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# Gram-positive bacterial cell envelopes: The impact on the activity of antimicrobial peptides☆

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ABSTRACT

A number of cationic antimicrobial peptides, effectors of innate immunity, are supposed to act at the cytoplasmic membrane leading to permeabilization and eventually membrane disruption. Thereby, interaction of antimicrobial peptides with anionic membrane phospholipids is considered to be a key factor in killing of bacteria. Recently, evidence was provided that killing takes place only when bacterial cell membranes are completely saturated with peptides. This adds to an ongoing debate, which role cell wall components such as peptidoglycan, lipoteichoic acid and lipopolysaccharide may play in the killing event, i.e. if they rather entrap or facilitate antimicrobial peptides access to the cytoplasmic membrane. Therefore, in this review we focused on the impact of Gram-positive cell wall components for the mode of action and activity of antimicrobial peptides as well as in innate immunity. This led us to conclude that interaction of antimicrobial peptides with peptidoglycan may not contribute to a reduction of their antimicrobial activity, whereas interaction with anionic lipoteichoic acids may reduce the local concentration of antimicrobial peptides on the cytoplasmic membrane necessary for sufficient destabilization of the membranes and bacterial killing. Further affinity studies of antimicrobial peptides toward the different cell wall as well as membrane components will be needed to address this problem on a quantitative level. This article is part of a Special Issue entitled: Antimicrobial peptides edited by Karl Lohner and Kai Hilpert.

## 1. Introduction

Antimicrobial peptides (AMPs) are part of humoral immunity of the innate immune response that is an old evolutionary defense strategy of organisms to defend against attack by other organisms/pathogens. They act as antibiotics or fungicides to potentially kill bacteria and fungi, but some of them are also active against viruses and cancer cells. Their mechanism of action mostly relates to targeting the microbial cytoplasmic membrane, interacting with the lipid matrix and subsequent permeabilization of the membrane [1–3]. Some peptides traverse the membrane and bind to intracellular targets [4,5] or exhibit, besides their antimicrobial activity, multifaceted immunomodulatory activities [6]. The mechanisms of membrane-active peptides [1,3,7] and the main characteristics of AMPs for high binding and selectivity toward microbial membranes [8] have been extensively reviewed.

It was suggested that the amino acid composition determining the physicochemical properties of the peptide in respect to charge, amphipathicity, hydrophobicity, flexibility and H-bonding capacity are key factors for their mode of action and selectivity toward microbial cells [9]. Upon contact with microbial membranes AMPs often undergo structural changes adopting defined secondary structures or oligomerize into aggregates that also account considerably for the diversity of antimicrobial mode of action [8]. Amphipathicity resulting from segregation of apolar and polar residues upon secondary structure formation favors internalization of the peptide and in turn membrane perturbation. Thereby, the presence of hydrophobic amino acids leads to stronger partitioning into membranes. Nevertheless, there is consensus that the positive charge of the peptide is essential for initial binding to the negatively charged bacterial membrane surface, which allows discrimination between bacterial and host cell membrane, and its hydrophobicity is needed for insertion into and perturbation of the membrane [10,11].

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*Abbreviations:* AMP, antimicrobial peptide; BSA, bovine serum albumin; CD, circular dichroism; CEME, a hybrid of silk moth cecropin and bee melittin; CL, cardiolipin; DGDG, diglycosyl-1,2-diacylglycerol; DPPG, 1,2-dipamitoyl-*sn*-glycero-3-phospho-rac-glycerol; DSC, differential scanning calorimetry; ERG, ergosterol; IL-6, interleukin 6; LPS, lipopolysaccharide; LTA, lipoteichoic acid; LUV, large unilamellar vesicle; LPC, lysylphosphatidylcholine; LPG, lysylphosphatidylglycerol; LPE, lysylphosphatidylethanolamine; MLVs, multilamellar vesicles; NAM, N-acetyl glucosamine; NAG, N-acetyl muramic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PGN, peptidoglycan; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-rac-glycerol; PS, phosphatidylserine; PGRPs, peptidoglycan recognition proteins; PI, phosphatidylinositol; TNF, tumor necrosis factor; TLR, toll like receptor; WTA, wall teichoic acid.

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However, the mode of action of AMPs is also strongly related to cellular envelope constituents that are different and variable through diverse microbial families (Fig. 1, Table 1). In contrast to higher living organism and mycoplasma, microbial plasma membranes are surrounded by a cell wall of a tight and flexible layer composed of polysaccharides, peptidoglycan (PGN) in bacteria and glucosamine polymer chitin and ß-glucan in fungi. The cell wall of Gram-positive and the outer membrane in Gram-negative bacteria contain anionic lipid molecules, lipoteichoic acid (LTA) and lipopolysaccharide (LPS) that may compete with the plasma membrane for the interaction with AMPs. Not only the cell walls, but also the plasma membrane, which matrix is formed by a phospholipid bilayer differing in headgroup and fatty acid composition contributes to mechanistic diversity of AMPs against microbial cells. Whereas bacterial plasma membranes are negatively charged due to the presence of anionic phospholipids, fungal membranes are more similar to neutral and rigid eukaryotic membranes because of their zwitterionic phospholipid constituents and ergosterol. The strong affinity to microbial membranes is also due to the transmembrane potential determined by the differences in inner and outer leaflet composition of microbial membranes and different charge density of phospholipids that promotes peptides insertion [8,12].

Although electrostatic interaction of AMPs with plasma membrane phospholipids, insertion and in turn membrane disruption is widely accepted for explaining the bacterial killing mechanism by a number of antimicrobial peptides, the pertinent question arising is to which extent antimicrobial peptides interact with microbial cell wall components that may affect the extent of their activity and functionality. Freire et al. [13] concluded that in the end the role of bacterial cell wall components as electrostatic barriers capturing AMPs and hence preventing their interaction with the cytoplasmic membrane is a matter of concentrations of AMPs and membrane components as well as of affinities of AMPs toward the different membrane components. In this context, Roversi et al. [14] showed an extremely high coverage of both leaflets of the outer and inner *Escherichia coli* membranes by PMAP-23, a cationic amphipathic helix from the cathelicidin family. Bacterial killing started at a molar ratio of bound peptide per lipid of about 1:30 and all bacteria were killed at a molar ratio of 1:4, corresponding closely to the numbers estimated by Castanho and co-workers [8] for other peptides, based on the partition constants derived from binding studies on model membranes. Therefore in this review, we will discuss the role of bacterial cell wall components interfering with antimicrobial activity either as molecules that may entrap AMPs to prevent their interaction with the inner lipid bilayer or in case of aggregation of AMPs to facilitate membrane interaction by accumulating AMPs on the surface and act via a "sponge like effect" to attract them onto the membrane interface.

