Role of antimicrobial peptides in human innate defense against bacteria

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Abstract

Antimicrobial peptides (AMPs) are important components of the innate immune system of many vertebrate and invertebrate species. These small peptides directly kill microorganisms by inducing microbial cell death. This review focuses on the mechanisms of direct antimicrobial effects against Gram positive and Gram negative bacteria of the main human AMPs, α-defensins, β-defensins, the cathelicidin LL-37 and the histatins. Effectiveness of the AMPs is dependent on both the properties of the AMPs and the target microbe. The size, cationic charge and amphipathicity allows AMPs to target and disrupt the membrane integrity of several bacteria. Different mechanisms of lipid bilayer disruption by AMPs are described by the 'barrel-stave', the 'toroidal-pore' and the 'carpet' model. Although these models are generally accepted to clarify direct antimicrobial killing by AMPs, the precise mechanisms of the main human AMPs remains to be clarified. In addition, the microbicidal activity of some AMPs might be explained by other mechanisms.

Introduction

Although the adaptive immune response is very effective in human defense against infection, it can take several days before this system is activated. Therefore, the innate immune system has an important responsibility as it directly fulfils the important role of providing general protection against a broad range of microbes prior to activation of the adaptive immune system (reviewed in ref.[1]).

The innate immune system is comprised of several mechanisms, including both physical and chemical barriers. The first line of innate defense against infection is formed by the physical barriers of the human body including epithelial surfaces which protect all tissues from the external environment. In addition, cells from mucosal epithelia produce mucus in which microbes can be trapped and thereby prevented from invading the human body.[1] Once microbes manage to overcome the physical barriers, the cellular compartment of the innate immune system is important. Neutrophils are considered to be the most important cells involved in microbial killing. Together with macrophages, these cells are the main phagocytic cells of the human body. Upon recognition of invaders, they engulf and subsequently destroy the microbes. Granules (lysosomes) within the cytoplasm of neutrophils and macrophages contain enzymes and proteases which are responsible for this microbial killing (reviewed in refs.[1,2]). In addition, neutrophils produce other microbicidal compounds including reactive oxigen species (ROS), generated by a multi-step enzymatic process which is called the respiratory burst.[2] Another essential component of innate immunity is the complement system. The complement system is a non-cellular based system, which consists of various proteins present in the blood plasma. Upon activation, these proteins either mediate phagocytosis by opsonization of the microorganism or they participate in enzymatic processes leading to destruction of the microbe.[1]

The production, either constitutively or pathogen-induced, of antimicrobial peptides (AMPs) assembles another important chemical barrier. A wide range of diverse peptides with the activity to kill or inhibit growth of pathogens, are collectively called AMPs. Toxicity of antimicrobial peptides lies within their microbial membrane interfering capacity. AMPs are able to interfere with microbial membranes to introduce pores, consequently leading to cell death. They are produced in several tissues and cell types of plant, invertebrate and vertebrate species. Since AMPs are already known for several decades, their variety of different structures and activities are studied and reviewed extensively.[3-8] The broad collection of different AMPs are classified based on their structural characteristics (reviewed in refs.[5,9]). Apart from their differences, AMPs share some collective features. The peptides are small, less then 60 amino acids long, and have an overall positive charge. Moreover, the peptides are amphipathic, meaning that they have both hydrophobic and

hydrophilic regions. The ability of AMPs to interact and disrupt microbial membranes is thought to be dependent of this latter characteristic.[5,9] In human, the main groups of AMPs are the defensins, cathelicidins and histatins.[1,4,7]

AMPs show antimicrobial activity against a broad spectrum of microorganisms including Gram positive and Gram negative bacteria, fungi and viruses. Several models are proposed for direct antimicrobial activity of AMPs; however the precise mechanisms are still not completely understood. Moreover, the peptides are not solely effective in defense against microbial infections by inducing microbial cell death. AMPs have an important additional function by influencing the overall immune response as they act as immune regulatory factors. Different AMPs can chemoattract several types of immune cells from both the innate as well as the adaptive immune system, including neutrophils, monocytes and T lymphocytes.[10-12] In addition, AMPs are able to influence the adaptive immune response by having adjuvant properties. For example, α-defensins influence the cytokine production and antibody response of the adaptive immune response.[13,14]

This review focuses on the precise mechanisms of direct antimicrobial effects against Gram positive and Gram negative bacteria by the important human AMPs, α-defensins, β-defensins, the cathelicidin LL-37 and, to a lesser extent, the histatins. Although the immune modulating functions of AMPs are essential for an optimal immune response, a discussion on this topic is beyond the scope of this review in which direct antimicrobial mechanisms of AMPs are described. Effectiveness of the different AMPs is considered to be dependent on properties of the peptide itself as well as properties of the target membrane.[7,15] Therefore both aspects of human AMPs and bacterial cell wall characteristics are discussed in order to define precise mechanisms of direct antimicrobial defense by human AMPs.

Antimicrobial peptides (AMPs)

A large variety of evolutionary conserved proteins that have the ability to kill or inhibit growth of various microbes are collectively called antimicrobial peptides. Many plant and animal species, including human, have the ability to produce multiple different AMPs. In this review the main human AMPs are discussed (see table 1) although many more AMPs have been identified. Also in other species than man, different classes of AMPs are present and although the peptide sequences can vary significantly, they share some common structural characteristics and mechanisms of action.[6,8] In particular the site of expression and the structural features of AMPs are conserved. AMPs are present at sites of the human body where they are needed the most; the places were the host is in direct contact with the external environment and where microorganisms are present.

