reason for the discrepancy between the two systems is the different geometry of LPS in bacteria, in which it is present as planar outer leaflet of the outer membrane, and in isolated form in which it forms non-lamellar curved bilayer aggregates probably cubic geometry. Furthermore, in the bacterial outer membrane there are a lot of OMPs (outer membrane proteins), many of which are known to directly interact with LPS such as the phosphoporin PhoE [59] and the outer membrane protease OmpT [60]. This more complex organization of the outer membrane does not allow to establish a simple model for AMP–bacteria interaction. Regarding the role of the secondary structure of the peptides on the efficiency of antibacterial and/or anti-LPS action, there are no unequivocal correlations. Experimental data seem to favor the neutralization of LPS, when AMPs assume a β-folded structure, but a general statement is presently not possible. It is even unclear which length of the polypeptide is necessary for β-sheets or α-helical structures to be expressed similar as in proteins. Short peptides are usually devoid of a defined secondary structure, but can adopt defined conformation and unique folds in membrane environment or upon binding to LPS as demonstrated for LF11 and its acylated form [61].

6. Conclusions and critical assessment of the AMP–LPS interaction data

For a comparison and a general assessment of the presented data, these must be critically reviewed with respect to the used AMP and LPS, the applied physical and biological techniques, and the cellular and microbiological systems. One essential point is the use of the inflammation system, i.e. the cytokine-secreting cells. Frequently used systems are cell lines such as RAW (from mouse macrophages) and HEK (human embryonic kidney) cells due to simplicity of handling. Cell lines, however, may be a problem, since they may be hypo- as well as hyper-reactive as compared to primary cells, and may change their responses over longer times of cultivation. These cells are of course comfortable and allow to study via transfection the receptor dependence of stimulation such as TLR2 and TLR4 [62]. Quantitative statements, however, should be taken with caution. The use of primary cells such as mononuclear cells or macrophages can be assumed to give more reliable results. A further problem is the use of murine cells. This relates to the mentioned RAW cell lines as well as primary murine monocytes/macrophages, because it is well-known that mice are much less sensitive to the action of LPS, affording the galactosamine treatment for sensitization [63].

As a measure of recognition of LPS, with which frequently the efficiency of its neutralization is monitored, the *Limulus* amebocyte lysate test may be used. It must be noted, however, that the recognition structures of the LAL test are the 4′-phosphorylated diglucosamine backbone of the LPS, independently of the presence of the 1-phosphate and the pattern of acyl chains [18]. In the cytokine test the situation is different; for a complete inflammation in the human system a hexa-acylated lipid A part with both phosphate groups must be present [18]. From these considerations, the use of human primary immune-relevant cells is urgently proposed.

The purity of AMPs as well as of LPS must also be considered. For the former, frequently no clear analytic data are given in most reports, but peptidic contaminations in the synthesis process may lead to non-assessable problems. LPS is in most cases from commercial origin. These samples are frequently contaminated with other molecules such as lipoproteins, which give rise to TLR-2 activity and which is absent for pure LPS. Furthermore, as outlined above, the use of wild-type LPS such as *E. coli* O111:B4 and O55:B5 is not helpful for getting quantitative structure–activity-relationships. This is due to the fact that the heterogeneous wild-type LPS consists of various fractions [37]. Within these fractions, the bioactive part is a Ra- or Rb-LPS. Therefore, it is strongly recommended to use purified rough mutant LPS. Furthermore, in the mouse model of endotoxemia many groups use LPS from *P. aeruginosa* as sepsis-inducing endotoxin. The reason for this must be a historical one, because the lipid A moiety of LPS from *P. aeruginosa* is essentially penta-acylated [64] and with that, at least one order of magnitude less active than the LPS from enterobacterial strains such as *Salmonella* and *Escherichia*. A direct comparison of the mouse models showed, that septic shock was elicited by 25 ng LPS from *Salmonella*, and 150 ng LPS from *Pseudomonas* [33].