## 2. Bacterial envelopes

Beyond the classification of bacteria according to Gram staining of PGN, Gram-positive bacteria distinguish in many features from Gram-negative bacteria [15,16] (Fig. 1, Table 1). Characteristic for both classes is that their cytoplasmic membrane is surrounded by a cell wall. Between those two compartments is the periplasmatic space or periplasm containing a wide variety of ions and proteins that are needed for numerous functions involving cellular (electron) transport, substrate hydrolysis, degradation and detoxification. In Gram-negative bacteria the periplasm occupies the space between the plasma membrane and the outer membrane. The presence of the outer membrane in Gram-negative bacteria adjacent to the periplasmatic space is the major difference between those bacterial classes as it does not exist in Gram-positive bacteria. This outer membrane is a lipid bilayer, where the inner leaflet is composed of phospholipids and the outer leaflet of lipopolysaccharides (LPS) [17–19]. In both lineages, the cell wall contains PGN layers that stabilize the cell membranes. The cell wall of Gram-positive bacteria is made of many PGN layers of about 40–80 nm that is drastically thicker than the single layered 7–8 nm thick cell wall of Gram-negative bacteria

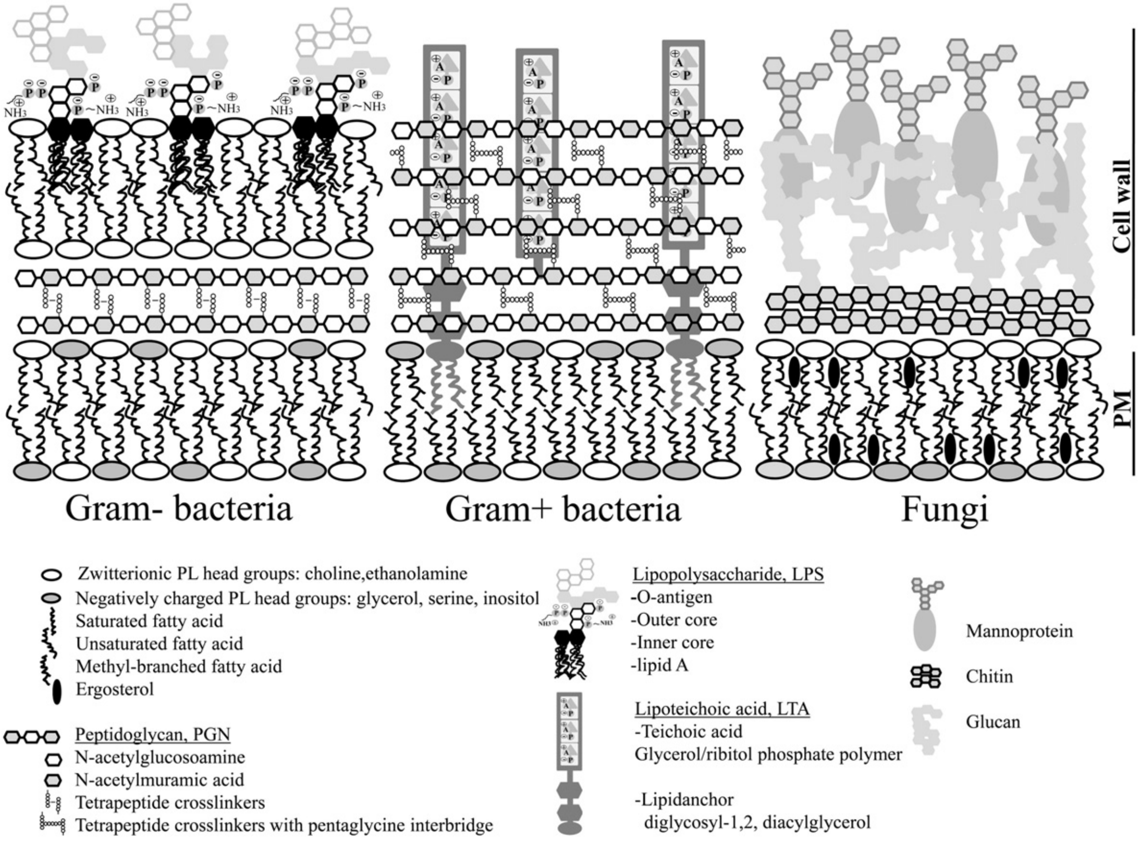

**Fig. 1.** Cell envelopes of various microbial families.

**Table 1**
Major lipid species identified in plasma membranes of selected organisms.
Abbreviations used: CL, cardiolipin; PG, phosphatidylglycerol; ERG, ergosterol; LPC, Lysylphosphatidylcholine; LPG, lysylphosphatidylglycerol; LPE, lysylphosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositole; SP, sphingolipid.

| | | Gram − bacteria | | Gram + bacteria | | Fungi | | | Human | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *E. coli* | *P. aeuriginosa* | *S. aureus* | *B. subtilis* | *C. albicans* | *A. niger*[a] | *S. cerevisae* | *RBC* | *Fibroblasts* |
| Phospholipid (%) | PG | 25 | 21 | 57 | 70 | – | – | – | – | – |
| | LPG | | | 38 | | | | | | |
| | PE | 75 | 60 | – | 12 | 26 | 37 | 19.5 | 27.5 | 31.8 |
| | LPE | | | | | | 31 | | | |
| | CL (DPG) | – | 11 | 5 | 4 | – | – | – | – | – |
| | PS | – | – | – | – | 21 | – | 40.4 | 14.8 | 5.9 |
| | PI | – | – | – | – | 8 | 6 | 9.6 | 0.6 | 2.5 |
| | PC | – | – | – | – | 15.5 | 25 | 15 | 29.2 | 43.7 |
| | LPC | | | | | 5.5 | | | 1.0 | |
| | SP | – | – | – | – | n.a. | n.a. | n.a. | 26.2 | 13.8 |
| | PA | – | – | – | – | 16.7 | n.a. | 2.3 | n.a. | 2.4 |
| Sterol | ERG/PL | – | – | – | – | 0.66 | 1.17 | 0.34–3.31 | 0.8 | 0.16 |
| Fatty acid | – | C16:0<br>C16:1<br>C18:1 | C16:0<br>C16:1<br>C18:1 | a-C15:0<br>C18:0<br>a-C17:0<br>C20:0 | a-C15:0<br>i-C17:0 | C16:0<br>C18:1<br>C18:2 | C16:0<br>C18:1<br>C18:2 | C16:0<br>C18:1<br>C18:2 | C16:0<br>C18:1<br>C18:2<br>C20:4 | C16:0<br>C18:1<br>C18:2<br>C20:4 |
| Reference | – | [92,93] | [94–96] | [97–99] | [100–102] | [103,104] | [105] | [106–108] | [109] | [110] |

n.a. not available.

[a] Total lipid contents of *A. niger*.

[20]. Therefore, the periplasmic space between the inner and outer membrane in Gram-negative bacteria is much larger than the narrow periplasm of Gram-positive bacteria. This gives different staining intensity by the Gram technique and results into classification of bacteria in two major groups, Gram-negative and Gram-positive bacteria. Also specific for Gram-positive bacteria is the occurrence of teichoic acid in the cell wall that can be linked via a glycolipid anchor with the plasma membrane. Cell wall does not exist in mycoplasma, L-form bacteria and some archaebacteria [21]. The plasma membrane is a phospholipid bilayer consisting of an inner and an outer leaflet that varies amongst the species not only in phospholipid composition (Fig. 2) but also in composition of their headgroups and fatty acid moieties. Basically, Gram-positive bacteria have larger fraction of negatively charged phosphatidylglycerol (PG) whereas Gram-negative bacteria contain larger proportions on zwitterionic phosphatidylethanolamine (PE) in addition to PG. Furthermore, the sn-1 position of Gram-negative phospholipids is primarily built up by saturated mostly C16:0 fatty acid, whereas the sn-2 position may vary by C16:1 and C18:1 substitution. In contrast, the Gram-positive bacteria contain branched fatty acids with major distribution of anteiso C15:0 and C17:0 chains (see Table 1). The influence of fatty acid composition will be discussed by A. Pokorny within this special issue. Here, we focus on the impact of the Gram-positive cell wall components, PGN and LTA, on the mode of action of AMPs.