In general, analysis of protein size, charge and structure leads to a better understanding of the proteins function. The amino acid sequence is unique for each protein which determines its charge, hydrophobicity and structure. The main structural properties of AMPs, which are the overall positive charge and the amphipathicity of the peptides, are considered to be responsible for the direct antimicrobial activity. These features allow interaction with the relatively negative charged microbial membranes resulting in membrane disruption and microbial killing. There are more specific characteristics of AMPs considered to be responsible for targeting microbial invaders selectively which are discussed later on in this review.

To be able to understand the mode of action of the main human AMPs structural, expressional and functional properties of the peptides are described in more detail below.

Defensins

The characteristic feature of the defensins molecule is the presence of six cysteine residues that form disulfide bonds. These bonds contribute to the specific folding of the protein into their functional β-sheet structure. The exact role of the disulfide bonds remains to be established. It was thought that the bonds are required for antimicrobial function of defensins. Conversely, other data demonstrated that defensin analogues without disulfide bonds are able to maintain their antimicrobial function. Therefore, it was proposed that the disulfide bonds protect the defensins from proteolytic cleavage.[16-18]

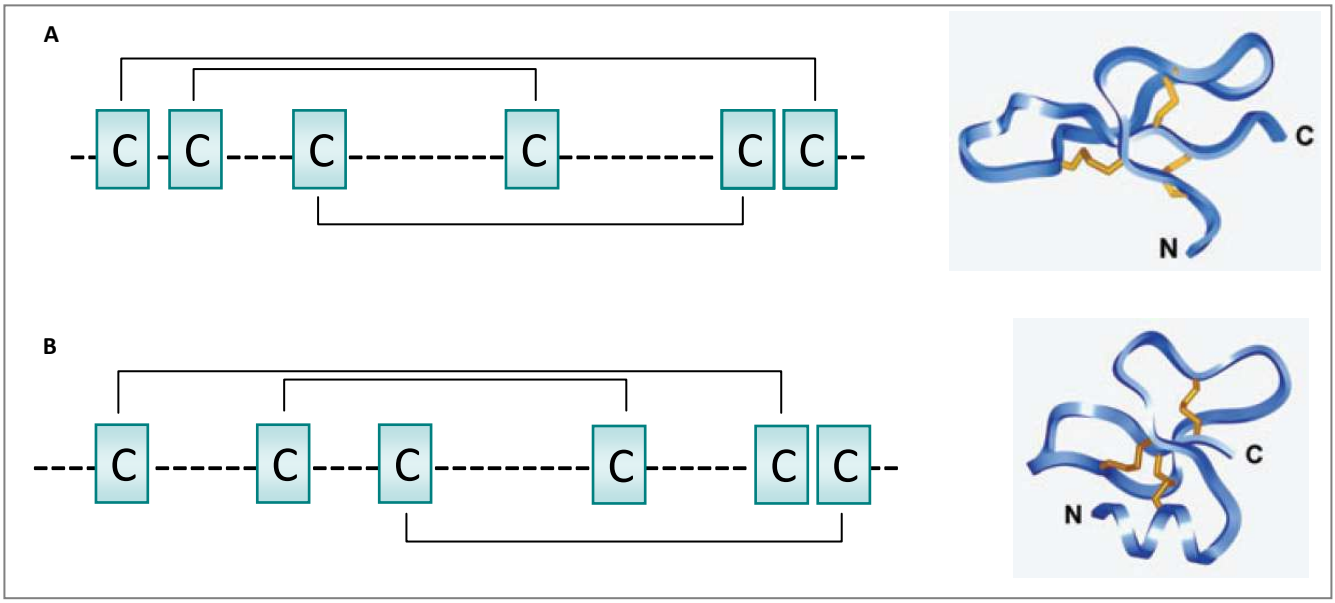

Figure 1 | Defensin structure
A) α-defensins **B)** β-defensins. On the left, a representation of the disulfide bridges between cysteine residue 1-6, 2-4 and 3-5 for α-defensins and 1-5, 2-4 and 3-6 for β-defensins is shown. Cysteine residues are indicated by a C, other amino acids by dashes. On the right a representation of the three-dimensional structure of defensins is depicted in which the disulfide bridges are indicated in yellow. This figure is adapted from M.E. Selsted and A.J. Ouellette (Nat Immunol. 2008).

The distinction between α- and β-defensins was originally made based on the location of the cysteine residues and their specific disulfide binding pairs. The cysteine residues of α-defensins form binding pairs at cysteine position 1-6, 2-4 and 3-5 whereas the cysteine residues of β-defensins are formed between residues 1-5, 2-4 and 3-6. The difference in folding results in different protein structure as is depicted in figure 1 (reviewed in refs.[7,9,17,19,20]). Expression of defensin genes results in synthesis of prepropeptides which are further processed into active peptides depending on the type of defensin and the site of expression (reviewed in refs.[17,21]).

α-Defensins

On average, human α-defensins have a length of 29-35 amino acids in which arginine is the predominant amino acid. Six different α-defensins are described. Four were initially isolated from the cytoplasmic granules in neutrophils. Therefore these α-defensins are called Human Neutrophil Peptides (HNP-1 to -4) (reviewed in refs.[4,9,17]). However, later it was shown that these peptides are also expressed by human natural killer cells and monocytes.[22,23] The final two α-defensins are expressed by Paneth cells and granulocytes within the epithelium of the intestinal tract and are called Human Defensin 5 and 6 (HD-5 and HD-6) or crypt defensins / cryptdins (reviewed in refs.[9,17]). HNPs in neutrophils are stored in the intracellular granules and are considered to be important for killing of phagocytosed microorganisms. The granular molecules are only secreted when neutrophils are stimulated. In contrast, HD-5 and HD-6 are constitutively expressed in Paneth cells and released in the intestinal lumen (reviewed in refs.[4,9,17]).