A severe problem may be the applied binding assays. As described above, frequently the competitive binding of AMP and LPS to RAW cells is taken as binding assay, or methods such as surface plasmon resonance, in which one of the reaction partners must be prepared as a multilayer. Here, usually LPS is immobilized on a surface and the binding partner such as AMPs flows over this surface and binds to the planar LPS multilayer. This is frequently performed in a similar way with fluorescence labeling. Binding constants from such experiments, however, are not exact. Therefore, the only precise method allowing a reliable determination of K_A is ITC.

The analyses of LPS and LPS–peptide, or bacteria–LPS morphologies are further critical points. Normal light microscopy or transmission electron microscopy is not adequate, AFM gives at least a qualitative impression of the interaction process. For a quantitative analysis, techniques such as cryo- or freeze-fracture EM must be applied, in which the preparation method of the LPS aggregates and complexes with the peptide guarantees the maintenance of the aggregates, when brought into the vacuum. For the determination of the kind of aggregate structure of LPS or the complex with the AMPs only highly sophisticated techniques such as SAXS and SANS are suitable, for which a synchrotron or a high-flux neutron source must be available. Although the data above show that the determination of the supramolecular aggregate structures is necessary for an understanding of the binding processes, due to restricted availability of such sources most groups do not have the opportunity to perform such experiments.

Conflict of interest

None of the authors have a conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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References

[1] K. Brandenburg, T. Schürholz, Lack of new antiinfective agents: passing into the pre-antibiotic age? World J. Biol. Chem. 6 (2015) 71–77.
[2] R.E. Hancock, Collateral damage, Nat. Biotechnol. 32 (2014) 66–68.
[3] A. Izadpanah, R.L. Gallo, Antimicrobial peptides, J. Am. Acad. Dermatol. 52 (2005) 381–390 391–382.
[4] D.A. Devine, Antimicrobial peptides in defence of the oral and respiratory tracts, Mol. Immunol. 40 (2003) 431–443.
[5] B. Schittek, M. Paulmann, I. Senyurek, H. Steffen, The role of antimicrobial peptides in human skin and in skin infectious diseases, Infect. Disord. Drug Targets 8 (2008) 135–143.
[6] M.E. Evans, M. Pollack, Effect of antibiotic class and concentration on the release of lipopolysaccharide from *Escherichia coli*, J. Infect. Dis. 167 (1993) 1336–1343.
[7] E.T. Rietschel, H. Brade, O. Holst, L. Brade, S. Möller-Loennies, U. Mamat, U. Zähringer, F. Beckmann, U. Seydel, K. Brandenburg, A.J. Ulmer, T. Mattern, H. Heine, J. Schletter, S. Hauschildt, H. Loppnow, U. Schönbeck, H.D. Flad, U.F. Schade, F. Di Padova, S. Kusumoto, R.R. Schumann, Bacterial endotoxin: chemical constitution, biological recognition, host response, and immunological detoxification, Curr. Top. Microbiol. Immunol. 216 (1996) 39–81.
[8] G. Martinez de Tejada, L. Heinbockel, R. Ferrer-Espada, H. Heine, C. Alexander, S. Barcena-Varela, T. Goldmann, W. Correa, K.H. Wiesmüller, N. Gisch, S. Sanchez-Gomez, S. Fukuoka, T. Schürholz, T. Gutsmann, K. Brandenburg, Lipoproteins/peptides are sepsis-inducing toxins from bacteria that can be neutralized by synthetic anti-endotoxin peptides, Sci. Report. 5 (2015) 14292.
[9] M. Trautmann, R. Zick, T. Rukavina, A.S. Cross, R. Marre, Antibiotic-induced release of endotoxin: in-vitro comparison of meropenem and other antibiotics, J. Antimicrob. Chemother. 41 (1998) 163–169.
[10] A.L. Hilchie, K. Wuerth, R.E. Hancock, Immune modulation by multifaceted cationic host defense (antimicrobial) peptides, Nat. Chem. Biol. 9 (2013) 761–768.
