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Mammals' humoral immune proteins and peptides targeting the bacterial envelope: from natural protection to therapeutic applications against multidrug-resistant Gram-negatives

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ABSTRACT

Mammalian innate immunity employs several humoral 'weapons' that target the bacterial envelope. The threats posed by the multidrug-resistant 'ESKAPE' Gram-negative pathogens (*Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) are forcing researchers to explore new therapeutic options, including the use of these immune elements. Here we review bacterial envelope-targeting (peptidoglycan and/or membrane-targeting) proteins/peptides of the mammalian immune system that are most likely to have therapeutic applications. Firstly we discuss their general features and protective activity against ESKAPE Gram-negatives in the host. We then gather, integrate, and discuss recent research on experimental therapeutics harnessing their bactericidal power, based on their exogenous administration and also on the discovery of bacterial and/or host targets that improve the performance of this endogenous immunity, as a novel therapeutic concept. We identify weak points and knowledge gaps in current research in this field and suggest areas for future work to obtain successful envelope-targeting therapeutic options to tackle the challenge of antimicrobial resistance.

Key words: multidrug-resistant Gram-negatives, ESKAPE pathogens, bacterial envelope, cell wall, peptidoglycan, bacterial membranes, mammalian humoral innate immunity, lysozyme, peptidoglycan recognition proteins, cationic antimicrobial peptides

CONTENTS

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I. INTRODUCTION

New therapeutic solutions are urgently needed to counter the clinical–epidemiological threat posed by the 'ESKAPE' Gram-negative species [*Klebsiella pneumoniae* (KP), *Acinetobacter baumannii* (AB), *Pseudomonas aeruginosa* (PA), and *Enterobacter* species (ES)] which have high levels of virulence, intra-hospital dissemination, morbi-mortality and antibiotic resistance (Mulani *et al.*, 2019; Tacconelli & Pezzani, 2019). As our therapeutic arsenal is becoming obsolete and the release of new antibiotics is increasingly rare (Provenzani *et al.*, 2020), the design of successful therapies must be approached from all perspectives, including learning from our own immune system (Theuretzbacher *et al.*, 2020). The design of treatments mimicking the modes of action of the immune system against infection is an old idea, but has only been partially exploited, with many research lines discontinued for a variety of reasons (Zelechowska, Agier & Brzezińska-Błaszczyk, 2016; Kumar, Kizhakkedathu & Straus, 2018). However, given our current need for new therapeutic options, recent research has resumed several of these projects. In addition, novel approaches involving natural immune defences, linked to scientific–technical advances such as the development of artificially modified proteins/peptides, nanoparticles, etc., and to recently discovered immune components, now together provide a large body of encouraging evidence (Sola *et al.*, 2020; Theuretzbacher *et al.*, 2020; Upert *et al.*, 2021). In this review we gather and discuss these advances, specifically regarding bacterial envelope-targeting humoral innate immunity.

The cell wall (comprising *sensu stricto* the outer membrane, periplasm, and peptidoglycan) of Gram-negative bacteria is the structure most exposed to the host's innate immunity and therefore, this weapon–target interplay is an obvious source of therapeutic potential (Mishra *et al.*, 2017). However, although many of the immune components reviewed herein are believed to target the cell wall (outer membrane and/or peptidoglycan), at least some of these proteins/peptides may also target the bacterial inner membrane and hence, the more inclusive concept of 'bacterial envelope'-targeting immunity is used herein (the 'bacterial envelope' includes the inner and outer membranes as well as the peptidoglycans) (Silhavy, Kahne & Walker, 2010). The humoral 'weapons' referred to here generally act by disturbing bacterial membranes and/or peptidoglycan through a variety of mechanisms, causing loss of the permeability barrier, cytosolic content, osmotic balance and resistance to osmotic pressure, leading to cell lysis. Moreover, several bacterial envelope-targeting peptides/proteins also display immunomodulatory features that may enhance their therapeutic potential. Among these, the capacity for lipopolysaccharide (LPS) neutralization (i.e. anti-endotoxin activity) is of particular interest because of its anti-inflammatory and septic-shock-preventive effects (Mishra *et al.*, 2017; Pachón-Ibáñez *et al.*, 2017; Theuretzbacher *et al.*, 2020).

Here we review mammalian Gram-negative envelope-targeting immune components that display the greatest antimicrobial therapeutic potential, including anti-biofilm applications. Although the complement system could fit into this definition because of its membrane perforation-mediated mode of action, given the complexity of its activation/regulation pathways and its cascade nature, we do not include it herein. Moreover, to our knowledge there is no research on compounds mimicking the complement membrane-attack complex as a therapeutic treatment.

Following our discussion of the general features and activity in the host of the main bacterial envelope-targeting innate humoral components, we review recent research regarding experimental therapeutics harnessing this immunomodulatory and especially bacteriolytic potential. We review studies based on exogenous administration (including artificially modified derivatives and combinations with antibiotics) but also the discovery of bacterial/host targets that enhance the performance of our endogenous immunity. We discuss the knowledge gaps, obstacles to overcome and avenues for further development to enable these treatments to advance towards clinical practice. We specifically focus this review on data regarding the ESKAPE Gram-negative pathogens, for which the development of new therapeutic treatments is most urgent. This review is intended to be a resource for researchers investigating bacterial envelope-targeting immunity and for those working in the fields of antimicrobial resistance and the development of new therapies, with the goal of defeating one of the greatest health threats of the 21st century: antibiotic resistance.

II. BACTERIAL ENVELOPE-TARGETING MAMMALIAN HUMORAL INNATE IMMUNITY: GENERAL FEATURES AND NATURAL PROTECTIVE ACTIVITY

Bacterial envelope-targeting immunity could be classified according to the predominant target (peptidoglycan *versus* membranes), but the presence of immune elements with simultaneous modes of action on both makes this classification complex (Raheem & Straus, 2019). In fact, although these immune elements interact, at least initially, with the bacterial envelopes as part of their mechanism of action, the concept that this interaction always leads to permeabilization and cell lysis is no longer valid. Moreover, some of these immune elements act in parallel on cytoplasmic targets and/or by disturbing cell division (Graf & Wilson, 2019; Raheem & Straus, 2019). We do not include in this review a discussion of immune elements that act exclusively against cytosolic targets (Cardoso *et al.*, 2019), those that act exclusively against Gram-positive species, nor artificially synthesized compounds that attack bacterial envelopes. A general overview of the elements included in this review is provided in Fig. 1.

(1) Peptidoglycan-targeting proteins

(a) *Lysozyme*

Although three major types of lysozymes have been described in the animal kingdom (c-type, g-type, and i-type), this review focuses on the conventional (c-type) form, predominant in mammals and considered the archetypical variant (Callewaert & Michiels, 2010). c-type lysozymes (see Table 1) are produced by several tissues and leukocytes that release the protein into body fluids (Callewaert & Michiels, 2010). c-type lysozymes display a globular structure (*ca.* 140 amino acids in length) with a long cleft accommodating the active site. Their classically attributed mechanism of action is peptidoglycan enzymatic disruption (Fig. 2): cleavage of the β-(1,4)-glycosidic bond between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) of the peptidoglycan's sugar chains, disturbing its structure and causing osmotic lysis (Juan *et al.*, 2018; Torrens *et al.*, 2020). Some authors propose instead that this muramidase enzymatic activity functions in the clearance of free peptidoglycan fragments to exert anti-inflammatory effects [i.e. avoiding their detection by certain pathogen-associated pattern recognition receptors (PRRs)], hence arguing against a significant antimicrobial action. Lysozyme bactericidal capacity then was suggested to be driven by membrane permeabilization, achieved through a positively charged helix–loop–helix motif (amino acids 87–115 in the human protein). This region enables the establishment of electrostatic interactions, allowing c-type lysozyme to cross the external bacterial membrane *via* self-promoted uptake and the formation of channels (Ibrahim, Thomas & Pellegrini, 2001; Ganz *et al.*, 2003; Nash *et al.*, 2006; Ragland & Criss, 2017; Juan *et al.*, 2018; Torrens *et al.*, 2020). This remains controversial however, since some studies suggest that this self-translocation allows lysozyme to reach the Gram-negative peptidoglycan, enabling its enzymatic breakdown. This activity would obviously be enhanced in the presence of immune elements that act as outer membrane permeabilizers, as happens *in vivo* (Ellison & Giehl, 1991; Callewaert & Michiels, 2010; Heesterbeek *et al.*, 2021).

Regardless of its mode of action, this protein provides an efficient early defence against ESKAPE Gram-negatives; lysozyme gene deletion and/or protein depletion was clearly related to increased severity of PA infection, and transgenic mice with higher expression levels of lysozyme in their airways exhibited milder infection-derived parameters (mortality, systemic dissemination and bacterial load) (Akinbi *et al.*, 2000; Markart *et al.*, 2004a; Cole *et al.*, 2005). Similar results were observed even with a muramidase activity-deficient lysozyme variant against KP (Markart *et al.*, 2004b; Nash *et al.*, 2006). It is important to note that lysozyme has additional functions besides its bactericidal effects. Its involvement in processes such as wound healing, immune modulation, inflammatory regulation and angiogenesis highlight this protein as a potential target for interventions to ameliorate the outcomes of infection, such as avoiding excessive inflammation that can be detrimental to the patient in