β-Defensins

Human β-defensins are slightly larger than α-defensins with an average length of 35 amino acids. The four different β-defensins are called Human Beta-Defensins (HBD-1 to -4). B-Defensins are mainly expressed by epithelial cells that are directly exposed to the environment (reviewed in refs.[4,9]). Although in most studies four human β-defensins are discussed, the existence of more human β-defensins is plausible.[24] HBD-1 to -4 are constitutively expressed in epithelial cells but expression can also be induced in other cell types. Expression of HBD-1 can be stimulated in monocytes by exposure to bacterial components including lipopolysaccharide (LPS) in the cell wall of Gram negative bacteria and cytokines including interferon-γ (IFN-γ). In addition, expression of HBD-2 to -4 in keratinocytes is promoted by inflammatory stimuli like cytokines produced by immune cells in response to bacterial exposure (reviewed in ref.[17]).

Cathelicidin, LL-37

Cathelicidins are positively charged peptides and, like defensins, derived after cleavage of a precursor peptide. LL-37 is present at the C-terminus of the precursor peptide hCAP18 (human cationic antimicrobial protein of 18kDa). hCAP18 has on the N-terminus a domain homologue to cathelin (reviewed in ref.[4,5]) (see figure 2). In human only one cathelicidin is characterized, LL-37, whereas other mammalian species possess multiple cathelicidins. LL-37 derived its name from the fact that the peptide is 37 amino acids long with two leucine residues at the N-terminus of the molecule. After cleavage of the precursor peptide, the active form of LL-37 forms amphipathic α-helices (reviewed in refs.[4,25]). In addition, the cathelin domain, released after cleavage of the precursor peptide, has distinct antimicrobial activity.[26] LL-37 is expressed after an inflammatory stimulus in many cell types including epithelial cells and neutrophils, monocytes, NK, T and B cells [4,26]. In neutrophils, the full-length precursor peptide is synthesized and stored in granules. Only upon inflammatory stimulation, the precursor is proteolytically processed resulting in active antimicrobial peptides including LL-37.[26] In keratinocytes and granulocytes in the skin, LL-37 expression is upregulated after skin injury.[27] Moreover, LL-37 is found to be constitutively expressed in sweat glands[28] and to be further processed into smaller peptides by other proteases (see figure 2). These smaller peptides have additional antimicrobial activity.[29]

Histatins

Histatins are constitutively produced and secreted by cells of the salivary glands. There are about 12 different histatin peptides of which the most abundant histatins are 1, 3 and 5. Histatin-1 and -3 are direct products of the two histatin genes whereas histatin-5 is formed after further processing of histatin-3 (reviewed in ref.[4]). Like the other human AMPs, histatins are cationic and small, respectively containing 38, 32 and 24 amino acids.[4,30] Histatin-5 is considered to have the strongest antimicrobial activity and therefore most research is focused on this particular peptide. In aqueous environments, histatin-5 forms random coiled structures whereas in non-aqueous solvents it adopts an α-helical structure (reviewed in ref.[4]) similar to the structure of LL-37. Although histatins have antibacterial activity they primarily show anti-fungal capacity.[4,30]