## 3. Peptidoglycan, a cell wall mesh

PGN because of its rigidity determines the strength and cellular shape of bacteria. Without PGN as it was shown in a production of spheroplast in *E. coli* [22] and L-form bacteria from *Bacillus subtilis* [23], cells lose their characteristic shape. PGN as a multi-gigadalton bag-like molecule accounts for around 90% of dry weight in Gram-positive and 10% in Gram-negative bacteria. The molecular weight of single layered *E. coli* PGN sacculus is $3 \times 10^9$ Da, which is in the same range as a chromosome ($2.32 \times 10^9$ Da) of this bacteria [20]. In Gram-positive bacteria PGNs make up to 40–80 layers. PGN is composed of alternating units of disaccharide N-acetyl glucosamine – N-acetyl muramic acid (NAM – NAG) cross-linked by a pentapeptide side chain (stem) [22] (Fig. 3). The pentapeptide has usually the sequence L-alanyl-γ-D-glutamyl-diaminopimelyl (or L-lysyl)-D-alanyl-D-alanine. In Gram-positive bacteria an inter-bridge structure of five amino acid residues that varies between the species (e.g. five glycine molecules in *Staphylococcus aureus*) links two disaccharide-pentapeptide moieties [24]. PGN synthesis starts on the cytosolic side of bacterial cell membrane from the common building block, lipid II (for graphic illustrations see review [25]) that consist of a polyisoprenoid anchor of C55 carbon chain (11 subunit long) attached to one disaccharide–pentapeptide subunit via pyrophosphate linkage. Lipid II monomer is translocated to the periplasmic (exterior) side of the bacterial cell membrane for incorporation into the growing PGN network. Different findings and models for organization of the PGN murein sacculus have been proposed and it has been a matter of debate, if murein glycans and peptides are arranged parallel (layered model) or perpendicular (scaffold model) to the membrane [20,26,27]. Recent NMR studies revealed that the disaccharide backbone of Gram-positive bacteria adopts 4-fold screw helical symmetry with disaccharide unit periodicity of 4 nm, where each PGN stem is oriented 90° in respect to the previous stem [24]. The lattice of cross-linked stems has parallel orientation.

### 3.1. Peptidoglycan, a cell wall “sponge” attracting antimicrobial peptides in Gram-positive bacteria

The role of PGN in respect of interaction with antimicrobial peptides is not well understood. The literature often reports studies performed with proteins, which also exhibit antimicrobial activity and use PGN as a target for pathogen recognition. This was reported for proteins as lectins and natural or semi-synthetic antibiotics like glycopeptides bearing unusual amino acids or modifications [28,29]. They bind to multiple sites in the PGN and in turn interfere with further enzymatic processes resulting in inhibition of PGN synthesis. Examples also include the branched tricyclic glycopeptide vancomycin [30] and lipoglycodepsipeptides like the macrocyclic ramoplanin derived from *Actinoplanes sp.* [29]. Vancomycin was developed in the 1950s and was viewed by many as a gold standard for treatment of methicillin resistant *S. aureus* (MRSA) infections and its analogues [30].

The mode of action on glycolipids can be exemplified by oritavancin, a semisynthetic lipoglycopeptide analogue of vancomycin, which displays a set of sequential mechanisms ranging from inhibition of PGN synthesis, perturbation of the membrane integrity to bactericidal activity against Gram-positive organisms. Oritavancin binds to the alanine–