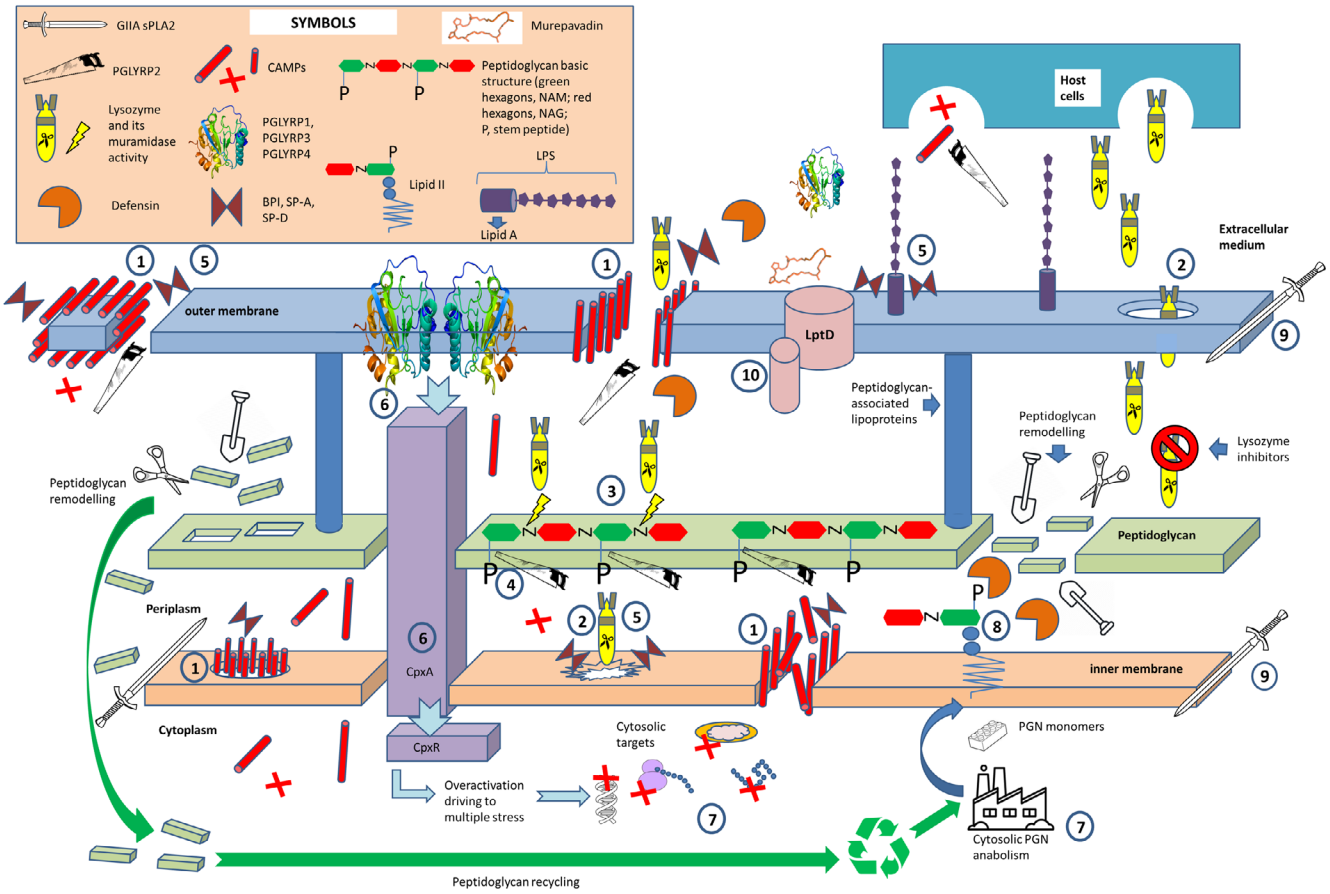

Fig. 1. Overview of the major bacterial envelope-targeting components of the mammalian innate humoral immune system, and of the artificial derivative murepavadin, and the specific bacterial structures/processes that they attack. Numbers within circles represent different targets and/or mechanisms of action: (1) disturbance of outer and inner membranes; (2) lysozyme-driven self-translocation and membrane permeabilization; (3) lysozyme muramidase activity; (4) cleavage site for PGLYRP2 enzymatic (amidase) activity; (5) binding of BPI, SP-A, and SP-D to LPS and participation in membrane disturbance; (6) sensing of outer membrane-bound PGLYRPs (monomers or dimers) by the two-component CpxA sensor system and signal transduction towards the cytosolic effector CpxR, leading to overactivation, multiple stressors, and thereby to cell suicide; (7) antimicrobial peptides and CpxAR overactivation-driven alteration of cytoplasmic processes such as nucleic acid synthesis, protein synthesis, protein folding, energy metabolism, and cytosolic steps of peptidoglycan synthesis; (8) although they are CAMPs, the particular mode of action of specific defensins (HNP-1 and hBD-3) is shown: blockade of lipid II-mediated incorporation of new building units into peptidoglycan; (9) enzymatic degradation of membrane phospholipids by GIIA sPLA2; (10) murepavadin specifically binds PA LptD membrane protein and thereby blocks LPS component translocation and outer envelope biogenesis. Abbreviations: BPI, bactericidal permeability increasing protein; CAMPs, cationic antimicrobial peptides; GIIA sPLA2, Group IIA secretory phospholipase A2; hBD-3, human β–defensin 3; HNP-1, human neutrophil peptide 1; LPS, lipopolysaccharide; NAG, N-acetylglucosamine; NAM, N-acetylmuramic acid; PGLYRP, peptidoglycan recognition protein; PGN, peptidoglycan; SP-A/-D, surfactant-associated protein A/D.

some contexts (Ganz *et al.*, 2003; Markart *et al.*, 2004b; Ragland & Criss, 2017).

(*b*) *Peptidoglycan recognition proteins (PGLYRPs)*

Four PGLYRPs (see Table 1) have been described in mammals: PGLYRP1, 2, 3, and 4, formerly named PGLYRP-S, −L, -Iα, and -Iβ, respectively (Torrens *et al.*, 2020). Although these proteins were initially named after their capacity for peptidoglycan binding *in vitro* and their role as peptidoglycan recognition receptors, they display additional domains to bind other molecules such LPS. Later, PGLYRPs were shown not only to be PRRs but also to display antibacterial activity through different modes of action (Dziarski & Gupta, 2018). The largest variant is PGLYRP2, with an approximate length of 550–650 amino acids depending on the species, and which is mainly produced in the liver and enters the serum as a monomer (Wolf & Underhill, 2018). This protein displays amidase activity, i.e. cleavage of the bonds between N-acetylmuramic acid and the stem peptides (Fig. 2) (Torrens *et al.*, 2020). The role of this activity is controversial. Some authors argue that causes only minor

Table 1. Overview of mammalian peptidoglycan-targeting innate humoral immune proteins and their therapeutic potential. See main text for additional references

Immune element	Main features and examples/variants	Mode of action	Activity (*in vitro*/*in vivo*) against ESKAPE Gram-negatives	Artificially derived variants, research lines, therapeutic potential
Lysozyme (c-type)	*Ca.* 140 aa. Produced in the skin, liver, granulocytes and monocytes and secreted into fluids. Highly concentrated in milk and tears. Production inducible upon multiple trauma or infection. Displays important immunomodulatory properties.	(*i*) Muramidase activity: cleavage of β-(1,4)-glycosidic bonds between NAM and NAG. (*ii*) Membrane-disrupting capacity, linked to its cationic helix–loop–helix motif establishing electrostatic interactions with external membrane, self-promoted uptake, and formation of channels through cytoplasmic membrane.	Variable (usually mild, with the exception of AB) *in vitro* activity against ESKAPE Gram-negatives unless certain adjuvants (polymyxins, docosahexanoic acid) are added. Different murine models of infection show encouraging results in terms of bacterial burden and reduction of inflammatory variables. High anti-biofilm activity against KP, poor against PA if not combined with cephalosporins (Eladawy *et al.*, 2020).	Modified versions created to avoid electrostatic sequestration (e.g. 2-3-7), and conjugate/nanoparticle formulations (e.g. LYZOX) to protect against proteolysis. Artificially designed variants to circumvent bacterial lysozyme inhibitors (Dostal *et al.*, 2015). Bacterial targets leading to increased susceptibility: PA PGN recycling, BamB and FabY; AB capsule. Boosting the expression of host lysozyme could increase antimicrobial activity and avoid harmful over-inflammation.
Peptidoglycan recognition protein 2 (PGLYRP2)	*Ca.* 90 kDa. Constitutively produced by the liver and released into blood as a monomer. Inducible in certain epithelia.	N-acetylmuramoyl-L-alanine amidase activity: breaks down PGN by cleaving stem peptide-NAM bonds.	Modest *in vitro* activity against PA. Addition of a permeabilizer increases activity up to 100-fold, more so in mutants with impaired PGN recycling. No data for other ESKAPE Gram-negatives.	Depending on the pathogen and context exerts a protective effect. In other cases, it triggers an inflammatory excess that is harmful to the host.
PGLYRP1, 3, and 4	PGLYRP1 (*ca.* 20 kDa.) secreted by granulocytes as monomer or homodimer. PGLYRP3/4 (both *ca.* 45 kDa) are secreted by skin/mucous epithelia as monomer, homodimer, or heterodimer.	Binding to LPS and outer membrane causing over-activation of the stress-sensing two-component CpxAR system. This induces the cessation of nucleic acid, protein, and PGN synthesis, leading to cell suicide.	Same as PGLYRP2.	Participation in microbiome homeostasis and inflammatory regulation. PGLYRP1-null mice display enhanced severity compared to wild-type animals in PA keratitis model.

aa, amino acid; ESKAPE, *Klebsiella pneumoniae* (KP), *Acinetobacter baumanii* (AB), *Pseudomonas aeruginosa* (PA) and *Enterobacter* species (ES); LPS, lipopolysaccharide; NAG, N-acetyl glucosamine; NAM, N-acetyl muramic acid; PGN, peptidoglycan.

impacts to intact peptidoglycan sacculi and thus would function to degrade free fragments, hence exerting a scavenger anti-inflammatory function [theoretically reducing stimulation of nucleotide-binding oligomerization domain-like receptors (NOD)] (Boneca, 2009). Others propose that it exerts bactericidal activity through peptidoglycan disruption, mainly against Gram-positives (Gelius *et al.*, 2003; Lee *et al.*, 2012; Bobrovsky *et al.*, 2016). Its large size likely hinders penetration through the external Gram-negative envelope, and thus its lytic activity is probably modest in the absence of a permeabilizer (Torrens *et al.*, 2017; Torrens *et al.*, 2020). Therefore, PGLYRP2 has been described as largely dispensable in terms of mammalian protection against Gram-negative infection (Xu, Wang & Locksley, 2004).

PGLYRP1 (*ca.* 200 amino acids), 3, and 4 (*ca.* 350–400 amino acids) do not show an enzymatic mode of action, nor do they interact directly with peptidoglycan when challenged with live Gram-negative bacteria, which appears

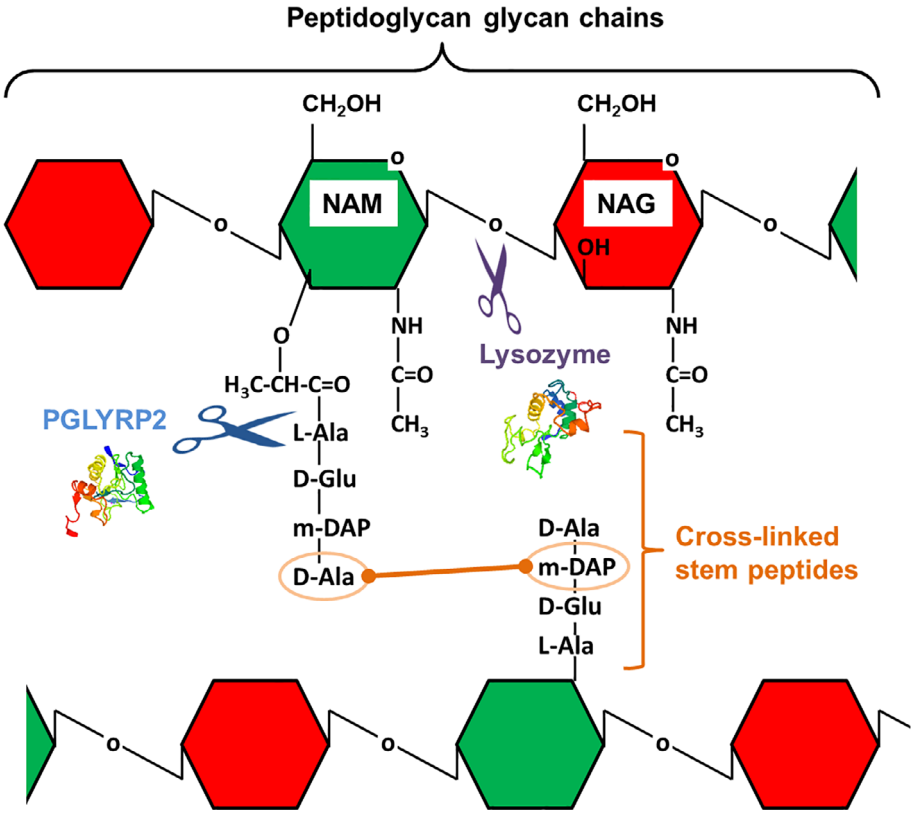

Fig. 2. Mechanism of action (cleavage sites) of two peptidoglycan-lytic mammalian immune proteins, lysozyme and peptidoglycan recognition protein 2 (PGLYRP2). The Gram-negative peptidoglycan, also known as murein sacculus, is composed of glycan chains (consisting of repeated NAG–NAM units) connected to other such chains through crosslinked stem peptides bound to the NAM units. Lysozyme (purple scissors, muramidase activity) cleaves the β-(1,4)-glycosidic bond between the NAM and NAG molecules, whereas PGLYRP2 (blue scissors, amidase activity) cleaves the bonds between NAM and L-Ala in the peptide chains. Abbreviations: Ala, alanine; Glu, glutamic acid; M-DAP, meso-di- amino-pimelic acid; NAG, N-acetylglucosamine; NAM, N-acetylmuramic acid.