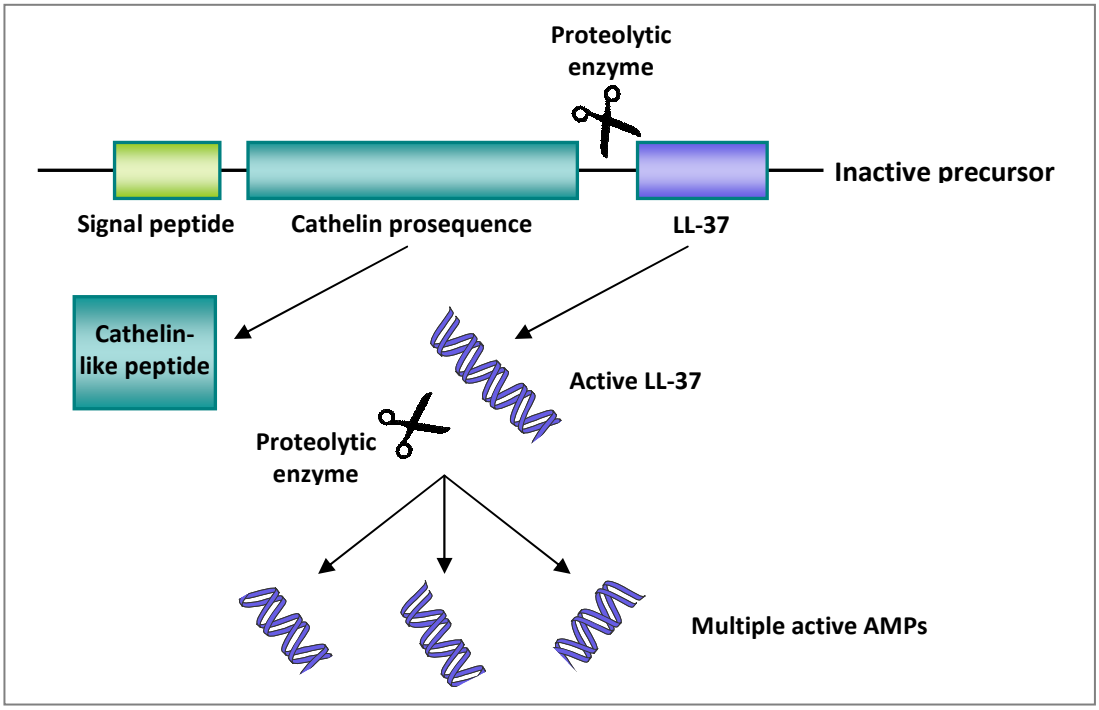

Figure 2 | LL-37 processing
After proteolytic cleavage of the inactive precursor peptide hCAP18, LL-37 as well as the Cathelin-like peptide are derived. Subsequently, LL-37 can be further processed into smaller AMPs with increased microbial activity. This figure is adapted from A. Izadpanah and R.L. Gallo (J Am Acad Dermatol. 2005).

In vivo evidence for the importance of AMPs

The importance of AMPs for the protection of microbial infections *in vivo* is underlined by studies investigating the relationship between pathogenesis of chronic inflammatory diseases and AMP production. There are several diseases in which pathogenesis is associated with impaired AMP levels (reviewed in ref.[31]). Furthermore, the *in vivo* relevance is revealed in several studies using mouse models.

In cystic fibrosis (CF) patients, pulmonary infections occur frequently. It was proposed that human β-defensin-1 molecules, secreted by lung epithelial cells, are inactivated in the airway surface fluid (ASF) due to the high salt concentration in the ASF of these patients. As a result, absence of AMPs in the lung contributes to chronic inflammation.[32] Moreover, proteases found in the ASF of CF patients are shown to degrade human β-defensin-2 and 3.[33] In inflammatory skin diseases like atopic dermatitis, pathogenesis is complicated by persistent microbial infections. Inflammatory skin lesions in the skin of these patients correlated with impaired expression of the skin AMPs, β-defensin-1 and LL-37.[34] These examples highlight the importance of AMP production and secretion in human. In mice, a homologue for the human LL-37 was identified, named CRAMP. Genes encoding these peptides share homology and the peptides have similar helical structures. Therefore, mice lacking CRAMP can be used to study the function of LL-37 *in vivo*. It was found that mice deficient for CRAMP showed significant larger areas of infection that persisted longer after subcutaneous injection of bacteria. These results indicate an essential role for LL-37 in defense against bacterial infection.[35]

Structural features of Gram positive and Gram negative bacteria

Bacteria can be roughly divided into Gram positive and Gram negative bacteria. This distinction is based on staining properties of the bacteria, specifically affected by their cell wall. Already in 1884 the Gram stain was developed by the physician Hans Christian Gram. Due to the fact that Gram positive bacteria possess a ticker peptidoglycan layer, the dye stains Gram positive bacteria more compared to Gram negative bacteria. The cytoplasmic cell membrane of Gram negative bacteria is covered with a thin peptidoglycan layer and an additional outer membrane. The cell envelope of Gram positive bacteria is comprised of a thicker peptidoglycan layer, lacking the outer membrane, see figure 3.[36] In addition, the cell envelope of most bacteria has additional surface structures with distinctive functions.[37]

Membranes of eukaryotic cells differ from bacterial membranes in several aspects. Accordingly, antimicrobial peptides can selectively target bacterial cells.[15,38,39] Here, several structural features of Gram positive and Gram negative bacteria are discussed in relation to their interaction with antimicrobial peptides.

Lipid bilayer

Antimicrobial peptides can specifically interact with bacterial membranes due to some fundamental differences in eukaryotic and prokaryotic membranes. In eukaryotic membranes phosphatidylcholine (PC) lipids are the predominant lipids. More importantly, the membrane has a neutral charge on the outside because the negatively charged phospholipids are located on the inner leaflet of the lipid bilayer. In contrast, bacterial membranes have an overall negative charge with on the outer leaflet of the membrane negatively charged phospholipids including phosphatidylglycerol (PG) and cardiolipin (CL) (reviewed in refs.[15,39]). Furthermore, sterols which are abundantly present in eukaryotes, are hardly present in bacterial membranes. In addition, the presence of the sterol, cholesterol, was shown to be protective for membrane disruption by AMPs in human erythrocytes (reviewed in ref.[15]).

Peptidoglycan layer

The peptidoglycan layer is essential for bacterial viability as it maintains cell structure and resists high osmotic pressure. Moreover, it is used as a scaffold for direct or indirect attachment of surface proteins or glycopolymers.[40,41] The importance of this peptidoglycan layer is further reflected by the fact that many antimicrobials interfere efficiently with the synthesis or maintenance of the peptidoglycan layer.[42,43] A lot of research has been done to reveal the chemical structure and biophysical properties of peptidoglycan layers of many bacteria (for a review, see ref.[41]).

As its name already reveals, peptidoglycan is composed of linear sugar polymer strands that are cross-linked by short peptides. There is high diversity and variability of bacterial peptidoglycan layers, including glycan strain length, cross-linking, orientation and modifications of the glycan strains, peptidoglycan structure and biophysical properties.[41] The peptidoglycan layer is indispensable for bacterial viability and therefore

its synthesis, required during replication, and maintenance needs to be tightly regulated. Synthesis of peptidoglycan is a complex process that involves synthesis of the peptidoglycan precursors in the cytoplasm, synthesis of peptidoglycan intermediates linked to membrane lipids on the plasmatic side and peptidoglycan polymerization reactions on the outer side of the bacterial cytoplasmic membrane (reviewed in detail in refs. [44-46]).