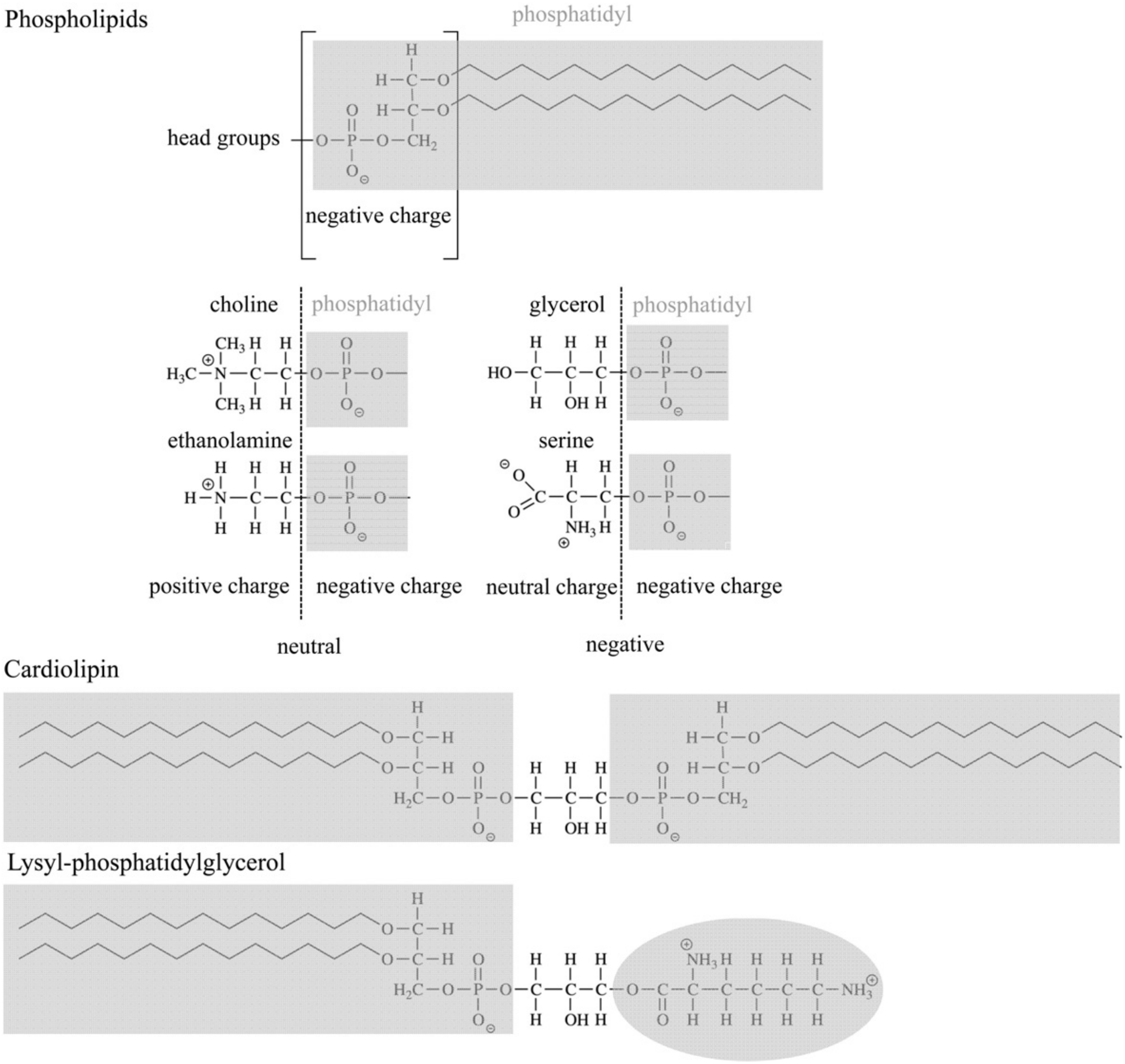

**Fig. 2.** Chemical structures of major phospholipids found in microbial membranes.

alanine stem of the pentapeptide moiety of lipid II and also to the pentaglycyl bridging segment that inhibits PGN synthesis via inhibition of transglycosylation and transpeptidation [31]. In contrast to its analogue vancomycin, the 4′chlorobiphenyl group of oritavancin allows interaction with lipid II and cell membrane anchoring, which results in perturbation of the cell membrane integrity in *S. aureus* and *Enterococcus faecalis* [32]. Using the fluorescence indicator 3,3′-dipropylthiacarbocyanine [32,33], membrane depolarization in *S. aureus* following the exposure to oritavancin was measured showing that oritavancin is able to depolarize the plasma membrane. In a “live and dead” assay, staining of *S. aureus* living cells using two fluorescent dyes, membrane permeable Syto 9 and membrane impermeable propidium iodide, showed that oritavancin treatment resulted in displacement of Syto9 by propidium iodide. This clearly indicates damage of the cell membrane by oritavancin resulting in increased permeability of the cell [33]. Furthermore, oritavancin induced rapid leakage of liposomes composed of lipids extracted from *S. aureus* [34], cardiolipin/POPE and POPG/POPE liposomes [35]. However, it is tempting to speculate if the ability of oritavancin to interact with glycerol backbones of phospholipids and to permeabilize those lipid vesicles at concentration where the oritavancin exert bactericidal activity toward bacteria makes the basis for its bactericidal effects. And, hence, to which extent the binding to PGN/inhibition of PGN contributes to bactericidal activity. Such multiple facets of molecular mechanism are often important to overcome bacterial resistance. Thus, high-level oritavancin resistance has not been reported neither in the laboratory nor in clinical studies [31,36].

One significant example is the antibacterial 34 amino acid long peptidic lantibiotic nisin derived from *Lactococcus lactus*, which primarily inhibits PGN synthesis. In addition, it is established that the membrane bound PGN precursor lipid II acts as a docking moiety to attract the nisin to the bacterial membrane and to promote peptide insertion into membrane leading to permeation [37–39]. This has been reconciled by a number of studies which will be discussed in detail by E. Breukink in this issue. Briefly, nisin binds to the pyrophosphate moiety of lipid II and can adopt a stable transmembrane orientation followed by pore formation. The concentration required for disruption of anionic model membranes is much higher than the effective concentration for bacterial killing. However, anionic liposomes become more susceptible to nisin in the presence of lipid II and pore formation is more stable in membranes containing lipid II [40] supporting the use of lipid II as a “docking moiety” to form pores and to disrupt the bacterial membrane.

In many cases it is not clear, which role the PGN may have regarding the interaction with antimicrobial peptides. Although in the following examples, interaction of AMPs with PGN has been reported, it is not obvious that the high binding affinity to PGN contributes to a determinant key event of bactericidal activity. A case in point is the human cationic polypeptide ECP (eosinophylic cationic protein), which in addition to its weak membrane disruptive capacity showed high binding affinity

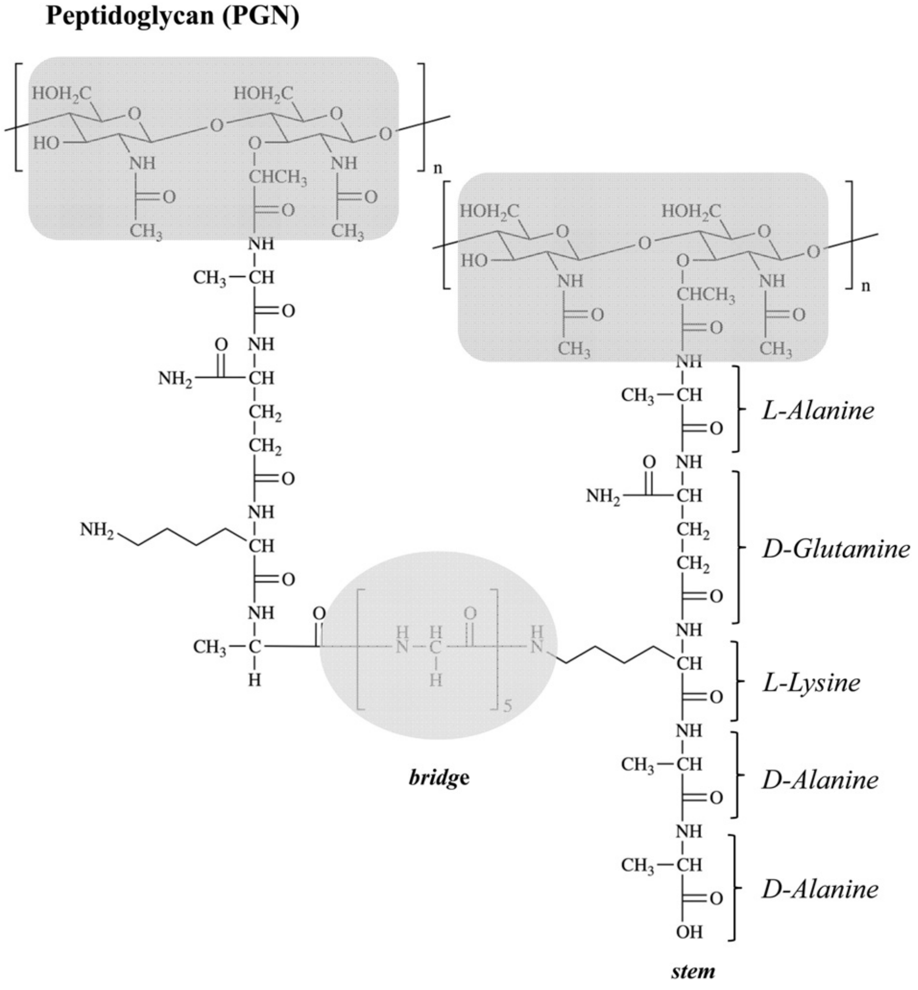

**Fig. 3.** Chemical structure of peptidoglycan.

for PGN [41]. ECP is an antimicrobial RNase participating in the inflammatory processes mediated by eosinophiles. It is ~155 amino acids long containing 19 arginine residues, which results in a high PI value of 11.4 and high cationicity [42]. This further confers high affinity to negatively charged surfaces, which is considered to be important for antimicrobial activity. However, ECP induced only weak leakage of negatively charged lipid vesicles composed of either POPG or POPG/POPC mixture at its bactericidal concentration toward *S. aureus* [43,44]. Moreover, electron micrographs did not show any damage of the cell wall and no detectable lysis processes on *S. aureus* cells in the presence of ECP [41]. Also omiganan, an antimicrobial peptide derivative of the bovine cathelicidin indolicidin, which has been in clinical III phase studies, showed strong partitioning toward a PGN mesh [45]. Although fluorescence quenching studies for peptide internalization showed higher partitioning constants for anionic model membranes, the peptide failed to induce leakage of those lipid vesicles. As omiganan strongly incorporates into anionic bilayers without inducing severe membrane perturbations, it was suggested as reported for indolicidin that the peptide translocates through the membrane and acts on an intracellular target such as DNA [46]. However, it is not clear, if ECP and omiganan actually have intracellular targets.