paradoxical. Instead, they seemingly bind to LPS and the outer membrane, where they stimulate the bacterial two component-system CpxAR, described to respond to envelope stress caused by changes in pH, composition, presence of misfolded proteins and β-lactams (Dziarski & Gupta, 2018; Masi, Pinet & Pagès, 2020). Over-activation of this system causes simultaneous and synergistic oxidative, thiol, and metal stresses, leading to bacterial suicide associated with the blockade of synthesis of nucleic acids, proteins, and peptidoglycan building units (Dziarski, Kashyap & Gupta, 2012; Kashyap *et al.*, 2014; Dziarski & Gupta, 2018) (Fig. 1). Interestingly, some authors proposed that PGLYRP2 may also drive CpxAR over-activation in certain species (Bobrovsky *et al.*, 2016). Whereas PGLYRP1 is mainly produced by granulocytes, PGLYRP3 and PGLYRP4 are principally secreted in skin and mucosal epithelia. These three variants can be found as monomers or disulfide-linked homodimers, although the formation of PGLYRP3:PGLYRP4 heterodimers is also common (Torrens *et al.*, 2020). PGLYRPs have other roles in addition to bacterial killing, such as participation in homeostasis of the microbiome and regulation of inflammation (Royet, Gupta & Dziarski, 2011; Dziarski *et al.*, 2012). This topic is very complex especially for PGLYRP2 which, depending on the pathogen and/or context, can either exert a protective anti-inflammatory effect (Saha *et al.*, 2010; Lee *et al.*, 2012) or trigger a harmful inflammatory excess (Boneca, 2009; Saha *et al.*, 2009; Gowda *et al.*, 2015).

Regarding the direct bactericidal capacity of PGLYRPs against ESKAPE pathogens *in vivo*, the available data refer only to murine models of PA keratitis. In these studies, the only protein shown to be significantly defensive against PA was PGLYRP1, since KO mice displayed enhanced infection severity. Whereas PGLYRP3 and PGLYRP4 displayed a minor protective role, PGLYRP2 was shown paradoxically to contribute to a poor clinical outcome, triggering an excessive inflammatory response and curbing the expression of other antimicrobial peptides (Ghosh *et al.*, 2009; Gowda *et al.*, 2015). There is an apparent lack of research assessing the natural activity of these proteins against KP, AB or ES, a gap in our knowledge worthy of filling to ascertain the therapeutic potential of these proteins against these species.

(2) Membrane disturbance-based immune components

In this section, we consider bacterial envelope-targeting immune components that, regardless of additional modes of action, at least initially interact with and disturb the bacterial membranes. We divide them into peptides (<100 amino acids) and proteins (>100 amino acids, usually with complex and/or oligomeric structures and enzymatic activity) (Haney, Straus & Hancock, 2019), with their main features listed in Tables 2 and 3, respectively.

(a) *Cationic antimicrobial peptides*

Although mammals display an important array of anionic antimicrobial peptides (Harris, Dennison & Phoenix, 2009), they seemingly do not work through bacterial envelope-targeting mechanisms and therefore are not discussed here. Conversely, the antimicrobial peptides that target bacterial envelopes generally have a strong positive charge, and are generically termed cationic antimicrobial peptides (CAMPs). Although other names for these humoral components, such as 'innate immune peptides', could be appropriate due to their immunomodulatory roles beyond direct antimicrobial action, we here retain 'CAMP' due to its widespread use (together with 'host defence peptide') in recent decades. CAMPs usually display an amphiphilic structure, which enables them to be soluble in aqueous solutions, facilitating their defensive functions (Boparai & Sharma, 2019). Although they can be classified in several ways, here we refer to their predominant structure: α-helical, β-sheet, loop, or those with no regular secondary structure (known as 'extended' or 'flexible') (Kumar *et al.*, 2018; Amerikova *et al.*, 2019). Regardless of structure, a general mode of action can be attributed to CAMPs: due to their positive charge, they establish electrostatic interactions with the negatively charged outer membrane moieties such as LPS or the head-groups of the lipid bilayer (Krishnakumari *et al.*, 2020). These interactions disturb the structure of the external envelope, enabling CAMP penetration into the periplasm where it can also affect the inner membrane, finally leading to indiscriminate permeabilization and lysis. Three classic models explain this process (Fig. 3): (*i*) carpet, (*ii*) barrel-stave pore, and (*iii*) toroidal pore, although other variations have recently been described (see recent reviews by Kumar *et al.* (2018) and Lee, Hall & Aguilar (2016)]. Table 2 provides a general overview of the main mammalian bacterial envelope-targeting CAMP groups: defensins, histatins, cathelicidins, and lactoferrin-derived peptides.

(i) Defensins. The extensive group of defensins show a molecular mass of *ca.* 3.5–4.5 kDa, a β-sheet structure, and the typical presence of three disulfide bridges between six cysteine residues. The exact location of these bridges enables their classification into three sub-classes: α, β, and θ (Amerikova *et al.*, 2019). Representatives of α and β defensins are found in different mammals, whereas θ defensins are only produced by certain primate species (Krishnakumari *et al.*, 2003; Pasupuleti, Schmidtchen & Malmsten, 2012; Amerikova *et al.*, 2019). In humans, we find six variants of α defensins: four of these are known as human neutrophil peptides 1–4 (HNPs; produced as active and processed mature peptides by these leukocytes), and the other two (HNP-5 and HNP-6) are mainly produced in the intestinal Paneth cells (stored as immature propeptides and sometimes known as cryptdins) and female genital epithelia (Pasupuleti *et al.*, 2012; Amerikova *et al.*, 2019). Closely related to these CAMPs but displaying five cysteines with a distinctive spacing pattern as opposed to the canonical α defensin six-cysteine motif, rattusin (isolated from rat intestines) shows strong *in vitro* activity against Gram-negatives, including antibiotic-resistant strains, but further research is needed (Min *et al.*, 2017). The human array of β defensins (hBDs) is even broader: over 30 variants have been described, although only hBD-1 to −4 have been deeply characterized (Amerikova *et al.*, 2019). β defensins usually show inducible expression and are almost ubiquitous, being found in urogenital epithelia, mouth mucosa, respiratory tract, skin, testicles, stomach, neutrophils, etc. (Bals *et al.*, 1999a; Sang *et al.*, 2005; Amerikova *et al.*, 2019).

Within the group of defensins, besides a general membrane permeabilization-based mode of action, some variants attack other related targets in parallel, such as blocking a specific step of peptidoglycan metabolism (Amerikova *et al.*, 2019; Malin & De Leeuw, 2019). In this last role, one key intermediary for peptidoglycan construction is lipid II (undecaprenyl-pyrophosporyl-N-acetyl-muramic acid-pentapeptide-N-acetyl-glucosamine), which enables the incorporation of new building units (NAG–NAM pentapeptides) into the murein sacculus (Malin & De Leeuw, 2019). Once translocated to the periplasmic side of the cytosolic membrane, both HNP-1 and hBD-3 have been shown to bind to lipid II, sequestering it from further reactions and therefore blocking the incorporation of new material into the nascent peptidoglycan, which drastically weakens the resistance of the cell wall to osmotic pressure, causing lysis (Fig. 1). These defensins were initially reported to act preferentially on Gram-positives, but they also retain activity against Gram-negatives because of their permeabilization capacity that enables their entry into the periplasm (Oppedijk, Martin & Breukink, 2016; Grein, Schneider & Sahl, 2019). Meanwhile, hBD-5 has been shown to translocate into the bacterial cytoplasm and accumulate in the surroundings of cell poles, interfering with cellular division (Chileveru *et al.*, 2015).

Some defensins have been shown to be essential for natural protection against ESKAPE Gram-negatives in animal and/or *ex vivo* models. For instance, the thrombocyte-secreted hBD-3 participates in the response against KP in the context of infected wounds (Tohidnezhad *et al.*, 2011). A very important protection capacity against PA has been demonstrated for murine β defensins (mBD) 3 and 4 (homologs of hBD-2 and -3 respectively) in the cornea (Huang *et al.*, 2007a; Augustin *et al.*, 2011). Conversely, mBD1 seems expendable for protection against PA keratitis (Wu *et al.*, 2009). Similarly, an antibacterial role of hBD-2 on the ocular surface has been questioned, since its activity is affected by high NaCl concentrations such as those in tears (Huang *et al.*, 2007b). Defensins contribute notably to natural protection against ESKAPE Gram-negatives in the lung

Table 2. Overview of mammalian bacterial membrane-targeting innate humoral immune peptides (<100 amino acids) and their therapeutic potential. See main text for references

Immune element	Main features and examples/variants	Mode of action	Activity (*in vitro*/*in vivo*) against ESKAPE Gram-negatives	Artificially derived variants, research lines, and therapeutic potential
Defensins	*Ca.* 3.5–4.5 kDa. Ubiquitous inducible expression. Three subclasses; α, β, and θ. In humans: six variants of α-defensins (HNP-1 to 6), and over 30 β-defensins (hBD-1 to -5 are the best studied).	Bacterial membrane permeabilization. HNP-1 and hBD-3 also block the incorporation of new material into PGN. hBD-5 targets cell division.	Notable *in vitro* anti-ESKAPE Gram-negative activity (including biofilms). Several murine models of infection with varied outcomes. Synergistic effects with ciprofloxacin, lysozyme, and erythromycin.	Modified (shorter, chimeric, dimeric) versions provide better access to targets and resistance to proteolysis (e.g. BAS00127538 and 6jc48-1). Bioengineered skin replacements for burn infections. hBD-1-loaded cotton gauzes for wound KP infection.
Histatins	Two human variants (20–40 amino acids): Hst1 and Hst3. They provide shorter derivatives (Hst2, Hst4–Hst12) after digestion with oral proteases. Also found in certain primates.	Mitochondrial targeting. Membrane disruption as a specific mechanism against AB and PA.	Notable *in vitro* activity against ESKAPE Gram-negatives (including biofilms), except KP. Synergy with β-lactams.	Hst5 derivatives with increased resistance against degradation in CF sputum and antipseudomonal efficacy. Safe in clinical trials for the treatment of oral candidiasis.
Cathelicidins	Mainly stored in neutrophils. Fewer than 50 amino acids. Main variants/homologues: LL-37, CRAMP, PR-39, protegrins, indolicidin, bactenecins, and MAPs.	Membrane disruption. Some variants attack cytosolic targets. Significant immunomodulatory activity.	Variable *in vitro* activity depending on the cathelicidin/pathogen. Variable synergy with conventional antibiotics and anti-biofilm capacity. Several animal infection models with various administration routes and outcomes (Chennupati *et al.*, 2009).	Artificially modified variants with improved activity: LL-37 derivatives (KS-30, KR-20, KR-12, LLKKK, GKE, P60.4, SAAP-148, 17BIPHE2DP7); Bac5(1-23), Bac5(1-25); omiganan, iseganan, and murepavadin (these latter three under clinical trials). Formulations for abiotic surface coating, immobilization on nanoparticles or magnetic beads.
Lactoferrin-derived peptides	Lactoferricin: 25–50 residues depending on the species), obtained mostly through digestion by host pepsin. LF1-11: obtained through artificial proteolysis of human lactoferrin (amino acids 1–11, which form the first cationic domain of the protein). Lactoferrampin: obtained through artificial proteolysis of human lactoferrin (amino acids 268–284).	LPS binding and bacterial membrane disruption. LF1-11 additionally targets mitochondria.	Lactoferricin: mild anti-ESKAPE-activity (including anti-biofilm and synergy with antibiotics). No bacterial burden reduction in murine PA keratitis. LF1-11: notable activity against KP and AB, and synergy with antibiotics. Successful in murine models. Lactoferrampin: mild antipseudomonal power *in vitro*.	Shorter/computationally designed Lactoferricin variants with increased permeabilization power. Acylated analogues with higher synergistic, LPS-neutralizing and antipseudomonal activities. Immobilization on surfaces for the design of anti-colonization strategies on catheters or implants (Chen *et al.*, 2017). Lactoferricin–lactoferrampin chimera: strong activity against planktonic PA and biofilms, also displaying virulence-attenuating effects (Xu *et al.*, 2010).