Cell wall glycopolymers (CWGs) in Gram positive bacteria

Lacking an outer membrane, Gram positive bacteria have provided additional protection by the presence of additional structures on their outer surface. Cell wall glycopolymers (CWGs) are highly diverse structures that are present on the cell wall of Gram positive bacteria. They can either be linked to the cytoplasmic membrane (Membrane-connected CWGs, M-CWGs) or to the peptidoglycan layer (Peptidoglycan-connected CWGs, P-CWGs). Often, bacterial cell walls contain one M-CWG and one P-CWG (reviewed in ref.[37]). CWGs have different structural characteristics and based on these properties they can be classified in three groups. Teichoic acids is a class of CWGs with zwitterionic properties meaning that these glycopolymers have a negatively charged phosphate group but have an overall neutral charge. Teichuronic acids are the negatively charged CWGs which lack the phosphate group. The third group of glycopolymers consists of uncharged polymers.[37] CWGs are of great importance for bacterial viability and are commonly present on all Gram positive bacteria. Although the functions of CWGs are not completely elucidated, it seems that CWGs have, besides their cell protective function, major implications for bacterial pathogenesis.[37]

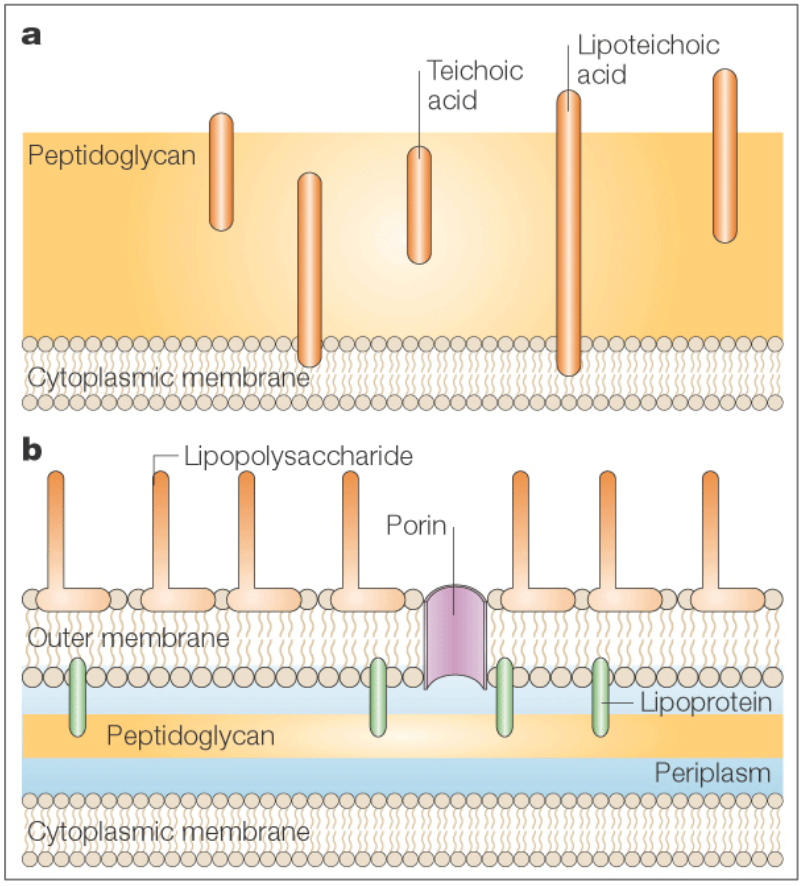

Figure 3 | Cell wall composition of Gram positive and Gram negative bacteria **A)** Gram positive bacteria possess a thick peptidoglycan layer covering the cytoplasmic membrane. Cell wall glycopolymers like teichoic acids and lipoteichoic acids are either membrane (M-CWG) or peptidoglycan bound (P-CWG). **B)** The cell wall of Gram negative bacteria is composed of a cytoplasmic (or inner) membrane and an outer membrane. In between, the periplasm is located containing a thin peptidoglycan layer. Additional surface structures can be lipopolysaccharide (LPS) which is attached to the outer membrane, proteins forming porins in the outer membrane or proteins attached to the peptidoglycan and outer membrane. The figure is from M.T. Cabeen and C. Jacobs-Wagner (Nat Rev. 2005)

Surface proteins

Different proteins are present on bacterial cell walls, each with its specific function. Surface proteins are bacterial specific and are therefore used to identify bacterial species. Functions of surface proteins include protection from the environment, nutrient uptake, attachment to host tissues, inter-bacterial interaction, cell growth and cell wall maintenance (reviewed in ref.[47]). In Gram positive bacteria, proteins are either attached directly to the peptidoglycan layer or to the CWGs. In Gram negative bacteria, the surface proteins are anchored the outer membrane (reviewed in ref.[40]). The proteins are produced in the cytoplasm after which they are targeted to the surface. Numerous different enzymes are required to covalently or noncovalently link the proteins to the outer membrane or peptidoglycan layer.[40,47]

Mechanisms of direct microbial killing by AMPs

Although the precise mechanisms are not completely understood, the general model of microbial killing achieved by AMPs is that these peptides disrupt microbal membranes, consequently leading to cell death. This can be nicely visualized by electron microscopy as in figure 4. This figure shows the effect of human β-Defensin-2 (HBD) on the membrane of *Streptococcus pneumoniae* cells (figure 4, panel B).