We investigated for the first time the mode of action of a synthetic antimicrobial peptide, OP-145, developed for a screen of the human cathelicidin LL-37 using model membranes composed of both PGN and phospholipids [47]. In this study we demonstrated that OP-145 can efficiently bind to PGN of *S. aureus* (Fig. 4) [47] as well as to PGN of other species like *Streptococcus epidermis* and *E. coli* (Malanovic, unpublished results). Thermodynamic studies performed with liposomes

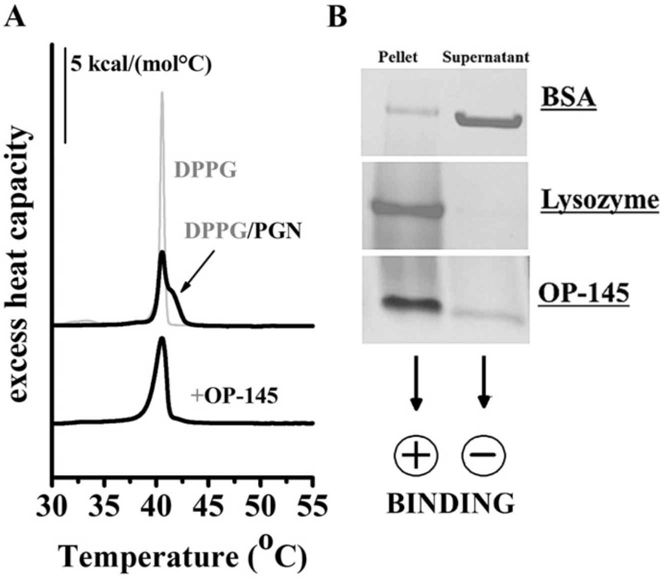

**Fig. 4.** Effect of OP-145 on PGN adopted from Malanovic et al. [48]. A) Thermotropic behavior of DPPG/PGN vesicles in the presence of 2 mol% OP-145 (lower curve). The corresponding DSC thermograms of pure DPPG with and without PGN (upper curves) are shown in black/grey. B) PGN binding of OP-145 as analyzed by gel electrophoresis. Lysozyme was used as a positive control and BSA as a negative control. Lysozyme or OP-145 bound to PGN is detected in the pellet, while unbound protein remains in the supernatant.

composed of DPPG and 0.1 wt% PGN showed that OP-145 interacts preferentially with the PGN enriched bilayer domains indicated by the disappearance of the characteristic shoulder in the thermogram (Fig. 4A). Furthermore, leakage experiments using liposomes composed of POPG in the presence and absence of 0.1 wt% PGN revealed that PGN did not affect membrane permeability of OP-145 [47]. In some other cases e.g. for peptides developed in a screen for OP-145 membrane permeability was even increased supporting the idea that PGN may assist in docking of the peptides on the bacterial surface and promoting them toward the membrane interface (unpublished, Malanovic). Thus, high binding affinity to PGN may serve as a general mechanism of the peptides entrance to the cell plasma membrane.

All these examples show the interplay between antimicrobial peptides, PGN and plasma membrane in Gram-positive bacteria and highlight the multiple facets of AMPs that may play a role for optimal activity. Designating PGN as a mesh may be misleading, but it can be assured that the PGN sacculus of both Gram-positive and -negative bacteria is relatively porous and does not represent a permeability barrier for particles of approximately 2 nm and globular hydrophilic molecules of a maximum of 50 kDa (AMPs are between 15 and 50 amino acids, <5 kDa) that do not bind to PGN [48]. Further, PGN is not negatively charged and hence is not considered to compete significantly with the membrane for interaction with AMPs so that they can pass freely through the PGN mesh of both lineages.

### 3.2. Peptidoglycan as a target for innate immune recognition not interfering with AMPs activity

PGN can rather be seen as a target for innate immune recognition by a family of pattern recognition molecules e.g. peptidoglycan recognition proteins (PGRPs) [49]. These proteins are evolutionary conserved and recognize the microbes via direct local attack against indwelling pathogens and induction of the acute inflammatory response and adaptive component of immune system. Thus, in such cases binding to PGN may serve as initial event for bacterial killing. An example includes human bactericidal RegIII lectins of the C-type lectin family with a molecular weight of ~16 kDa. Characteristic C type lectins possess a globular structure with four functional domains: (i) carbohydrate binding domain, (ii) neck repeat region of tandem helical repeats with exposed hydrophobic residues, which allows oligomerization, (iii) transmembrane domain and (iv) cytosolic domain promoting internalization into the membrane [50]. RegIII lectins recognize the bacteria by binding to PGN carbohydrate via a Glu–Pro–Asn (EPN) tripeptide motif located in the long loop region of the protein as was demonstrated by NMR spectroscopic studies [28]. This Glu–Pro–Asn motif is required for bacterial killing, as a point mutation of Glu (E) residue in Glu–Pro–Asn motif showed reduced affinity to *staphylococcal* PGN and a 6 fold decrease in antimicrobial activity against Gram-positive species *Listeria monocytogenes*. However, the mechanism by which lectins kill bacteria is not known. Just recently it was published that RegIIIα kills bacteria by oligomerization on the membrane forming a membrane-penetrating pore [51]. Interestingly, RegIIIα lectin exhibits a point mutation in the Glu-Pro-Asn motif and bears instead a Gln–Pro–Asn (QPN) motif, which prevents binding of RegIIIα to PGN [28]. But, RegIIIα lectin permeabilizes membranes of *Listeria monocytogenes*, as shown by an increased uptake of the membrane impermeable fluorescent dye SYTOX green. By measuring changes in intrinsic tryptophan residues upon contact to PC/PS liposomes as well fluorescence energy transfer between donor RegIIIα and dansyl-labelled PC/PS liposomes, it has been figured out that RegIIIα binds to negatively charged membrane phospholipids (PC/PS) but not to zwitterionic PC disrupting PC/PS membranes. In addition, electron microscopy in combination with crosslinking experiments demonstrated formation of a hexameric membrane-permeabilizing oligomeric pore with a diameter of about 100 Å in PC/PS liposomes [51]. Although it has to be emphasized that PS is not a typical bacterial lipid, but shares the negative charge with PG, it was concluded that bacterial killing resulted from uncontrolled ion efflux and subsequent osmotic lysis. All these events are not found for Gram-negative bacteria, as LPS has been identified to inhibit RegIIIα membrane permeabilization and disruption of liposomes composed of *E. coli* total lipid extracts or PC/PS in the presence of LPS [51]. This explains the lack of ability of RegIIIα to kill Gram-negative bacteria.

It is interesting that the ability to recognize and eliminate pathogens is versatile but still evolutionary conserved. Although proteins such as C-type lectins are bigger in size and more complex in structure than the small antimicrobial peptides, they often share the same mechanisms like membrane disruption to combat against the pathogen. One may wonder if this could be through evolution a strategy of nature to combine building blocks of known functions in domains and design more complex molecules (proteins) with increased potency to overcome the emerging resistance of microbes.

### 3.3. Modifications of peptidoglycan to evade innate immunity

An important strategy of pathogenic and commensal bacteria to evade innate immunity and to control autolysins involves modifications of stem peptide (amidation of D-Glu and mDAP, modification of L-ala by Gly and L-ornithine instead of meso-DAP) and glycan chains (O-acetylation of NAM, N-deacetylation of NAG and glycosylation of NAM) of PGN, documented and reviewed for diverse bacterial species elsewhere [52–54]. This widely leads to survival of the bacteria as a result of reduced recognition of the pathogen by the host receptors and hence decreased activation of the innate immune response. Modification of PGN and hence, increased antimicrobial resistance is also related to increased virulence of bacteria [55].