CF, cystic fibrosis; CRAMP, cathelin-related antimicrobial peptide; ESKAPE, *Klebsiella pneumoniae* (KP), *Acinetobacter baumanii* (AB), *Pseudomonas aeruginosa* (PA) and *Enterobacter* species (ES); LPS, lipopolysaccharide; MAPs, myeloid antimicrobial peptides; PR-39, proline-arginine-rich-39.

(Porro *et al.*, 2001) and when their levels decrease below certain thresholds, the risk of infection increases. This has been reproduced in a murine model of PA infection of burnt skin (Kawasaki *et al.*, 2015), but is also visible in patients with chronic respiratory pathologies such as chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF), in which conditions such as an abnormally acidic pH impair the activity of defensins, favouring infection (Taggart *et al.*, 2003; Dalcin &

Table 3. Overview of mammalian bacterial membrane-targeting innate humoral immune proteins (>100 amino acids) and their therapeutic potential. See main text for references

Immune element	Main features and examples/variants	Mode of action	Activity (*in vitro*/*in vivo*) against ESKAPE Gram-negatives	Artificially derived variants, research lines and therapeutic potential
Bactericidal/ permeability increasing protein (BPI) and related proteins	*Ca.* 60 kDa. The cationic N-terminal fragment harbours antimicrobial properties. Mainly found within neutrophil granules, although its expression is inducible elsewhere. Other members of the BPI superfamily with antibacterial activity include: SPLUNC1 and BPIFA2 (both *ca.* 25 kDa and secreted in nasal/ oral cavities)	Binding to LPS through electrostatic interactions and penetration into inner membrane, leadng to its disturbance and dissipation of electrochemical gradients. Several roles beyond bacterial killing: anti-endotoxin, anti-angiogenesis, chemotaxis, and opsonization.	Therapeutic approaches with fragments of human BPI: rBPI-23 (first 199 amino acids), rBPI-21 (first 193 amino acids with Cys132Ala substitution), rBPI (10–193) (lacks the first nine rBPI-21 amino acids), showed enhanced activity *in vitro*. Different animal models provided encouraging results in terms of bacterial burden and inflammation reduction. Additive (not synergistic) effects with antibiotics.	Synthetic BPI derivatives: Asp190Ala substitution in murine BPI5. Betapep 33mers: betapep-25 (and shorter derivatives, with LD_{50} at nM ranges against PA). Subcutaneous administration of SC-4 improved survival in murine PA intraperitoneal infection model. SPLUNC1-derivative α4-short (24 residues, increased positive charge): important anti-KP/ AB/PA activity (including biofilms), and encouraging results in a murine model of PA respiratory infection. GL13NH2, GL14K, and D-GL13K (141–153 residues from BPIFA2 with different modifications): notable antipseudomonal power (including biofilms), and efficacy in murine models of septic shock, PA burn wound infection, and *G. mellonella*.
Group IIA secretory phospholipase 2 (GIIA sPLA2)	*Ca.* 14 kDa. Constitutively expressed in the surroundings of infection gates: Paneth cells, prostate epithelial cells, lacrimal glands, etc. Inducible expression elsewhere. Metabolizes endogenous phospholipids to enable the synthesis of pro-inflammatory eicosanoids.	Enzymatic hydrolysis of bacterial membrane phospholipids.	Notable antipseudomonal power not affected by high protein or salt concentrations. Different animal models showed the potential of this weapon as an exogenous administered option (not tested against ESKAPE Gram-negatives).	Transgenic mice over-expressing human sPLA2-IIA are resistant to infection by different pathogens (not ESKAPE Gram-negatives), which suggests that increasing the expression of this protein could be a valid therapeutic/ prophylactic measure.
Surfactant-associated proteins (SP-A and SP-D)	*Ca.* 25 and 37 kDa, respectively. Mainly produced by bronchiolar exocrine cells and type II pneumocytes, and other locations such as the eye. SP-A: hexamer of *ca.* 600 kDa. SP-D: tetramer of *ca.* 500 kDa. Different associations (multimers, trimers, dimers, monomers) may also appear.	Membrane permeabilization relying on binding to LPS sugar moieties. Work as opsonins leading to enhanced phagocytosis and superoxidative burst.	Pivotal role in the defence of the cornea against PA infection. Exogenous administration (or artificial induction of expression) significantly enhanced survival of mice/rats with lung KP/PA infection, respectively.	SP-A4: not directly bactericidal, but its administration in a murine model of infection caused a significant decrease in bacterial burden, inflammation, oedema, and tissue damage, linked to immunomodulatory and opsono-phagocytosis-enhancing capacities.

BPIFA2, BPI fold containing family A member 2; ESKAPE, *Klebsiella pneumoniae* (KP), *Acinetobacter baumanii* (AB), *Pseudomonas aeruginosa* (PA) and *Enterobacter* species (ES); LD_{50}, lethal dose 50; LPS, lipopolysaccharide; SPLUNC1, short palate, lung, and nasal epithelium clone 1.

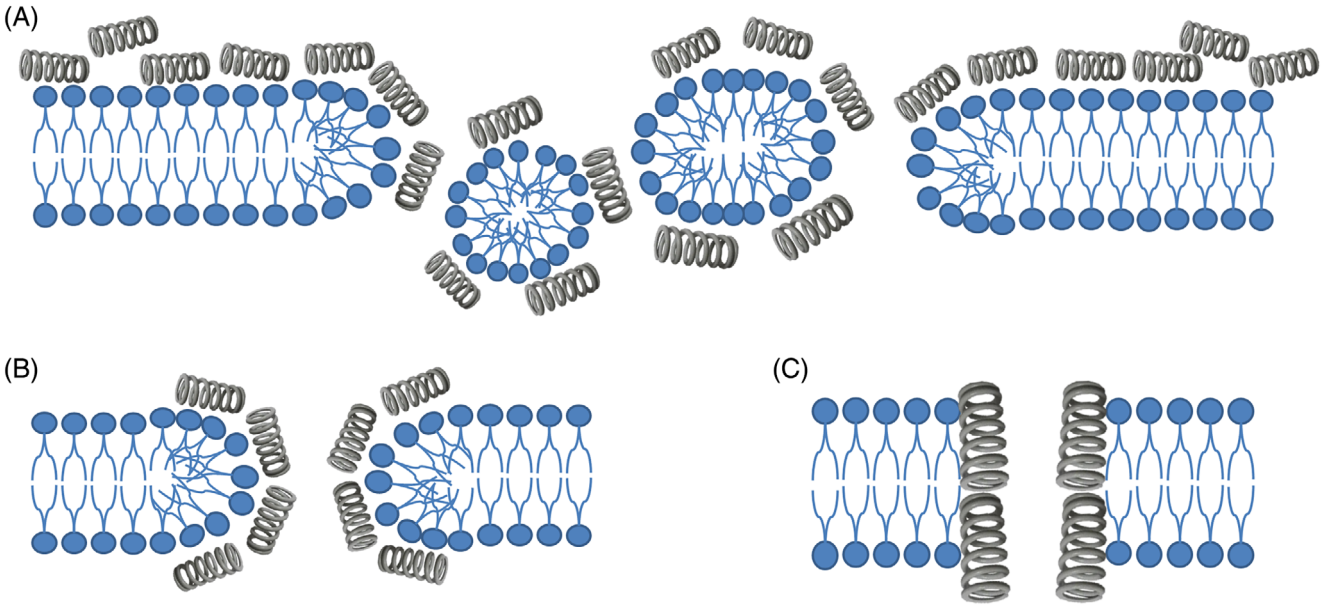

Fig. 3. The three main mechanistic models for the membrane disturbance-based activity of cationic antimicrobial peptides (CAMPs). (A) In the 'carpet model', CAMPs do not form a defined channel across the lipid bilayers but adhere to them in parallel, causing a detergent-like effect leading to the formation of micelles and membrane disintegration. Indolicidin and LL-37 both work through this mode of action. Some authors have proposed that this 'carpet' formation is also an essential step to trigger toroidal pore formation. (B) In the 'toroidal pore' model, CAMPs insert perpendicularly into the lipid bilayer and disrupt the regular segregation of polar (hydrophilic head-groups) and non-polar (hydrophobic hydrocarbon cores) segments of lipids, alternately exposing them in order to enable interaction with different sections of the CAMP. This leads to local curvature of the lipid bilayer, with the pores partially formed by the CAMP but also partially by the polar head-groups. No vertebrate CAMPs seem to act through this model. (C) In the 'barrel-stave pore' model, CAMPs are again inserted perpendicularly, but here their hydrophobic region only interacts with the membrane hydrocarbon core for self-assembly, thus not disorganizing the polar/non-polar distribution.

Ulanova, 2013; Alaiwa *et al.*, 2014; Arnason *et al.*, 2017). This has been demonstrated by a notable attenuation of hBD-2 production caused by certain contaminants on respiratory epithelia (Chen *et al.*, 2018b). The artificial overexpression of rat β-defensin 2 in rodent airways has been found to enhance natural protection against PA infection (Hu *et al.*, 2010; Shen *et al.*, 2014). Similar results have recently been obtained by using lentiviral vectors in mice to increase expression of hBD-2 and hBD-3, i.e. the mice showed ameliorated survival after skin flap infection by PA introduced through catheters (Casal *et al.*, 2019). Likewise, the overexpression of β-defensin 2 in rats improved the outcome of PA lung infection, demonstrating the important protection exerted by these CAMPs *in vivo* (Shen *et al.*, 2017). As recently demonstrated for diverse HNPs (Zheng *et al.*, 2018), besides their obviously beneficial bactericidal activity (McHugh *et al.*, 2019), defensins show pro-inflammatory properties which, depending on their levels and context of infection, can lead to excessive and harmful consequences for the host. Therefore, defensins have been suggested as a target for future personalized therapies (Crimi *et al.*, 2020).

(ii) Histatins. Another interesting group of bacterial envelope-targeting CAMPs is histatins, which were originally discovered in human saliva, although they are also produced by certain primates. Histatins are *ca.* 20–40 amino acids long and display an extended structure with a high proportion of histidines (Oppenheim *et al.*, 1988). There are two human variants (Hst1 and Hst3) that provide shorter derivatives (Hst2 and Hst4–Hst12) after digestion by oral proteases (Pasupuleti *et al.*, 2012). Interestingly, histatins seem to display different bactericidal mechanisms depending on the bacteria. For instance, Hst5-mediated killing of PA and AB appears to be exclusively linked to membrane permeabilization, whereas *Enterobacter cloacae* elimination requires mitochondrial targeting, therefore affecting energy metabolism (Du *et al.*, 2017). The scarcity of recent studies characterizing the natural protective activity of these CAMPs against ESKAPE pathogens is striking.