[11] M.G. Vallespi, J.C. Alvarez-Obregon, I. Rodriguez-Alonso, T. Montero, H. Garay, O. Reyes, M.J. Arana, A *Limulus* anti-LPS factor-derived peptide modulates cytokine gene expression and promotes resolution of bacterial acute infection in mice, Int. Immunopharmacol. 3 (2003) 247–256.
[12] W.C. Wimley, K. Hristova, Antimicrobial peptides: successes, challenges and unanswered questions, J. Membr. Biol. 239 (2011) 27–34.
[13] P. Pristovsek, J. Kidric, Peptides neutralizing lipopolysaccharide — structure and function, Mini-Rev. Med. Chem. 1 (2001) 409–416.
[14] L. Martin, A. van Meegern, S. Doemming, T. Schuerholz, Antimicrobial peptides in human sepsis, Front. Immunol. 6 (2015) 404.
[15] K.A. Brogden, Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. 3 (2005) 238–250.
[16] L.H. Vorland, Lactoferrin: a multifunctional glycoprotein, APMIS 107 (1999) 971–981.
[17] K. Brandenburg, G. Jürgens, M. Müller, S. Fukuoka, M.H.J. Koch, Biophysical characterization of lipopolysaccharide and lipid A inactivation by lactoferrin, Biol. Chem. 382 (2001) 1215–1225.
[18] T. Gutsmann, J. Howe, U. Zähringer, P. Garidel, A.B. Schromm, M.H. Koch, Y. Fujimoto, K. Fukase, I. Moriyon, G. Martinez-de-Tejada, K. Brandenburg, Structural prerequisites for endotoxic activity in the *Limulus* test as compared to cytokine production in mononuclear cells, Innate Immun. 16 (2010) 39–47.
[19] K. Brandenburg, M.H.J. Koch, U. Seydel, Phase diagram of lipid A from *Salmonella minnesota* and *Escherichia coli* rough mutant lipopolysaccharide, J. Struct. Biol. 105 (1990) 11–21.
[20] K. Brandenburg, A. David, J. Howe, M.H. Koch, J. Andrä, P. Garidel, Temperature dependence of the binding of endotoxins to the polycationic peptides polymyxin B and its nonapeptide, Biophys. J. 88 (2005) 1845–1858.
[21] V.M. Bhor, C.J. Thomas, N. Surolia, A. Surolia, Polymyxin B: an ode to an old antidote for endotoxic shock, Mol. Bio Syst. 1 (2005) 213–222.
[22] S. Farnaud, C. Spiller, L.C. Moriarty, A. Patel, V. Gant, E.W. Odell, R.W. Evans, Interactions of lactoferricin-derived peptides with LPS and antimicrobial activity, FEMS Microbiol. Lett. 233 (2004) 193–199.
[23] B.E. Haug, W. Stensen, M. Kalaaji, O. Rekdal, J.S. Svendsen, Synthetic antimicrobial peptidomimetics with therapeutic potential, J. Med. Chem. 51 (2008) 4306–4314.
[24] J. Andrä, K. Lohner, S.E. Blondelle, R. Jerala, I. Moriyon, M.H. Koch, P. Garidel, K. Brandenburg, Enhancement of endotoxin neutralization by coupling of a C12-alkyl chain to a lactoferricin-derived peptide, Biochem. J. 385 (2005) 135–143.
[25] K. Brandenburg, S. Sánchez-Gómez, P. Garidel, M. Roessle, J. Andrä, R. Jerala, D. Zweytick, K. Lohner, M. Rappolt, S. Blondelle, I. Moriyon, G. Martinez de Tejada, Effective antimicrobial and anti-endotoxin activity of cationic peptides based on lactoferricin: a biophysical and microbiological study, Anti-Infect. Agents Med. Chem. 9 (2010) 9–22.
[26] D. Zweytick, G. Deutsch, J. Andrä, S.E. Blondelle, E. Vollmer, R. Jerala, K. Lohner, Studies on lactoferricin-derived *Escherichia coli* membrane-active peptides reveal differences in the mechanism of N-acylated versus nonacylated peptides, J. Biol. Chem. 286 (2011) 21266–21276.