## 4. Lipoteichoic acid, an anionic polymer matrix

Most Gram-positive bacteria incorporate teichoic acid polymers into their cell envelopes that largely contribute to a bacterial negative surface charge. The basic structure of teichoic acid encompasses a soluble polymer of glycerolphosphate or ribitolphosphate repeating units that is either attached to the cytoplasmic membrane via a glycolipid anchor (lipoteichoic acid, LTA) [56] or covalently linked to N-acetylmuramic acid of PGN (wall teichoic acid, WTA) (Fig. 5). The glycolipid anchor in *S. aureus* is diglycosyl-1,2-diacylglycerol (DGDG) with two fatty acids of different composition [57], mostly C14:0 and branched C15:0 at the sn-2 position and C16:0,C18:0 and C20:0 at the sn-1 position of the glycerol moiety [58]. LTA deficient *ltaS* mutant of *S. aureus* exhibits aberrant cell growth and division and is synthetic lethal with *tagO* mutant defective in WTA synthesis indicating that LTA and WTA compensate for their activities and that complete loss of the anionic polymer matrix in bacterial envelopes affects the growth leading to inviability of the cells [59].

In the logarithmic phase of growth the concentration of LTA in the outer membrane layer ranges between 0.4 and 1.6% of the cell dry weight [60], which means that one LTA molecule contributes to every ninth to tenth lipid molecule [58,61]. In contrast to membrane lipids, LTA and not deacylated LTA does not form a stable monolayer structure [62], but forms a micellar supramolecular structure in aqueous dispersion [63]. X-ray structure of staphylococcal [63] and pneumococcal LTA micelles [64] was characterized with a total diameter of 22 nm. The core made of hydrocarbon chains of the glycolipid anchor is 5 nm, which is surrounded by an 8.5 nm shell of heavily hydrated hydrophilic chains. The critical micelle concentration of LTA from several bacterial species in phosphate-buffered saline ranged from 28 to 60 μg/ml [65]. Systematic thermodynamic studies on the miscibility of LTA with DPPG revealed stable mixtures up to LTA concentrations of 20 mol% [62]. Increasing the LTA concentration in the DPPG matrix leads to an increase of the phase transition temperature indicating a stabilizing effect on lipid membranes within the head group region [58]. At higher concentrations than 20 mol% separation of both lipids

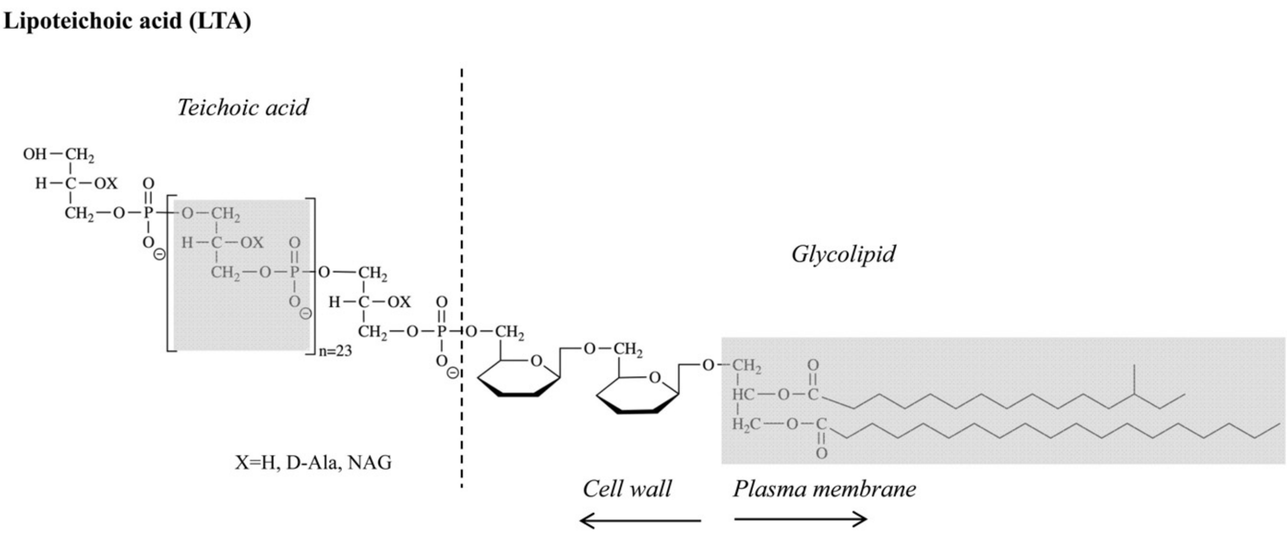

**Fig. 5.** Chemical structure of lipoteichoic acid.

occurs, accompanied by a destabilization of the lamellar aggregation of DPPG and segregation of LTA into the sub-phase, presumably in the form of micelles owing to the small cross-sectional parameter of the diacylglycerol moiety and the resulting conical shape of LTA [58].

### 4.1. Interaction of lipoteichoic acid with AMPs

Gram-positive bacteria e.g. *S. aureus* contain on average 24 glycerolphosphate repeating units of 6200 g/mol weight LTA of which 70% are substituted by D-alanine [57,66]. The length of the glycerolphosphate chain varies between Gram-positive species from 15 to 50 residues. Each repeating unit of LTA contains one negative charge from the phosphate group, which potentially can attract positively charged AMPs [67,68]. Obviously, many bactericidal peptides bind with high affinity to LTA, but may in addition exhibit membrane disruptive properties like mellitin, cecropin [69] and LL-37 [67] contributing to their bacterium-killing activity. Thus, it has been postulated that binding and attraction of the AMPs to LTA may initiate bacterial killing by AMPs mediating peptide's entry into the bacteria. In other words, by building polyanionic ladder LTA and WTA may help polycationic peptides to traverse from outside to the cytoplasmic membrane.

Koprivnjak et al. [70] suggested that the killing activity of both the 14 kDa mammalian group II phospholipase A2 (gIIA PLA$_2$) and the highly basic 45 amino acid human ß-defensin 3 toward *S. aureus* depends on initial electrostatic interaction with WTA, as the *tagO* mutant lacking WTA was highly resistant to these antimicrobial peptides. Detailed analysis with highly positively charged (+12 to +17) gIIA PLA$_2$, as deduced from recovery of [1-$^{14}$C]oleate radiolabeled bacterial *tagO* strain, revealed that phospholipid degradation was reduced in the presence of gIIA PLA$_2$. Moreover, the peptide was able to hydrolyze phospholipids from cell-wall depleted protoplasts from both wild type and the *tagO* mutant and their radiolabeled lipid pattern resembled that of the intact bacteria. As the binding of the peptide to the surface of *tagO* mutant was not reduced, the authors concluded that the binding of the gIIA PLA$_2$ to WTA is important to facilitate cell wall penetration and to gain access for membrane phospholipid degradation.