(iii) Cathelicidins. Peptides belonging to this large group are mainly stored in neutrophils and are formed by the enzymatic cleavage of the C-terminus of protein precursors showing homology with a porcine cysteine protease inhibitor called cathelin (Kościuczuk *et al.*, 2012; Haney *et al.*, 2019). Cathelicidins are highly heterogeneous and representatives are known that belong to any of the three predominant structures (α-helical, β-sheet and extended), many of them with notable membrane-permeabilizing activities (Travis *et al.*, 2000; Kumar *et al.*, 2018). Examples include the human LL-37 (Dürr, Sudheendra & Ramamoorthy, 2006), known to

act synergistically with other innate immune system components such as neuropeptides (Ohta *et al.*, 2011); cathelin-related antimicrobial peptide (CRAMP) in rats and mice (Kovach *et al.*, 2012); cationic antimicrobial protein 18 (CAP-18) in rabbits (Larrick *et al.*, 1994); porcine antibacterial peptides (PMAPs) (Scheenstra *et al.*, 2019), prophenins (PFs) (Wang *et al.*, 2004), protegrins (PGs) (Lazaridis, He & Prieto, 2013), salivary pig E (SP-E) (Ciociola *et al.*, 2018), tritrpticin, and proline-arginine-rich 39 (PR-39) in pigs (Mishra *et al.*, 2018); bovine myeloid antimicrobial peptides (BMAPs) and indolicidin in cattle (Mishra *et al.*, 2018); ChMAPs in goats (Panteleev *et al.*, 2018); SMAPs in sheep (Brogden *et al.*, 2001); bactenecins (a group that includes proline-rich Bac5 and Bac7 peptides, for instance) in several ruminants (Shamova *et al.*, 2016); an extraordinary diversity in marsupials (Peel *et al.*, 2016); etc. Since an exhaustive description of all of these would be excessive, only those used for experimental therapeutics are reviewed in Sections III.2 and III.3. As explained above, some immune components are known to interfere with certain intracellular processes in parallel to their envelope-targeting activity and similar examples can be found within cathelicidins: indolicidin, which inhibits DNA synthesis; the proline-rich Bac5/Bac7 peptides that inhibit protein synthesis by binding to ribosomes; and PR-39 which blocks both processes (Cardoso *et al.*, 2019).

(iv) Lactoferrin-derived peptides. Another example of a protein precursor that can produce different CAMPs depending on the proteolytic process is lactoferrin, which is a secreted globular glycoprotein (≈80 kDa) with an iron sequestration-based (non-cell wall lytic) antimicrobial activity (Cutone *et al.*, 2019; Gruden & Poklar Ulrih, 2021). Besides the two bacterial envelope-targeting peptides obtained from lactoferrin by artificial digestion (LF1-11 and lactoferrampin, see Section III.2*b.iv*), the loop-structured lactoferricin is naturally released mainly through proteolysis by host pepsin (Bellamy *et al.*, 1992; Van Der Kraan *et al.*, 2004; Bruni *et al.*, 2016; Amerikova *et al.*, 2019). Human lactoferricin is a single chain corresponding to residues 1–49 of whole lactoferrin [including two-disulfide bridges (Hunter *et al.*, 2005)] or residues 17–41 with an inner disulfide bridge in the case of bovine lactoferricin (Gifford, Hunter & Vogel, 2005). Human lactoferricin has also been proposed to comprise lactoferrin residues 1–47, separated into two chains linked through a disulfide bond (Bellamy *et al.*, 1992; Wakabayashi, Takase & Tomita, 2005). The capacity of lactoferricin for LPS binding and membrane alteration lies in a well-defined 11-amino acid amphipathic α-helical region (Gifford *et al.*, 2005; Wakabayashi *et al.*, 2005; Bruni *et al.*, 2016), but its translocation to reach cytosolic targets has also been proposed (Haukland *et al.*, 2001). Given this evidence, although the power of lactoferricin cannot easily be differentiated from that of lactoferrin *in vivo*, it is likely that lactoferricin also contributes to natural protection in the host (Gruden & Poklar Ulrih, 2021).

Apart from the abovementioned works for defensins, there is a notable scarcity of publications assessing the natural protective activity of the bacterial envelope-targeting CAMPs listed in this section, particularly against ESKAPE Gram-negatives. The few examples that can be cited include the artificially induced expression of LL-37 in rodent lungs, which enhanced natural protection against PA infection (Bals *et al.*, 1999b), and the well-documented defensive role of CRAMP in the rodent cornea and lung (Huang *et al.*, 2007c; Kovach *et al.*, 2012; Wang *et al.*, 2013). Finally, the so-called keratin-derived antimicrobial peptides (KDAMPs) have been shown to be essential for protection of the cornea against PA infection (Tam *et al.*, 2012). These 13- to 26-amino acid long glycine-rich peptides derived from the C-terminus of human cytokeratin 6A (a fibrous protein contributing to the cytoskeleton of epithelial cells) seemingly exert their antibacterial activity through initial interference with bacterial adherence to the ocular surface but also by direct membrane permeabilization, but no further therapy-oriented research has been published (Tam *et al.*, 2012).

(*b*) *Antimicrobial proteins*

Mammals display a wide array of membrane-targeting antimicrobial proteins, but only representatives with activity against at least some of the ESKAPE Gram-negatives, and/or publications regarding their therapeutic potential are discussed here (Table 3, Fig. 1): Bactericidal/permeability increasing protein (BPI) and related proteins, Group IIA secretory Phospholipase A 2 (GIIA sPLA2), and the surfactant-associated proteins SP-A and SP-D, which have been reviewed recently not only in terms of their antimicrobial power but also for other interesting immunomodulatory features and implications (Floros *et al.*, 2021; Letsiou, Htwe & Dudek, 2021; Theprungsirikul, Skopelja-Gardner & Rigby, 2021). Some antimicrobial proteins are not addressed here, either because they have poor (or untested) activity against ESKAPE Gram-negative pathogens [for example, human skin chemerin (Banas *et al.*, 2013)], or because there is uncertainty about their mechanistic action. This is the case for certain C-terminal fragments derived from filaggrin-2, a pleiotropic protein involved in the maintenance of the skin barrier function, which shows high activity against PA apparently not mediated by membrane permeabilization (Hansmann, Schröder & Gerstel, 2015). Another example is psoriasin, which has a well-known membrane permeabilizer activity involved in skin defence, but has not been approached for therapeutic exploitation against ESKAPE Gram-negatives (Michalek *et al.*, 2009).

(i) Bactericidal/permeability increasing protein (BPI). The multifunctional cationic BPI has several roles beyond bacterial killing, including LPS neutralization, anti-angiogenesis, chemotaxis, opsonization, etc. (Balakrishnan *et al.*, 2013). This protein has a molecular weight of *ca.* 60 kDa and has two functional domains: the N-terminal harbours antimicrobial properties, whereas the C-terminal region is responsible for its other activities. BPI is found mainly within neutrophil granules, although it is also produced by monocytes or eosinophils. Expression of BPI is highly inducible at least in mice, through the interaction of different toll-like receptors (TLRs)

with their agonists (Eckert *et al.*, 2006). The bactericidal activity of BPI is restricted to Gram-negatives and is initially mediated by electrostatic interactions with the lipid A moiety of LPS, which enables the penetration of BPI into the periplasm, reaching the inner membrane in a time-dependent fashion and leading to its disturbance, dissipation of electrochemical gradients and death (Fig. 1) (Mannion, Weiss & Elsbach, 1990). BPI has been shown to act synergistically with other CAMPs but the murine protein seems to be incapable of inhibiting PA growth by itself (Wittmann *et al.*, 2008). Although BPI was classically considered a bactericidal protein, its superfamily has additional representatives with non-negligible antimicrobial potential: the bovine salivary proteins BSP30a and BSP30b, which display some degree of antipseudomonal activity (Haigh *et al.*, 2008); the human parotid secretory protein (PSP or BPIFA2), from which synthetic derivatives displaying direct bactericidal activity have been obtained [see Section III.2*b.v* (Abdolhosseini *et al.*, 2012)]; and the short palate, lung, and nasal epithelium clone 1 (SPLUNC1). Some studies proposed that SPLUNC1 acts as a permeability-increasing agent in Gram-negatives together with other roles such as an immunomodulator or chemoattractant (Balakrishnan *et al.*, 2013; Sayeed *et al.*, 2013; Wei *et al.*, 2016). SPLUNC1 exerts an important natural protective role *in vivo*, since mice in which this protein was deleted showed an increased severity of infection by KP or PA. Similarly, individuals with decreased levels or an impaired performance of this protein are at increased risk of PA infection (Tsou *et al.*, 2013; Nichols *et al.*, 2015; Moore *et al.*, 2019). The mechanisms involved are likely muti-factorial, including anti-biofilm and inflammatory regulation properties, and are not only based on the direct bacteriolytic power of SPLUNC1 (Liu *et al.*, 2013a, b). Likewise, BPI exerts an essential protection *in vivo*; its malfunction dramatically increases susceptibility to infections, as for example in CF patients. In fact, chronic infection by PA is a hallmark of these individuals, whose increased susceptibility to PA colonization is explained, at least in part, by the well-documented expression of anti-BPI autoantibodies that dramatically impair its activity in the lung (Carlsson *et al.*, 2011).

(ii) Group IIA secretory phospholipase A2 (GIIA sPLA2). Within the phospholipase A2 (PLA2) superfamily, secretory PLA2 (sPLA2) enzymes are the largest family, with 10–12 different variants depending on the species. Members of the sPLA2 family display quite specific tissue and cellular distributions, as well as particular enzymatic behaviours (Murakami *et al.*, 2015). Nevertheless, some common traits can be found, such as a low molecular weight and a shared activity in the degradation of phospholipids. The goal of this degradation is variable: production of inflammatory mediators, membrane remodelling, breakdown of food lipids or the destruction of microbes. GIIA sPLA2 clearly stands out in terms of antibacterial activity. GIIA sPLA2 has a molecular weight of *ca.* 14 kDa and its expression is induced during inflammatory processes linked to infection, in response to the stimulation of certain host PRRs by microbial ligands. Consequently, GIIA sPLA2 levels are correlated with the severity of inflammation (Wu *et al.*, 2010; Pernet *et al.*, 2014; Dore & Boilard, 2019). For this reason, GIIA sPLA2 is often known as an inflammatory sPLA2, related to its capacity to generate inflammatory intermediaries: once synthesized in the cytosol, GIIA sPLA2 degrades some intracellular phospholipids, and then is released outside the cell, exerting this degradation over phospholipids from extracellular vesicles. The arachidonic acid derived from this activity is used for the synthesis of pro-inflammatory eicosanoids, which enable the amplification of inflammation and drive the recruitment of additional immune components (Nevalainen, Graham & Scott, 2008; Murakami *et al.*, 2015; Dore & Boilard, 2019). In accordance with this protective role, GIIA sPLA2 is constitutively expressed in the surroundings of potential infection sites, i.e. intestinal Paneth cells, prostate epithelial cells and lacrimal gland cells. Its expression also can be induced in other sites such as vascular endothelium, hepatocytes, platelets, etc., but GIIA sPLA2 is not released by neutrophils (Dore & Boilard, 2019).