[27] S. Dankesreiter, A. Hoess, J. Schneider-Mergener, H. Wagner, T. Mietke, Synthetic endotoxin-binding peptides block endotoxin-triggered TNF-α production by macrophages in vitro and in vivo and prevent endotoxin-mediated toxic shock, J. Immunol. 164 (2000) 4804–4811.
[28] C.A. Weiss III, K.R. Wasiluk, T.A. Kellogg, D.L. Dunn, Bactericidal and endotoxin neutralizing activity of a peptide derived from *Limulus* antilipopolysaccharide factor, Surgery 128 (2000) 339–344.
[29] D.B. Leslie, P.S. Vietzen, V. Lazaron, K.R. Wasiluk, D.L. Dunn, Comparison of endotoxin antagonism of linear and cyclized peptides derived from *Limulus* anti-lipopolysaccharide factor, Surg. Infect. 7 (2006) 45–52.
[30] J.D. Ren, J.S. Gu, H.F. Gao, P.Y. Xia, G.X. Xiao, A synthetic cyclic peptide derived from *Limulus* anti-lipopolysaccharide factor neutralizes endotoxin in vitro and in vivo, Int. Immunopharmacol. 8 (2008) 775–781.
[31] J. Andrä, M. Lamata, G. Martinez de Tejada, R. Bartels, M.H. Koch, K. Brandenburg, Cyclic antimicrobial peptides based on *Limulus* anti-lipopolysaccharide factor for neutralization of lipopolysaccharide, Biochem. Pharmacol. 68 (2004) 1297–1307.
[32] J. Andrä, J. Howe, P. Garidel, M. Rossle, W. Richter, J. Leiva-Leon, I. Moriyon, R. Bartels, T. Gutsmann, K. Brandenburg, Mechanism of interaction of optimized *Limulus*-derived cyclic peptides with endotoxins: thermodynamic, biophysical and microbiological analysis, Biochem. J. 406 (2007) 297–307.
[33] T. Gutsmann, I. Razquin-Olazaran, I. Kowalski, Y. Kaconis, J. Howe, R. Bartels, M. Hornef, T. Schürholz, M. Rössle, S. Sanchez-Gomez, I. Moriyon, G. Martinez de Tejada, K. Brandenburg, New antiseptic peptides to protect against endotoxin-mediated shock, Antimicrob. Agents Chemother. 54 (2010) 3817–3824.
[34] L. Heinbockel, S. Sanchez-Gomez, G. Martinez de Tejada, S. Dömming, J. Brandenburg, Y. Kaconis, M. Hornef, A. Dupont, S. Marwitz, T. Goldmann, M. Ernst, T. Gutsmann, T. Schürholz, K. Brandenburg, Preclinical investigations reveal the broad-spectrum neutralizing activity of peptide Pep19-2.5 on bacterial pathogenicity factors, Antimicrob. Agents Chemother. 57 (2013) 1480–1487.
[35] E.T. Rietschel, T. Kirikae, F.U. Schade, U. Mamat, G. Schmidt, H. Loppnow, A.J. Ulmer, U. Zähringer, U. Seydel, F. Di Padova, M. Schreier, H. Brade, Bacterial endotoxin: molecular relationships of structure to activity and function, FASEB J. 8 (1994) 217–225.
[36] Y. Kaconis, I. Kowalski, J. Howe, A. Brauser, W. Richter, I. Razquin-Olazaran, M. Inigo-Pestana, P. Garidel, M. Rössle, G. Martinez de Tejada, T. Gutsmann, K. Brandenburg, Biophysical mechanisms of endotoxin neutralization by cationic amphiphilic peptides, Biophys. J. 100 (2011) 2652–2661.
[37] B. Jiao, M. Freudenberg, C. Galanos, Characterization of the lipid A component of genuine smooth-form lipopolysaccharide, Eur. J. Biochem. 180 (1989) 515–518.
[38] T. Schuerholz, K. Brandenburg, G. Marx, Antimicrobial peptides and their potential application in inflammation and sepsis, Crit. Care 16 (2012) 207.