Another bactericidal protein, the phospholipoglycoprotein vitellogenin, which is a major precursor of the yolk proteins in oviparous organisms, kills bacteria via binding to LTA and not via membrane disruption [68]. Results from scanning electron microscopy showed that 450 kDa vitellogenin from fish *Hexagrammos otakii* causes damage of the cell wall of *S. aureus* whole cells with the appearance of collapsed architecture, but does not induce changes in the morphology of *S. aureus* protoplasts, which are depleted of cell wall and hence LTA. This was also confirmed in a lysis assay, where significant reduction of OD$_{420}$ indicated severe cell lysis in vitellogenin treated *S. aureus* whole cells, but not in cell wall depleted protoplasts. In addition, cell-wall destroying activity of vitellogenin toward *S. aureus* is abolished, when vitellogenin was preincubated with LTA before applying to the *S. aureus* cells, which concomitantly resulted in loss of antibacterial activity of the peptide. These observations suggest that the binding of vitellogenin to LTA is lethal to *S. aureus*. It has also been reported that the antibacterial cell-permeable peptide PBP 10, LL-37 and melittin efficiently bind to LTA inhibiting their antimicrobial activity [67]. Although it is obvious that for instance the antimicrobial activity of LL-37 is maintained by its action on the plasma membrane [71], LTA possesses inhibitory effect on its bacterium-killing activity [67]. It is most likely however that some peptides may be entrapped by LTA through an increase of peptide adsorption to the bacterial surface, which results in a decrease in local peptide concentration on the cytoplasmic membrane.

Using a fluorescence assay our group showed that OP-145, a derivative of LL-37, also binds to LTA [47]. Analyzing the thermotropic behavior of liposomes composed of DPPG and LTA characterized by two overlapping phase transitions corresponding to DPPG and DPPG/LTA domains also indicated that OP-145 interacts preferentially with DPPG/LTA domains. Further, the bilayer permeability of large unilamellar vesicles composed of POPG and LTA at a biologically relevant molar ratio

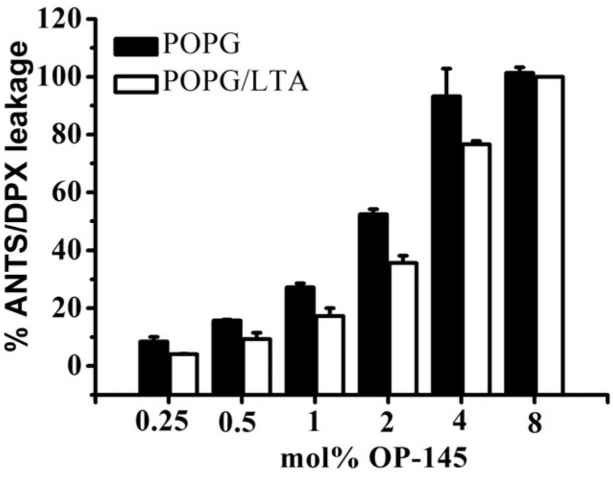

**Fig. 6.** Effect of LTA on membrane permeability of OP-145 adopted from Malanovic et al. [48]. Leakage of POPG LUVs (black bars) and POPG/LTA (white bars) at respective OP-145 concentration.

of 9:1 was reduced by about 25% in the presence of OP-145. This indicates a slightly hindered penetration of OP-145 toward the membrane interface owing to the presence of LTA in the liposome (Fig. 6). Notwithstanding, OP-145 is able to induce full leakage of DPPG/LTA vesicle although at higher concentrations. Thus, the molecular mechanism of bacterial killing by OP-145 can rather be explained by incorporation of the peptide into the phospholipid matrix and consequent membrane perturbation. To the best of our knowledge this was the first report using a model system composed of phospholipids and LTA to study the impact of both components on the interaction of AMPs. OP-145 induces membrane permeability of POPG or POPG/LTA liposomes between 2 and 4 μM, which is in the range of the effective lethal concentrations for *S. aureus* bacteria at 1.6–3.2 μM. Referring to Roversi et al. [14] and Castanho and co-workers [8], who described bacterial killing via membrane disruption as an event taking place only when bound AMPs completely saturate the bacterial membrane, one may consider that in the case of OP-145, binding to LTA may in fact reduce the total concentration of the peptide on the membrane interface, but not sufficiently enough to prevent significant membrane coverage to kill bacteria.

In addition, it has been demonstrated for resistant group B *Streptococcus* bacteria, which via D-alanylation have decreased anionic charge of LTA and decreased susceptibility to AMPs e.g. LL-37, magainin 2, polymyxin B and colistin, that the resistance to AMPs may not necessarily be attributed to decreased amounts of bound peptide to bacteria, but it may alter conformation of the LTAs [72]. Consequently, this result in increased cell wall density hindering AMPs to reach the plasma membrane through compact heavily coiled conformation of staphylococcal LTA [72]. Another example includes ß-bungarotoxin B chain, an antibacterial cationic polypeptide from snake venom that upon binding to LTA undergoes conformational changes resulting in inhibition of its active site that abrogates its membrane-damaging activity and inhibits its bactericidal activity toward *S. aureus* [73]. ß-bungarotoxin is the main presynaptic phospholipase $A_2$ neurotoxin consisting of ~14 kDa A chain that shows similarities to phospholipase 2 and a 7 kDa B chain peptide more similar to toxin I, trypsin inhibitor and dendrotoxin. The ß-bungarotoxin B chain exerts membrane-damaging activity as shown by calcein release from liposomes composed of PG and PE but also of mixtures of PG and cardiolipin. The B chain exerts its damaging activity without involvement of A chain and because of its abundant positively charged amino acid residues it is more likely that the B chain displays bactericidal action via a membrane-damaging activity [74]. However, the peptide was unable to inhibit growth or induce membrane permeability of *S. aureus*. As the membrane permeability of propidium iodide fluorescent dye was induced in *E. coli* cells treated with ß-bungarotoxin B chain but not in *S. aureus* cells, it was clear that components of the Gram-positive cell wall may contribute to these negative results. Indeed, calcein release from PG/cardiolipin vesicles was absolutely abolished, when the peptide was preincubated with LTA. CD spectra indicated conformational changes of the peptide in the presence of 4.5 mg LTA suggesting that LTA efficiently blocks the B-chain functional site or conformation on damaging membrane.

### 4.2. Role of lipoteichoic acid in immune response

LTA is released spontaneously into the culture medium during growth of Gram-positive bacteria [75] but the release can be enhanced upon treatment with antibiotics like penicillin [76] or after bacteriolysis induced by cationic peptides from leucocytes (for review see [77]). Released LTA is believed to stimulate production of inflammatory mediators, immune response to infection in host organism to fight the invading bacteria. Given this one can consider if the high concentration of LTA or exposure of the hydrophilic fatty acid chains of LTA micelles are initializing the activation of the signaling cascade of inflammatory response. Interestingly, the activation of the inflammation pathway, among others occurs specifically via interaction of LTA with the host CD14 [77] and TLR2 ligand [78] most probably via insertion of the two fatty acids into the binding pocket of TLR2 [79,80] followed by induction of complement cascade to activate production of cytokines such as tumor necrosis factor (TNF) and interleukin 6, chemokines and various other genes [81,82]. As a consequence, a set of circulating problems ranging from beneficial pro-inflammatory responses and fever to organ failure may lead even to the death [83]. A number of AMPs, effectors of innate immunity [82] are potent in prevention of sepsis and inflammation as they particularly can neutralize LTA and inhibit LTA-induced cytokine release. Examples include among others human cathelicidin LL-37 and its derivative OP-145 [84] as well as CEME related peptides [69] derived from a hybrid of silk moth cecropin and bee melittin. CEME related peptides inhibit LTA stimulated production of TNF and IL-6 by murine macrophage cells RAW 264.7 *in vitro* as well as in whole blood samples of human volunteers [69]. However, the relative ability to bind/neutralize LTA did not correspond to their MICs as they exhibited only moderate activity against Gram-positive bacteria. Thus the high binding affinity to LTA seems likely not to be important for their antimicrobial activity. This was also observed for OP-145 [47], which showed high binding affinity to LTA, but was able to induce significant permeabilization of the bacterial model membranes, vesicles composed of membrane phospholipid PG and LTA but also of pure PG vesicles. It is more likely that the high binding affinity to LTA can be important to “mask” the binding sites of LTA necessary for induction of inflammation processes. Accordingly, OP-145 [47,84] but also its parent peptide LL-37 [84] is able to efficiently neutralize LTA and inhibit production of cytokines known to induce the inflammation. However, the interaction of LTA and antimicrobial peptides (intrinsic or therapeutic) during immune response and inflammation remains largely not understood and requires further efforts. In a recent publication, Brandenburg and coworkers [85] disclaimed the role of LTA in cytokine induction. They demonstrated that lipoproteins/lipopeptides which usually contaminate commercial LTA products and bacterial isolates are the most potent pro-inflammatory toxins of bacterial cell wall not only *in vitro* but also when inoculated into mice.