The bactericidal capacity of GIIA sPLA2 relies on the enzymatic hydrolysis of phospholipids in bacterial membranes, whose high content of phosphatidylglycerol and phosphatidylethanolamine make them excellent substrates for this enzyme (Fig. 1). GIIA sPLA2 is bactericidal only against Gram-positives at physiological concentrations, and hence it requires the collaboration of additional immune elements that disturb LPS, such as BPI or complement, to enable access to Gram-negative membranes (Nevalainen *et al.*, 2008; Murakami *et al.*, 2015; Heesterbeek *et al.*, 2021). The concentrations of GIIA sPLA2 required to kill Gram-negative bacteria are not attainable *in vivo*, although these have been shown to be bactericidal for PA *in vitro* (Dubouix *et al.*, 2003; Nevalainen *et al.*, 2008). Obviously, not all pathogens will exhibit the same level of susceptibility to GIIA sPLA2. PA infection has been shown to alter the expression of GIIA sPLA2 in the lungs of CF patients, increasing its levels by 2.5-fold compared with healthy individuals. This increased concentration is sufficient to kill *Staphylococcus aureus*, another typical colonizer of CF patients, but does not kill PA. These facts have been interpreted as a strategy of PA to eliminate competitors, and may be one of the reasons why PA is more associated with CF in patients during adulthood, whereas *S. aureus* is most prevalent in younger individuals. These phenomena have been related to the PA exotoxin S (ExoS), which is responsible for the promotion of GIIA sPLA2 expression through different pathways (Pernet *et al.*, 2014; Dore & Boilard, 2019). These data suggest an important role of GIIA sPLA2 in natural protection against infection and, indeed, overproduction of human GIIA sPLA2 in transgenic mice was demonstrated to protect against various pathogens in different models (Wu *et al.*, 2010).

(iii) Surfactant-associated proteins. Pulmonary surfactant is a lipid–protein complex whose classically attributed function is to decrease the surface tension of the air–water interface of alveoli, avoiding their collapse during exhalation and being essential for correct pulmonary function. Other roles

have been attributed to its protein fraction, including immunomodulation and direct microbicidal action (Han & Mallampalli, 2015). The proteins that take part in these functions are known as surfactant-associated proteins. They are produced by bronchiolar exocrine cells and type II pneumocytes, and their classical representatives are SP-A, SP-B, SP-C, and SP-D. SP-B and SP-C (but also the recently discovered SP-G and SP-H) are hydrophobic proteins without significant antimicrobial capacity and therefore, we do not discuss them further here, although some immunomodulatory functions related to LPS-binding have been attributed to them (Ketko & Donn, 2014; Han & Mallampalli, 2015).

Structurally, SP-A and SP-D usually appear as oligomeric complexes: SP-A is a hexamer in which the different subunits associate to form a *ca.* 600 kDa macromolecule, whereas human SP-D is assembled into a *ca.* 500 kDa tetrameric structure. However, different associations, i.e. multimers, trimers, dimers, and monomers are also known (Nayak *et al.*, 2012). SP-A and SP-D are hydrophilic collectins capable of binding to sugars on the microorganisms' surfaces through their C-terminal lectin domains, thus working as opsonins. This results in the aggregation of pathogens, enhanced phagocytosis and induction of superoxidative bursts by phagocytes, which together enable increased bacterial killing (Han & Mallampalli, 2015; Vieira, Kung & Bhatti, 2017). Once SP-A and SP-D are bound to their microbial ligands, they interact through their collagen-like N-terminal end, with certain surface receptors [cluster of designation 14 (CD14), toll-like receptor 4 (TLR4), etc.] on different immunocytes in order to initiate and/or regulate inflammatory and clearance responses (Jakel *et al.*, 2013). Therefore, SP-A and SP-D are often considered as soluble PRRs. They also bind to different humoral proteins (such as the complement C1q or immunoglobulins), exerting an additional aggregative role on pre-opsonized pathogens and other regulatory functions (Nayak *et al.*, 2012; Jakel *et al.*, 2013).

Regarding their direct bactericidal capacity, SP-A and SP-D display strong membrane permeability-increasing activity mainly related to LPS binding, although several additional ligands have been suggested (Kuzmenko *et al.*, 2005; Jakel *et al.*, 2013). For instance, SP-A also binds to capsular polysaccharides of *Klebsiella* species, which could contribute to permeabilization (Nayak *et al.*, 2012). SP-A seems to bind strongly to rough-type LPS (variants lacking the O-antigen usually found in pathogenic bacteria) and lipid A, but not to complete LPS (Kuzmenko, Wu & McCormack, 2006). By contrast, SP-D apparently binds to both rough LPS and smooth LPS containing mannose-rich O-polysaccharides. SP-A-mediated permeabilization involves the formation of calcium-mediated three-dimensional lipid–protein aggregates that extract lipids from their correct location within bacterial membranes, thus disrupting their natural structure (Cañadas *et al.*, 2008). SP-A and SP-D are highly susceptible to oxidative stress, and therefore pathological conditions that enhance the formation of reactive oxygen species such as CF, functionally impair these proteins. This may contribute to the enhanced susceptibility of CF patients to PA infection, demonstrating the natural anti-infective role that these proteins play (Kuzmenko *et al.*, 2005; Starosta & Griese, 2006). Other data also support these two proteins as being essential for defence against infection: animal models in which they were deleted showed increased severity of lung infection by PA or KP (LeVine *et al.*, 1998; Giannoni *et al.*, 2006; Mikerov *et al.*, 2008; Ali *et al.*, 2010; Du *et al.*, 2018; Thorenoor *et al.*, 2018). SP-A and SP-D also are secreted in locations other than the lung and apparently play an important role in defence of the cornea against PA infection as shown in different animal and cell-culture models (Mun *et al.*, 2009; Alarcon *et al.*, 2011; Nayak *et al.*, 2012; Vieira *et al.*, 2017).

III. BACTERIAL ENVELOPE-TARGETING MAMMALIAN HUMORAL INNATE IMMUNITY: THERAPEUTIC POTENTIAL

In this section we address the most relevant and recent work investigating the therapeutic potential of mammalian bacterial envelope-targeting immunity, not only studies on 'conventional' treatments based on the external administration of a bactericidal compound, but also *in vitro* activity studies, animal models, and clinical trials. We additionally provide insights into new concepts based on host/bacterial targets that could increase the performance of bacterial envelope-targeting immunity and/or reduce the resistance of pathogens to these approaches, as a necessary perspective for the future development of such treatments (Bergman *et al.*, 2020). To avoid saturation with numerical data, only the most relevant values of minimum inhibitory concentrations (MICs), survival percentages and related parameters are provided below.

(1) Bacterial resistance: a non-negligible threat for treatments based on envelope-targeting immune proteins/peptides

An initial question is the potential for the development of bacterial resistance against bacterial envelope-targeting treatments, which could be a serious threat. However, one strength that has been classically attributed to such an approach is the unlikely development of resistance. The main reason for this is that many of the immune components targeting the envelope work through non-specific modes of action, making it less likely that there will be selection for bacterial resistance mechanisms (Dziarski & Gupta, 2018; Lewies, Du Plessis & Wentzel, 2019). Whereas classic antibiotics usually have one specific target and mechanism (enabling the selection of single mutations conferring resistance), envelope-targeting immune components often interact with different ligands, with parallel and relatively unspecific effects on bacterial envelopes. Therefore, the acquisition of resistance would require the simultaneous selection of different adaptations, which is less likely to occur.

Since these immune components target highly conserved essential structures, the possible selection of viable mutants with alterations in these structures is limited (Lewies *et al.*, 2019). Selection of resistance is even less likely if the immune-based treatment is combined with a classic antibiotic, a strategy followed in several studies, as described below [Sections III.2*a* and III.2*b.iv* (Zharkova *et al.*, 2019)]. Another advantage of envelope-targeting immunity in comparison with antibiotics is that bacteria seemingly have not developed mechanisms/platforms for the dissemination of genes conferring resistance to these immune elements (Kintses *et al.*, 2019), contrary to the situation for the horizontal transfer of β-lactamases for example, which can take place through integrons, transpons, and/or plasmids (Juan *et al.*, 2017). The minimal potential for environmental dissemination of free and active immune compounds (given their low stability outside the organism), in contrast with antibiotics (usually found at sub-lethal concentrations in the environment), is another factor that may limit the selection of mutations and the appearance/transmission of resistance genes against bacterial envelope-targeting immune elements (Lazzaro, Zasloff & Rolff, 2020).

One circumstance that suggests caution when considering bacterial envelope-targeting immunity as therapeutic option is the identification of cross-resistance phenomena between certain antibiotic treatments and exogenously administered CAMPs, potentially affecting the effectiveness of endogenous CAMPs (Kádár *et al.*, 2015; Fleitas & Franco, 2016). Such cross-resistance episodes often involve polymyxins and CAMPs, perhaps understandably given their biochemical and mechanistic similarities (Kádár *et al.*, 2015; Fleitas & Franco, 2016; Crémieux *et al.*, 2019). It is also of interest that a dramatically increased susceptibility to lysozyme appearing in tandem with colistin resistance mediated by LPS loss in AB has been reported, as well as an increase in KP or PA susceptibility to CAMPs after avibactam/quinolone treatments (Campos, Morey & Bengoechea, 2006; Ulloa *et al.*, 2019; Kamoshida *et al.*, 2020). A lack of association between antibiotic *versus* defensin bacterial resistance has also been suggested, indicating the complexity of this topic (Supp *et al.*, 2009). The aforementioned cross-resistance phenomena may be caused by point mutations in different genes related to LPS and/or synthesis of other surface structures (Napier *et al.*, 2013), but they are also intimately related to the induction of adaptive resistance phenotypes. These latter are transitory states with no mutational basis, usually mediated by regulatory networks including two-component systems such as CprRS, which are ultimately activated by the presence of CAMPs (Fernández *et al.*, 2012). Interestingly, these metabolic regulatory networks have also been shown to facilitate bacterial adaptation by regulating the expression of virulence factors, which could be a negative implication of using some CAMPs as therapies. This threat could be even greater if we consider that mutations in the abovementioned two-component systems themselves could be selected to increase resistance to CAMPs (Llobet *et al.*, 2011; Limoli *et al.*, 2014; Rodríguez-Rojas *et al.*, 2015). Therefore, while the therapeutic potential of bacterial envelope-targeting immunity is encouraging, unwanted problems may arise if they are used without control or foresight (Kádár *et al.*, 2015; Fleitas & Franco, 2016; Crémieux *et al.*, 2019; Lazzaro *et al.*, 2020; Vitale *et al.*, 2020). Among these, we include recently reported phenomena of non-classical antagonism in combined treatments of lysozyme with certain CAMPs against PA, a worrying development that deserves detailed investigation (Blumenthal *et al.*, 2021). Therefore, specific strategies to prevent/block such phenomena and potential mechanisms of cross-resistance/adaptation must be studied in parallel with the development of envelope-targeting immunity-based therapies (Shang *et al.*, 2020).