Selectivity of AMPs for microbial membranes

An important aspect for the function of AMPs is the ability to specifically target microbial cells leaving host cells unaffected. Electrostatic attraction between AMPs and microbial membranes is important for this selectivity. The positive charged AMPs are prone to interact with the more negatively charged microbial cell walls.[4,5,8,9,15,24,25,38,39] This overall negative charge of bacterial cell walls is a result from the negatively charged phospholipids in the bacterial membranes but also from the negatively charged lipopolysaccharides in Gram negative and CWGs in Gram positive bacterial cell walls (reviewed in ref.[9]) *In vivo* evidence for microbial selectivity of AMPs comes from studies using radioactive labeled AMPs in animal models. Comparing bacterial and fungal lesions in mice and rabbits with animals with a sterile induced inflammation showed significantly higher accumulation of AMPs in the microbial infected lesions. This indicates that also *in vivo* AMPs are able to distinguish between microbial and host cells.[48]

Interaction bacterial membrane – mechanisms of microbial killing by AMPs

After initial interaction of AMPs with the bacterial cell wall, the peptides are able to interact with the lipid bilayer and subsequently disturb its structure. As lipids are composed of a hydrophilic head group and a hydrophobic tail,

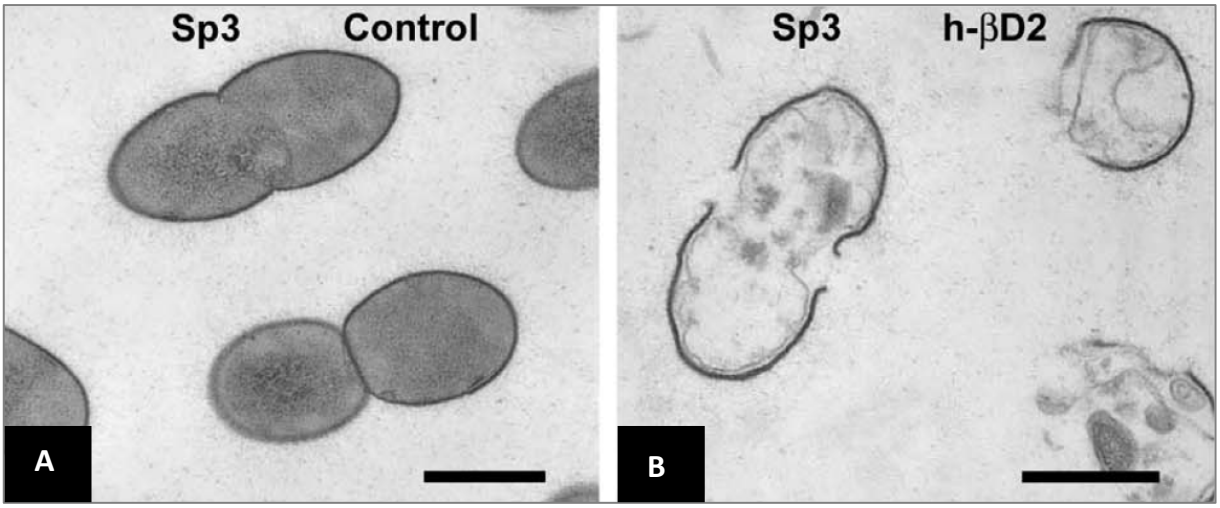

Figure 4 | Electron micrographs of *Streptococcus pneumoniae* cells
This figure shows results from experiments with the Gram positive bacteria *Streptococcus pneumoniae 3*. Untreated cells are depicted in panel **A)** and cells treated with human β-Defensin-2 (HBD) in panel **B)**. The bacterial cells are highly damaged in the presence of HBD-2. It can be nicely seen that the cell membranes of the bacteria are disrupted. This figure is from H.Y. Lee *et al.* (BMC Infect Dis. 2004)

membranes have a hydrophilic outer and a hydrophobic inner region. The amphipatic nature of AMPs allows them to interact with lipid bilayers. The hydrophobic parts of the peptide can face the inner core whereas the hydrophilic regions remain in contact with the lipid head groups on the surface of the membrane.[15]

Three basic models for membrane disruption by AMPs are proposed and discussed extensively, explaining how AMPs are able to permeabilize bacterial membranes after initial binding. Models are often based on data from studies with model AMPs, for example synthetically designed peptides or peptides originating from other species than man.[3,5,8,15,49,50] However, the precise mechanisms are still not completely elucidated and some AMPs possibly act via other mechanisms.

The barrel-stave model is formed by α-helical peptides that bind to the microbial membrane followed by insertion into the inner core of the membrane. A pore is formed when multiple peptides are recruited to form a bundle of α-helices crossing the membrane. In this model, the hydrophobic regions of the peptides face the inner side of the lipid bilayer whereas the hydrophilic regions form the inner side of the pore (see figure 5a).[3,8] This model was already proposed by Baumann and Mueller in 1974, describing how the fungal peptide alamethicin forms transmembrane pores. However, this is still considered as the only peptide inducing membrane permeabilization via the barrel-stave model.[51]

The toroidal-pore model is somewhat similar to the barrel-stave model since this model describes multiple peptides binding and insertion into the microbial membrane. However, these peptides induce bending of the membrane lipids leading to the formation of a pore in which the inner side of the pore is formed by peptides as well as head groups of the lipids (see figure 5b).[3,8] This model is first described by K. Matsuzaki *et al.* based on pore formation in lipid bilayers by the helical AMP Magainin, isolated from amphibians.[52]