### 4.3. Modifications of lipoteichoic acid to promote antimicrobial resistance

These complex amphiphilic molecules underlay diverse modifications of the repeating units providing diversity across the various bacterial strains. One of these modifications is the decoration with a variety of sugars (α-galactose, NAG) and esterification with D-alanine [56]. The latter remain to be unstable and e.g. in *S. aureus* can continuously be re-esterified. In addition, a number of environmental factors have been shown to influence D-alanylation of LTA. An increase in pH [86], temperature [87] or NaCl [66] leads to decrease in D-alanine ester content of LTA. Esterification/re-esterification with alanine of the negative charge from phosphate of LTA's glycerolphosphate repeating units induces zwitterionic properties of teichoic acids via the positively charged free amino groups of alanine. This is one of the protective mechanisms of bacteria in order to reduce negative charge in the cell envelope and, hence susceptibility to antimicrobial agents/peptides [88,89]. Accordingly, *dlt* mutants derived from gallidermin-sensitive *S. aureus* are devoid of D-alanylester from teichoic acid and have increased sensitivity to positively charged AMPs such as defensins, protegrins, magainin and diverse lantibiotics but not to neutral gramicidin B indicating reduced electrostatic interaction between *S. aureus* and cationic molecules [89]. This is also reflected in a mouse model of *S. aureus* sepsis, where a *dltABCD* mutant was impaired in disease progression with significantly reduced rates of septic arthritis and mortality and reduced bacterial load in the kidney [90]. Similarly, it was the reason that *dlt* mutant in mouse model of Group B streptococcus (GBS) disease which is normally characterized by invasive infections from pneumonia to meningitis, was cleared from lungs too quickly to cause pneumonia and was unable to survive bloodstream or colonize the brain [91].

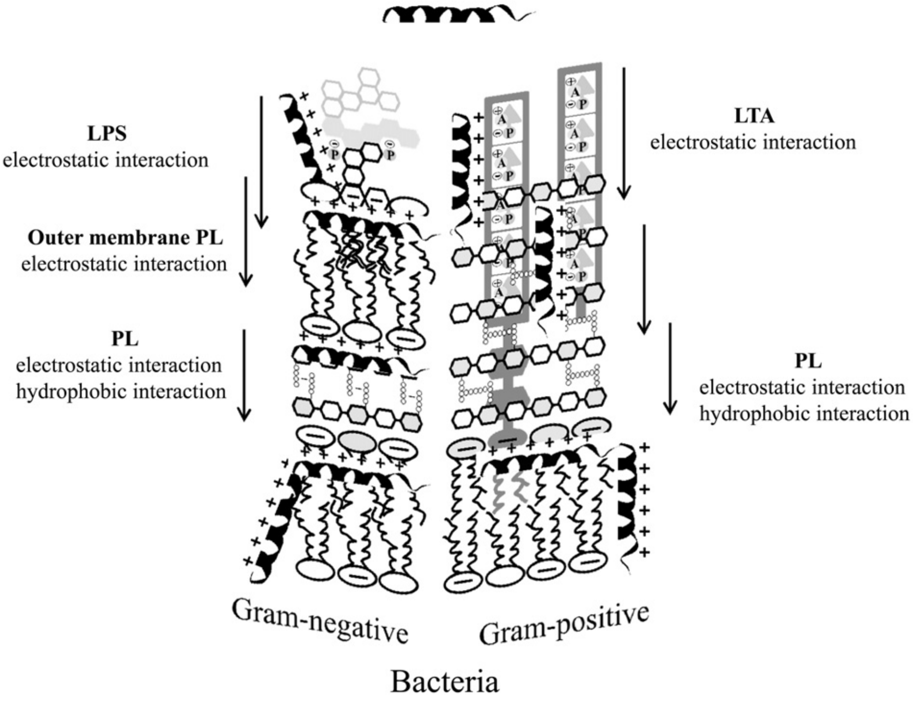

**Fig. 7.** Schematic illustration of AMPS interactions.

## 5. Concluding remarks

So far limited quantitative data have been reported on the interaction of AMPs with Gram-positive cell wall components, which may be partly due to the fact that their extraction from bacterial cells is problematic and are often not pure enough to test it on a single molecule level. Moreover, it is not possible to isolate the intact PGN wall [13] and in addition commercially available PGN preparations often contain proteolytic enzymes. Similarly, LTA can be contaminated also with proteins and endotoxins, which may interfere with experimental interpretation. One strategy to overcome these problems is to perform experiments on live cells as extensively discussed by Castanho and coworkers [8]. Nevertheless, studies on membrane–mimetic systems, which definitely will become more complex in future, have revealed interesting insights into the interaction of AMPs with these components. These studies provided evidence that membrane-active cationic antimicrobial peptides on their way to cytoplasmic membranes are exposed to different interaction partners, to which they exhibit different affinities, which may reduce their effective concentration on the membrane surface (Fig. 7).

Taking into account that PGN is relatively porous and freely penetrable for small molecules like AMPs and that permeability assays on simple membrane model systems were not impaired in the presence of PGN, one would assume that the role of PGN is not in entrapping AMPs but might rather act as sponge facilitating the penetration of the cell wall and in turn interaction with the phospholipid bilayer. In contrast, LTA as an anionic polymer has a strong potential to attract positively charged molecules and may act as both entrapper of AMPs or ladder for a route to the plasma membrane. As one resistance mechanism of Gram-positive bacteria is modification of LTA by incorporation of alanine reducing the negative net charge, it is tempting to speculate that rather the latter role is of importance. The same may be the case for Gram-negative bacteria, where LPS, besides of its immune-orchestrating effects may have similar functions as its counterpart in Gram-positive bacteria, LTA (Fig. 7). To conclude, the question raised by Castanho [8] and other colleagues in the field, whether cell wall components act as electrostatic barriers preventing membrane-active AMPs from their lethal action on the cytoplasmic membrane, can only be answered once we have more quantitative data on the affinity of AMPs and cell wall components. The current data suggest that AMPs may "use" these components to enrich at the membrane surface, but we may well end up that it is a matter of concentration [8].

## Transparency document

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

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