(2) *In vitro* activity, experimental treatments and animal models

(*a*) *Peptidoglycan-targeting proteins*

Several studies have analysed the *in vitro* activity of lysozyme against different ESKAPE Gram-negatives, generally showing modest effectiveness ranges (Torrens *et al.*, 2020). However, powerful activity against AB (MIC 1.2 μM), potentiable through polymyxin B, has been reported (Zharkova *et al.*, 2019). From *in vivo* assays, the early instillation of human lysozyme into the lungs of PA-infected mice has been shown to significantly reduce bacterial load (by *ca.* 1 log unit compared to controls) (Epaud *et al.*, 2019). Milder results had been obtained previously with aerosolized lysozyme in hamster models of PA respiratory infection, with this difference probably due to differences in timing, dosage, and administration route. However, a significant decrease in inflammation parameters and a significant synergy with tobramycin was observed (Bhavsar *et al.*, 2011, 2010). Initiatives to improve the performance of aerosolized lysozyme and increase its effectiveness have been published (Ferrati *et al.*, 2018; Nieto-Orellana *et al.*, 2018). Modified variants of lysozyme with a reduced positive charge to avoid electrostatic sequestration by various mucus polyanions, including DNA, mucin, and alginate (all of which are typically increased in CF patients), have been developed, with encouraging results in murine respiratory infection models (Teneback *et al.*, 2013; Griswold *et al.*, 2014). In a search for greater stability, solubility, and reusability, synthetic conjugates or nanoparticles have been developed, demonstrating improved activity against PA and AB (Liu *et al.*, 2013c; Saito *et al.*, 2019). This is particularly interesting for lysozyme immobilized in chitosan nanoparticles through ionic gelation, which is very active against KP, with MICs around 10 mg/l and significant bactericidal capacity (a 2 log unit decrease in viable bacteria in 120 min) (Wu *et al.*, 2017a; Wang *et al.*, 2020). Although lysozyme barely inhibits PA biofilm formation, application against KP seems exploitable, since concentrations of 5 mg/l

completely eradicated 24 h-old biofilms (Sheffield *et al.*, 2012; Hukić *et al.*, 2018).

(*b*) *Membrane disturbance-based immune weapons*

(i) Defensins. Several studies have analysed the *in vitro* activity of defensins against AB, KP, or PA, mostly using hBD-2/-3, and providing data that suggest significant bactericidal power [e.g. a MIC of 5 mg/l for hBD-1 against KP (Sahly *et al.*, 2006; Huang *et al.*, 2007b; Maisetta *et al.*, 2008; Routsias *et al.*, 2010; Gomes *et al.*, 2015)]. Some defensins, such as hBD-2, also have interesting anti-biofilm potential, as tested using classical *in vitro* assays but also in novel electrochemical catheters (designed to avoid infections linked to these devices), in which the defensin is administered synergistically together with nitric oxide (NO), with reports of a 5 log unit reduction in viable cells from 7-day-old biofilms (Ren *et al.*, 2016; Parducho *et al.*, 2020). The natural dimerization of human α-defensin 5 has been demonstrated under certain conditions, enhancing its protease resistance and activity against AB (Wommack *et al.*, 2014). Different variants from species including monkeys (θ-defensins), rats, dogs, and even a defensin-like peptide from platypus have been tested for antimicrobial activity *in vitro*, with notable results against PA or KP although with similar performance to human variants (Sang *et al.*, 2005; Torres *et al.*, 2014; Gomes *et al.*, 2015; Tai *et al.*, 2015; Beringer *et al.*, 2016).

The search for artificially modified defensin derivatives with improved properties is ongoing. Defensin-related peptide 1 (Defr1), a 34-amino-acid fragment with only five of the canonical six cysteine residues, works as a covalent dimer with enhanced bactericidal power (Taylor *et al.*, 2009); some simplified hBD-3 or HNP-1 scaffolds show increased anti-ESKAPE activity (Hoover *et al.*, 2003; Varney *et al.*, 2013; Chauhan *et al.*, 2016; Nigro *et al.*, 2017); some synthetic peptides corresponding to the carboxy-terminal segment of bovine β-defensin-2 retained interesting antibacterial activity (Krishnakumari *et al.*, 2003); the β-hairpin loop of the 33-amino-acid α-defensin rabbit neutrophil peptide 2 (NP-2) exhibits high antipseudomonal power (Thennarasu & Nagaraj, 1999); a Cys2-4 bond-containing 32 residue derivative of HNP-5, with non-cationic/non-hydrophobic residues replaced by positively charged arginine (denominated HD5d5), showed enhanced activity against AB (Wang *et al.*, 2018); the fragment of the first 11 N-terminus residues obtained by tryptic digestion of HNP-4 (denominated HNP-$4_{\mathbf{1–11}}$) outperformed the original peptide and showed activity enhanced by acetylation/amidation (Ehmann *et al.*, 2020); certain chimeric/oligomeric variants with specific amino acid substitutions displayed significant activity against PA, etc. (Zhou *et al.*, 2011; Corrales-Garcia *et al.*, 2013; Scudiero *et al.*, 2015, 2013).

Fewer studies have assessed the administration of defensins as a treatment *in vivo*. Interestingly, injection of HNP-1 was found to cause a notable (*ca.* 2 log units) leukocyte-dependent KP burden reduction in the peritoneum and thigh in murine models (Welling *et al.*, 1998). Nebulized monkey θ-defensins provided significant inflammation and PA load reductions in rodent models of chronic respiratory infection (Beringer *et al.*, 2016; Bensman *et al.*, 2017). Finally, the artificially induced over-expression of different defensins in rodent lungs and in skin infection models provided encouraging results, as did the incorporation of hBD-1 into cotton gauzes to treat KP-infected wounds (Hu *et al.*, 2010; Gibson *et al.*, 2012; Park *et al.*, 2014; Shen *et al.*, 2014; Gomes *et al.*, 2015).

(ii) Histatins. Research has focused on Hst5, which shows powerful *in vitro* activity especially against PA, with over 99.9% of bacteria killed after 1 h incubation with 30 μM Hst5 in solution (Du *et al.*, 2017). By contrast, KP has been shown to remain mostly resistant to this peptide, likely owing to its capsule (Du *et al.*, 2017). The Hst5-derivative Dhvar-5, a synthetic analog based on residues 11–24, showed potent anti-PA and anti-AB activity and encouraging anti-biofilm potential for the development of preventative coatings against implant-associated infections (Barbosa *et al.*, 2019). The P-113D derivative (Hst5 residues 4–15) showed increased stability against proteases (Cirioni *et al.*, 2004), leading to 100% planktonic PA death after 10 min of exposure to ×2 MIC, an activity that was amplifiable through synergy with β-lactams (Giacometti *et al.*, 2005). Interestingly, P-113D was also shown to clear PA sepsis successfully in rats, with an activity comparable to that of imipenem/polymyxin B (Cirioni *et al.*, 2004). Unfortunately, these encouraging results with histatin derivatives have not been translated into ongoing research lines.

(iii) Cathelicidins. The human cathelicidin LL-37 has been the subject of intensive research [reviewed by Duplantier & van Hoek (2013) and Wang *et al.* (2019)]. This peptide comes from the proteinase 3-mediated cleavage of the inactive human precursor protein hCAP18 (human cationic antimicrobial protein 18, 170 amino acids). LL-37 is the accepted name for the mature CAMP derived from the last 37 residues of the hCAP18 C-terminus and is widely expressed in different tissues but mainly produced by neutrophils. It is believed to act through the carpet model (see Fig. 3), but the existence of intracytosolic targets has not been completely ruled out (Rozek, Friedrich & Hancock, 2000; Kumar *et al.*, 2018). The existence of a second cathelicidin, FALL-39, similarly derived from the proteolysis of hCAP18, but displaying two additional residues at the beginning of the peptide, was accepted some years ago; it is considered analogous to porcine PR-39, but has been little studied to date (Kuroda *et al.*, 2015). There are mixed results regarding the *in vitro* activity of LL-37, ranging from mild antipseudomonal activity (Pompilio *et al.*, 2011; Min *et al.*, 2012; Wang *et al.*, 2012; Chen *et al.*, 2018a) to high effectiveness against AB, with active concentrations below 4 μM, and a comparatively higher bactericidal efficiency than either hBD-3 or Hst5 (Lin *et al.*, 2015b; Jaśkiewicz *et al.*, 2019). Variable results regarding its anti-biofilm capacity are also reported (Overhage *et al.*, 2008; Dean, Bishop & Van Hoek, 2011; Dosler & Karaaslan, 2014; Gomes *et al.*, 2015; Jaśkiewicz *et al.*, 2019). Shorter LL-37-derived fragments such as FK-16 display similar activity to LL-37 and synergy with

conventional antibiotics against KP, AB, and PA, but their performance against ES is poor (Feng *et al.*, 2013; Lin *et al.*, 2015a; Rajasekaran, Kim & Shin, 2017; Mishra & Wang, 2017b; Geitani *et al.*, 2019; Mohammed *et al.*, 2019). Other artificially modified versions of LL-37 with cationicity and/or hydrophobicity changes outperform the original peptide regarding anti-ESKAPE Gram-negative activity *in vitro* (Table 2) (Wang *et al.*, 2014; Kim, Rajasekaran & Shin, 2017; Mishra & Wang, 2017a; De Breij *et al.*, 2018; Gunasekera *et al.*, 2020, 2018). The anti-biofilm action of LL-37 immobilized onto poly-hydroxyethylmethacrylate or of FK-16 on titanium surfaces suggests their potential for the development of anti-infective coatings (Mishra & Wang, 2017b; Su *et al.*, 2019).

In vivo and *ex vivo* data show that the LL-37 D-enantiomer increased the survival (from 10 to 60–70%) of PA-infected *Galleria mellonella* larvae (a typical invertebrate infection model) compared to ciprofloxacin, clearly outperforming the original peptide (Dean *et al.*, 2011; Duplantier & van Hoek, 2013). The use of LL-37 in wound dressings/ointments resulted in significant KP, AB, and PA load reductions in cell culture, *ex vivo* and in murine skin models (De Breij *et al.*, 2018; Su *et al.*, 2019). The artificial expression of LL-37 in rodents or in bioengineered skin provided greater AB and PA load reductions compared to exogenous administration in burn infections (Bals *et al.*, 1999b; Thomas-Virnig *et al.*, 2009; Thomas-Virnig & Allen-Hoffmann, 2012; Zhou *et al.*, 2020). The administration of LL-37 successfully helped to clear PA sepsis and lung infection in mice, although the proposed therapeutic mediator was neutrophil recruitment rather than direct bacteriolysis (Cirioni *et al.*, 2008; Beaumont *et al.*, 2014). Finally, $CAP18_{106–142}$ peptide (also known as CAP7, a fragment of 37 residues from rabbit CAP18 protein, homologous to LL-37) and some derivatives have shown *in vitro* antipseudomonal activity exceeding that of LL-37 (Travis *et al.*, 2000; Brogden *et al.*, 2001; Saiman *et al.*, 2001), but unfortunately they failed *in vivo* likely because of high susceptibility to proteolysis (Sawa *et al.*, 1998). Attempts to circumvent this limitation, and to increase its concentration through microgel/carboxylate anions have not yet been tested *in vivo* (Qin *et al.*, 2015; Nordström *et al.*, 2018).

The membrane-permeabilizing 13-amino-acid long bovine peptide indolicidin is believed additionally to inhibit DNA replication (Bera *et al.*, 2015), contributing to its notable anti-PA, anti-KP, and to a lesser extent anti-AB, activities (Giacometti *et al.*, 2000; Overhage *et al.*, 2008). Recently, it has been shown that this cathelicidin and its derivatives (called 'Indopts') are interesting in terms of their better synergy with antibiotics, compared to over 20 other CAMPs tested in ≈400 combinations (Ruden *et al.*, 2019). Proline-rich bactenecins are cathelicidins present in ruminant neutrophils, with those from goats and sheep displaying the strongest activity against Gram-negatives (Shamova *et al.*, 2009). Their effectiveness against PA seems limited (Runti *et al.*, 2017; Mardirossian *et al.*, 2019), but for AB and KP lower MICs are reported (Mardirossian *et al.*, 2018; Dolzani *et al.*, 2019). The bovine bactenecin dodecapeptide (Bac2A) and certain derivatives (DP7 and DP-7C) showed better antipseudomonal activity (Wu & Hancock, 1999; Hilpert *et al.*, 2006) and synergy with antibiotics (Wu *et al.*, 2017b). When used in a zebrafish model, DP-7C caused drastic reductions in PA loads linked to boosted monocyte recruitment (Zhang *et al.*, 2018). Very recently, several cetacean proline-rich antimicrobial peptides (cePrAMPs), which display high similarity to bovine Bac7 but nuances regarding their mechanisms of action (some inhibited protein synthesis without membrane disturbance, while others were very membrane-active), have shown significant bactericidal activity against ESKAPE Gram-negatives, with MICs ≤4 μM for PA (Sola *et al.*, 2020).