The carpet model describes a mechanism of membrane disruption in which peptides do not insert into the hydrophobic core of the membrane. Dermaceptin, also isolated from amphibians, orients itself parallel to the outer layer of the lipid bilayer.[53] Binding is caused by electrostatic interactions and multiple of these peptides cover the membrane as if it were a carpet. When the concentration of peptides reaches a certain threshold, membrane integrity is lost leading to disruption of the membrane in a detergent-like manner which can lead to the formation of micelles (see figure 5c).[3,8]

The group of Henzler Wildman *et al.* (2003) studied how LL-37 interacts and disturbs bacterial membranes. By the use of solid-state NMR and differential scanning calorimetry the interaction of LL-37 with lipid bilayer models was determined. The study indicated that LL-37 works via the toroidal pore mechanism. It interacts at the surface of the lipid bilayers where it is located between headgroups of phospholipids. Furthermore the interaction of LL-37 was much stronger to negatively charged lipid bilayers compared to zwitterionic bilayers. This illustrates the importance of the electrostatic interaction between membranes and AMPs.[38]

In contrast to LL-37, the mechanism of membrane disruption by defensins is not clarified. However, several studies are performed showing that defensins are able to disturb membrane integrity of negatively charged lipid bilayers.[3,54,55] A model is proposed in which defensins fuse lipid bilayers of different cells and thereby causing lysis.[54] Moreover, it was shown that defensins are able to form ion channels in model lipid bilayers which could also explain its microbicidal activity.[56]

Histatins show mainly antimicrobial activity against fungi. A complete different mechanism of antimicrobial action is defined for histatins compared with the models described above. Studying the effect of histatin-5 on *Candida albicans* cells, showed that the peptides are taken up into the cells where they are targeted to the mitochondria.[57] Mitochondrial respiration is inhibited but more importantly, reactive oxygen species are formed. ROS formation, induced by histatin cell entry, is the event leading to cell death rather than membrane rupture.[30] Besides fungi, histatins are found to be active towards bacteria.[58] However, whether these peptides act against bacteria via this specific mechanism remains to be elucidated.

Resistance to AMP activity

Bacteria have developed several strategies enabling them to invade multi-cellular organisms. These include proteins that mediate attachment to host cells, secretion of toxins that damage host tissues and factors mediating evasion of host defense mechanisms (reviewed in refs.[59,60]). Accordingly, several bacteria are able to resist AMP activity via diverse avoidance mechanisms.

In order to target the cytoplasmic membrane of Gram positive bacteria, AMPs have to attach and cross the thick peptidoglycan layer

of the bacterial cell wall. By modifying the negative charge of the cell wall, electrostatic affinity is lost for the positively charged AMPs (reviewed in ref.[60,61]). Several factors from Gram positive bacteria are identified that mediate CWG modification. In some bacteria, the negative charge of teichoic acids or lipotechoic acids are neutralized. This is done by modification with D-alanine residues which have a positively charged amino group. In many Gram positive bacteria, including Group A streptococcus, it was shown that D-alanylation of CWGs indeed resulted in resistance towards AMPs.[60-62] *Staphylococcus aureus* uses an additional strategy to neutralize its cell wall in order to establish AMP resistance. It has the ability to link the positively charged amino acid L-lysine to its CWGs.[63] Gram negative bacteria have developed similar strategies to avoid AMP electrostatic attraction. The membrane component of LPS has negatively charged phosphate groups and several Gram negative bacteria place positively charged amino sugars into this region of LPS thereby neutralizing the negative charge of the cell wall (reviewed in ref.[59,60]).

Another mechanism of AMP resistance by bacteria is the production of proteases that degrade AMPs. Both Gram positive and negative bacteria produce such proteases. In particular simple helical structured AMPs are susceptible for microbial proteases.[60] Accordingly, the disulfide bridges in defensins are considered to protect them from proteolytic degradation rather than that these bonds are required for their microbicidal function.[18,60] In addition, bacteria have evolved other strategies that impair the activity of AMPs. Most of these mechanisms are AMP specific because they depend on peptide recognition and inactivation. Some strains of *S. aureus* produce the protein staphylokinase which can interact and abrogate the activity of α-defensins. Via this mechanism, survival of S. aureus in the host is promoted.[64] Another Gram positive bacteria, *Streptococcus pyogenes*, secretes the protein SIC (streptococcal inhibiter of complement). This protein was identified to inhibit complement mediated killing and was later found to bind and inhibit the activity of AMPs in addition.[65]

Some Gram negative bacteria have the ability to actively export microbicidal components by the use of an efflux-pump in their cell wall. It was found that *Neisseria gonorrhoea* is more sensitive to the microbicidal activity of LL-37 in absence of this pump.[66] In addition, in *Neisseria meningitides* susceptibility for LL-37 is decreased in strains with an efflux pump.[67] LL-37 and perhaps other AMPs are substrates for efflux pumps and thereby prevented from membrane disruption. However, not all bacterial efflux pumps provide protection for AMP activity. Susceptibility for AMPs was determined in the Gram negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* and in the Gram positive *S. aureus* overexpressing their endogenous efflux pumps. Interestingly, overexpression of the efflux pump did not mediate AMP resistance.[68]

Discussion

Antimicrobial peptides provide an important innate barrier against a broad range of microorganisms. Although AMPs have a dual function, this review focuses on the direct antimicrobial function. Structural and functional properties of the human AMPs as well as structural features of bacterial cell walls are discussed in this review. Knowledge about a combination of these two components leads to a better understanding of how AMPs are able to kill microorganisms. However, there is a lot that remains to be established.