[39] T. Schuerholz, S. Doemming, M. Hornef, L. Martin, T.P. Simon, L. Heinbockel, K. Brandenburg, G. Marx, The anti-inflammatory effect of the synthetic antimicrobial peptide 19–2.5 in a murine sepsis model: a prospective randomized study, Crit. Care 17 (2013) R3.
[40] P. Garidel, K. Brandenburg, Current understanding of polymyxin B applications in bacteraemia/sepsis therapy prevention: clinical, pharmaceutical, structural and mechanistic aspects, Anti-Infect. Agents Med. Chem. 8 (2009) 367–385.
[41] S.M. Opal, J. Cohen, Clinical Gram-positive sepsis: does it fundamentally differ from Gram-negative bacterial sepsis? Crit. Care Med. 27 (1999) 1608–1616.
[42] Z. Wang, E. Choice, A. Kaspar, D. Hanson, S. Okada, S.C. Lyu, A.M. Krensky, C. Clayberger, Bactericidal and tumoricidal activities of synthetic peptides derived from granulysin, J. Immunol. 165 (2000) 1486–1490.
[43] X. Chen, J. Howe, J. Andrä, M. Rössle, W. Richter, A.P. da Silva, A.M. Krensky, C. Clayberger, K. Brandenburg, Biophysical analysis of the interaction of granulysin-derived peptides with enterobacterial endotoxins, Biochim. Biophys. Acta 1768 (2007) 2421–2431.
[44] J. Andrä, M.H.J. Koch, R. Bartels, K. Brandenburg, Biophysical characterization of endotoxin inactivation by NK-2, an antimicrobial peptide derived from mammalian NK-lysin, Antimicrob. Agents Chemother. 48 (2004) 1593–1599.
[45] M.U. Hammer, A. Brauser, C. Olak, G. Brezesinski, T. Goldmann, T. Gutsmann, J. Andrä, Lipopolysaccharide interaction is decisive for the activity of the antimicrobial peptide NK-2 against *Escherichia coli* and *Proteus mirabilis*, Biochem. J. 427 (2010) 477–488.

[46] W. Correa, M. Manrique-Moreno, E. Patino, C. Pelaez-Jaramillo, Y. Kaconis, T. Gutsmann, P. Garidel, L. Heinbockel, K. Brandenburg, *Galleria mellonella* native and analogue peptides Gm1 and DeltaGm1. I) Biophysical characterization of the interaction mechanisms with bacterial model membranes, Biochim. Biophys. Acta 1838 (2014) 2728–2738.
[47] W. Correa, M. Manrique-Moreno, J. Behrends, E. Patino, C. Marella, C. Pelaez-Jaramillo, P. Garidel, T. Gutsmann, K. Brandenburg, L. Heinbockel, *Galleria mellonella* native and analogue peptides Gm1 and DeltaGm1. II) Anti-bacterial and anti-endotoxic effects, Biochim. Biophys. Acta 1838 (2014) 2739–2744.
[48] Q.P. Lin, L.F. Zhou, N.N. Li, Y.Q. Chen, B.C. Li, Y.F. Cai, S.Q. Zhang, Lipopolysaccharide neutralization by the antibacterial peptide CM4, Eur. J. Pharmacol. 596 (2008) 160–165.
[49] M.J. Nell, G.S. Tiabringa, A.R. Wafelman, R. Verrijk, P.S. Hiemstra, J.W. Drijfhout, J.J. Grote, Development of novel LL-37 derived antimicrobial peptides with LPS and LTA neutralizing and antimicrobial activities for therapeutic application, Peptides 27 (2006) 649–660.
[50] T. Sigurdardottir, P. Andersson, M. Davoudi, M. Malmsten, A. Schmidtchen, M. Bodelsson, In silico identification and biological evaluation of antimicrobial peptides based on human cathelicidin LL-37, Antimicrob. Agents Chemother. 50 (2006) 2983–2989.
[51] K.H. Park, Y.H. Nan, Y. Park, J.I. Kim, I.S. Park, K.S. Hahm, S.Y. Shin, Cell specificity, anti-inflammatory activity, and plausible bactericidal mechanism of designed Trp-rich model antimicrobial peptides, Biochim. Biophys. Acta 1788 (2009) 1193–1203.