The interaction of AMPs with the bacterial cytoplasmic membrane is considered to be responsible for the direct bactericidal mechanism of AMPs. However, AMPs have to cross the peptidoglycan layer of Gram positive and the outer membrane of Gram negative bacteria prior to gain access to this cytoplasmic membrane. Although this is an essential concept, it is rarely addressed in mechanical studies.[3] To investigate the mechanical activity of direct antibacterial killing of AMPs, most studies use model membranes rather than bacterial cells.[38] Naturally, these model membranes are not an exact representation of bacterial cell walls as they lack cell wall proteins and the peptidoglycan layer.

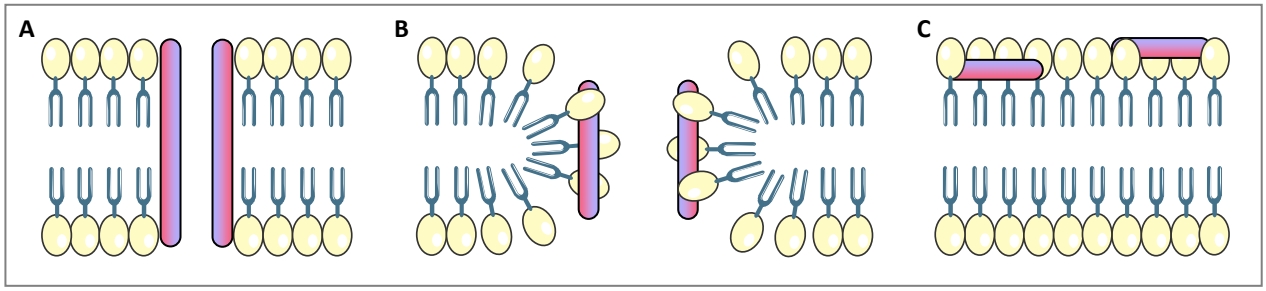

Figure 5 | AMP mechanism of membrane interference
This figure shows schematically the three main models of microbial membrane disturbance by AMPs. The lipid bilayers represent the cytoplasmic membrane of both Gram negative and positive bacteria, the blue-red colored rods represent AMPs in which hydrophilic regions are blue and hydrophobic regions are red. **A)** The barrel-stave model describes how helical peptides attach and insert into the membrane upon peptide aggregation. The peptides are aligned with the phospholipids of the membrane, with the hydrophobic region of the AMP facing the core of the lipid bilayer. This way, a pore is formed resulting in leakage of cellular components and subsequently cell death. **B)** The toroidal pore model differs from the barrel-stave model in a way that the inserting AMPs lead to bending of the phospholipids. The pore that is formed is lined with both the hydrophilic regions of the AMPs and the headgroups of the phospholipids. **C)** The carpet model describes how peptides are able to disrupt membrane integrity leading to complete disruption of the membrane. Peptides are attracted to the membrane by electrostatic interactions. They can integrate between the headgroups of the phospholipids with the hydrophobic regions facing the lipid tails. By this superficial integration of AMPs, microbial membrane integrity is lost.

Given the fact that AMPs are microbicidal *in vivo*, they must be able to cross these structures somehow to reach the cytoplasmic membrane subsequently to induce cell death. How the external structures are crossed remains to be resolved.

Three basic models of direct bacterial killing by AMPs are proposed. These models explain how microbial membranes are disturbed by rupture or pore formation. However, other models of microbial killing are likely to exist in addition to the three basic models. The mechanical studies on the mode of action of histatins for example revealed that in fungi mitochondria are targeted rather than the cytoplasmic membrane.[57] Moreover, one of the cathelicidin peptides from cows, bac7, was found to be unable to permeabilize the membrane of *E. coli* cells. Interestingly, bac7 was sufficient to cause a reduction in the number of bacteria (as reviewed in refs.[3,69]). Although the generally accepted models of microbial killing by AMPs are still based on their membrane interfering capacities, more and more studies reveal other microbial targets of AMPs.[3]

Table 1b lists the activity of human AMPs against a broad range of microorganisms. It seems like some defensins have activity towards very few microorganisms (HNP-4, HD-5 and HD-6). However, this is probably an effect of the lack of studies performed on these specific defensins. Many studies on human defensins rely on the purification of these peptides directly from tissues. Since HNP-4 and HD-6 are less abundantly present, it is more difficult to obtain an adequate amount of the specific AMP which could explain the lack of knowledge.[70] Currently, the synthesis of HNP-4, HD-5 and HD-6 is reported which could contribute to a better understanding of the function of these peptides.[70]

There is a growing need for new antibiotics since bacteria are able to acquire resistance against antibacterial drugs.[71,72] Knowledge on the antimicrobial mechanisms of AMPs can be of great importance. Naturally occurring peptides can be used therapeutically although this has not been a great success in clinical studies thus far.[39,73,74] Disadvantages of the use naturally occurring peptides can be that they cause toxicity by their ability to modulate the overall immune system or that they are not solely selective for the bacterial cell membrane. Moreover, these peptides can be substrates for proteases creating poor pharmacokinetics.[73] The disadvantages can possibly be overcome by the use of synthetic mimics of antimicrobial peptides (SMAMPs). SMAMPs possess the essential properties of AMPs including amphipathicity, overall negative charge and size. Additionally, they are selective for bacterial cells.[75] AMPs possess many potent properties to be used as new antibacterial drugs, however more studies need to be performed. The understanding of the direct antimicrobial activity of AMPs is far from complete as well as the immune modulating function.

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