[52] Y.R. Bommineni, H. Dai, Y.X. Gong, J.L. Soulages, S.C. Fernando, U. Desilva, O. Prakash, G. Zhang, Fowlicidin-3 is an alpha-helical cationic host defense peptide with potent antibacterial and lipopolysaccharide-neutralizing activities, FEBS J. 274 (2007) 418–428.
[53] I. Nagaoka, S. Hirota, F. Niyonsaba, M. Hirata, Y. Adachi, H. Tamura, S. Tanaka, D. Heumann, Augmentation of the lipopolysaccharide-neutralizing activities of human cathelicidin CAP18/LL-37-derived antimicrobial peptides by replacement with hydrophobic and cationic amino acid residues, Clin. Diagn. Lab. Immunol. 9 (2002) 972–982.
[54] L. Heinbockel, L. Palacios-Chaves, C. Alexander, E. Rietschel, J. Behrends, W. Correa, S. Fukuoka, T. Gutsmann, A.J. Ulmer, K. Brandenburg, Mechanism of Hbgamma-35-induced an increase in the activation of the human immune system by endotoxins, Innate Immun. 21 (2015) 305–313.
[55] Y. Rosenfeld, H.G. Sahl, Y. Shai, Parameters involved in antimicrobial and endotoxin detoxification activities of antimicrobial peptides, Biochemistry 47 (2008) 6468–6478.
[56] R.J. Ulevitch, P.S. Tobias, Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin, Annu. Rev. Immunol. 13 (1995) 437–457.
[57] W. Richter, L. Heinbockel, Y. Kaconis, F. Steiniger, T. Gutsmann, L. Brade, K. Brandenburg, Cellular distribution of lipid A and LPS R595 after in vitro application to isolated human monocytes by freeze-fracture replica immunogold-labelling, Innate Immun. 19 (2013) 588–595.
[58] G. Bello, J. Eriksson, A. Terry, K. Edwards, M.J. Lawrence, D. Barlow, R.D. Harvey, Characterization of the aggregates formed by various bacterial lipopolysaccharides in solution and upon interaction with antimicrobial peptides, Langmuir 31 (2015) 741–751.
[59] J. Andrä, H. de Cock, P. Garidel, J. Howe, K. Brandenburg, Investigation into the interaction of the phosphoporin PhoE with outer membrane lipids: physicochemical characterization and biological activity, Med. Chem. 1 (2005) 537–546.
[60] R.A. Kramer, K. Brandenburg, L. Vandeputte-Rutten, M. Werkhoven, P. Gros, N. Dekker, M.R. Egmond, Lipopolysaccharideride regions involved in the activation of *Escherichia coli* outer membrane protease OmpT, Eur. J. Biochem. 269 (2002) 1746–1752.
[61] B. Japelj, M. Zorko, A. Majerle, P. Pristovsek, S. Sanchez-Gomez, G. Martinez de Tejada, I. Moriyon, S.E. Blondelle, K. Brandenburg, J. Andrä, K. Lohner, R. Jerala, The acyl group as the central element of the structural organization of antimicrobial lipopeptide, J. Am. Chem. Soc. 129 (2007) 1022–1023.
[62] B. Beutler, A. Poltorak, Sepsis and evolution of the innate immune response, Crit. Care Med. 29 (2–6) (2001) S6–S7 (discussion).
[63] C. Galanos, M.A. Freudenberg, W. Reutter, Galactosamine-induced sensitization to the lethal effects of endotoxin, Proc. Natl. Acad. Sci. U. S. A. 76 (1979) 5939–5943.
[64] G. Bedoux, K. Vallee-Rehel, O. Kooistra, U. Zähringer, D. Haras, Lipid A components from *Pseudomonas aeruginosa* PAO1 (serotype O5) and mutant strains investigated by electrospray ionization ion-trap mass spectrometry, J. Mass Spectrom. 39 (2004) 505–513.