Another extensively studied group of cathelicidins is the myeloid antimicrobial peptides (MAPs), a generic denomination for various ruminant cathelicidins such as BMAPs (bovine), ChMAPs (caprine), and SMAPs (sheep). Of these, BMAP-27/-28 showed interesting antipseudomonal activity (including the prevention of/disruption of biofilms) with MICs ≈ 1 μM (Pompilio *et al.*, 2011, 2012). Unfortunately, MAPs generally display poor selectivity between bacterial and eukaryotic membranes (Lee *et al.*, 2011; Guo, Xun & Han, 2018), but BMAP-18 (a synthetic N-terminal 18-residue amphipathic peptide obtained from BMAP-27) showed low eukaryotic cytotoxicity due to its different mode of action: formation of well-defined channels as opposed to massive membrane perturbation (Lee *et al.*, 2011). Sadly, BMAPs failed *in vivo*, likely because of susceptibility to proteolysis and/or sequestration by tissues (Mardirossian *et al.*, 2016, 2017). Ovine MAPs, especially SMAP-29 and its derivatives ovispirins, displayed increased activity against PA and KP compared to LL-37 or Bac7, although their anti-biofilm power was low (Pompilio *et al.*, 2011). Interestingly, although CF sputum notably inhibits SMAP-29 activity *in vitro*, SMAP-29 successfully prevented endotoxin-induced mortality in rat models of septic shock (Travis *et al.*, 2000; Brogden *et al.*, 2001; Saiman *et al.*, 2001; Giacometti *et al.*, 2004; Pompilio *et al.*, 2012).

The porcine cathelicidins protegrins (usually 16–18 amino acids long) act through a barrel-stave pore mechanism (see Fig. 3) (Lazaridis *et al.*, 2013), although some synthetic peptidomimetics (e.g. murepavadin, see Section III.3) act through interference with membrane biogenesis (Shankaramma *et al.*, 2003; Srinivas *et al.*, 2010; Zerbe, Moehle & Robinson, 2017; Andolina *et al.*, 2018; Martin-Loeches, Dale & Torres, 2018). The protegrin PG-1 exhibited interesting MICs against several PA strains (up to 4 mg/l) (Steinstraesser *et al.*, 2001; Albrecht *et al.*, 2002), and notable *in vitro* activity of PG-1 against AB and strong synergy with colistin has also been reported (Steinstraesser *et al.*, 2001; Morroni *et al.*, 2019). PG-1 has been tested in porcine/murine wound infection models, where its inoculation/topical application produced a 4 log unit reduction in PA loads. This effectiveness, likely at least partially dependent on immunomodulatory effects, was very variable depending on the timing of administration: when PG-1 was inoculated

together with bacteria, a 10000-fold decrease in bacterial counts was observed but when the administration was 120 h post-infection, the reduction was only 10-fold. These results indicate the importance of early administration for a successful clinical outcome (Ceccarelli *et al.*, 2001; Steinstraesser *et al.*, 2001; Morroni *et al.*, 2019). Another porcine cathelicidin, tritrpticin, displayed the same MIC_{90} as colistin (8 mg/l) against several clinical strains of PA and good synergy with β-lactams, in a very encouraging research line that awaits further study (Cirioni *et al.*, 2006). Some tritrpticin derivatives with enhanced permeabilization capacity have been developed, but not tested on ESKAPE Gram-negatives to date (Arias *et al.*, 2016, 2018). Finally, some interesting cathelicidins with excellent anti-AB and anti-PA activity, e.g. WAM-1, have been found in marsupials (Wang *et al.*, 2011b; Spencer *et al.*, 2018).

(iv) Lactoferrin-derived peptides. The *in vitro* potential of lactoferrin-derived peptides have been widely investigated (Gifford *et al.*, 2005; Théolier *et al.*, 2014; Bruni *et al.*, 2016; Vega-Bautista *et al.*, 2019; Gruden & Poklar Ulrih, 2021). LF1-11 (an artificially obtained peptide corresponding to the initial 11 amino acids of human lactoferrin) showed strong activity, reducing the KP load by *ca.* 3 log units was observed in a murine thigh infection model (Nibbering *et al.*, 2001). Different synergistic combinations suggest that LF1-11 causes a sensitizing effect on KP especially to hydrophobic antibiotics, which are usually ineffective against it (Morici *et al.*, 2017). In another murine thigh infection model, a *ca.* 3 log unit reduction in AB count was also reported (Dijkshoorn *et al.*, 2004). Administration of LF1-11 is well tolerated in healthy individuals (van der Velden *et al.*, 2009), which enhances its possible use in clinical applications as a bactericidal/immunomodulatory agent, potentially improving AB infection outcomes as shown in mice (Dai *et al.*, 2018).

Lactoferricin is formed by digestion through proteases produced by the host (mainly pepsin) but likely also by microbes at infection sites (Gifford *et al.*, 2005; Théolier *et al.*, 2014; Bruni *et al.*, 2016). The bovine variant displays the strongest antimicrobial power *in vitro* among mammals (Bellamy *et al.*, 1992; Wakabayashi *et al.*, 2005), whereas some derivatives (undecapeptides, based on lactoferricin amino acids 17–27, especially undeca-9) have been found to outperform the original peptide, but not to avoid the appearance of resistant mutants *in vitro* (Strøm, Rekdal & Svendsen, 2002). The fragment between residues 17 and 30 exhibits strong activity, outperforming that of the whole protein (PA MICs: 3 *versus* 414 mg/l) (Van Der Kraan *et al.*, 2004). The use of bovine lactoferricin in a murine keratitis model was demonstrated to cause a significant decrease in inflammation, but barely reduced PA burden (Oo *et al.*, 2010). Other derivatives (linear, palindromic, branched, polymeric, etc.) based on bovine lactoferricin have been shown to outperform it only slightly in terms of MICs for KP or PA (Huertas *et al.*, 2017; Vega Chaparro *et al.*, 2018). Conversely, some derivatives such as C12LF11 (also known as lauryl-LF11), a N-terminally acylated analogue of LF11 (which corresponds to amino acids 21–31 of human lactoferricin), clearly outperformed the original molecule *in vitro* (PA MICs 10-fold lower) (Andrä *et al.*, 2005; Hunter *et al.*, 2005; Zweytick *et al.*, 2006). Other LF11 variants such as P-22 showed excellent synergies with antibiotics at subinhibitory concentrations, and were stable against well-known PA resistance mechanisms (Sánchez-Gómez *et al.*, 2015), although their cytotoxicity towards eukaryotic cells has not been solved (Harm *et al.*, 2019).

Finally, some studies have analysed the *in vitro* activity of multiple CAMPs in parallel, providing a useful comparison. Xie *et al.* (2020) reported that BMAP-27 was the strongest cathelicidin (PA, KP, and AB MICs below 2 μM) and that a combined treatment with LL-37 provided a 2.5 log unit PA load reduction in a murine lung infection model. Other single/combined treatments were recently tested (Zharkova *et al.*, 2019), and showed the CAMPs with the highest activity against AB, KP, and PA to be HNP-1, HNP-4, and PG-1 (MICs below 2 μM). The most synergistic combinations were polymyxin B plus hBD-2, and gentamicin plus hBD-3 or PG-1 against AB; and amikacin/erythromycin plus PG-1 or ChBac3.4 (a 26-amino-acid long proline-rich cathelicidin identified in goat leukocytes) against KP and PA (Zharkova *et al.*, 2019).

(v) Bactericidal/permeability increasing protein (BPI). Although the whole BPI protein is bactericidal, some shorter fragments with increased activity against ESKAPE Gram-negatives have received research interest (Domingues, Santos & Castanho, 2012; Domingues *et al.*, 2014) (Table 3). Among these, rBPI-21 (comprising the first 193 amino acids of BPI and displaying a Cys132Ala substitution) was the most stable and functional in a murine systemic PA infection model, increasing survival up to 10-fold (Ammons, Kohn & Kung, 1994). The use of rBPI-21 against KP or *E. cloacae* cells in blood/plasma suggested a leukocyte-independent activity, and therefore a direct permeabilization was proposed, although no synergy with antibiotics was seen (Levy *et al.*, 2000). A ≈ 2 log unit reduction in multidrug-resistant PA and AB viable cells was shown *in vitro* after 1 h with achievable doses of rBPI-21, although KP remained mostly resistant (Weitz *et al.*, 2013). The Asp190Ala variant of BPI5 (murine BPI fold containing family B, member 5; also known as Bpifb5 or Lplunc5) showed a significant increase in LPS-binding capacity and in *in vitro* antipseudomonal power (Wu *et al.*, 2016). Some synthetic constructs combining fragments of chemokines and functional residues of the BPI β-sheet have shown interesting results: Betapep-25 and its shorter dodecapeptide, SC-4, showed a lethal dose 50 (LD_{50}) at nanomolar range for PA (Lockwood *et al.*, 2004; Dings, Haseman & Mayo, 2008; Dings *et al.*, 2013). Interestingly, SC-4 also improved survival in mice intraperitoneally infected with PA from 9 to 45% (60% in combination with piperacillin-tazobactam) (Dings *et al.*, 2013).

Another BPI superfamily member, SPLUNC1 displays neutrophil chemoattractant activity and inhibits PA growth (Sayeed *et al.*, 2013), properties residing in its 30-residue α-helix called α4 (Walton *et al.*, 2016). Its α4 short derivative has shown even better characteristics, including: PA biofilm

preventive and disrupting activity, MICs against multidrug-resistant KP, AB, and PA between 2 and 4 μM, and a 2 log unit decrease in PA burden in murine respiratory infection models (Jiang *et al.*, 2019). GL13K (a fragment including residues 141–153 of BPIFA2, with specific lysine residues replacing the original amino acids and an increased positive charge) displayed direct antipseudomonal activity *in vitro* and reduced mortality (from 85 to 30%) in a murine model of LPS-induced shock (Gorr *et al.*, 2008; Abdolhosseini *et al.*, 2012). A combination of GL13K with tobramycin provided an excellent anti-PA biofilm activity. This was further improved by the D amino-acid version (DGL13K), which was found to be more resistant to proteases (Hirt & Gorr, 2013). DGL13K has been shown to kill PA efficiently in both *G. mellonella* and murine burn wound infection models (Gorr, Flory & Schumacher, 2019).

(vi) Group IIA secretory phospholipase A2 (GIIA-sPLA2). Although GIIA-sPLA2 displays non-negligible activity *in vitro*, it needs the collaboration of permeabilizers to enable its access to the bacterial inner membrane in order to kill Gram-negatives *in vivo* (Dubouix *et al.*, 2003; Nevalainen *et al.*, 2008; Pernet *et al.*, 2014; Dore & Boilard, 2019). Data regarding its potential therapeutic applications are still scarce, with additional research needed. Nevertheless, the available results are encouraging: GIIA-sPLA2 (probably largely through its pro-inflammatory properties) has been shown significantly to increase protection against respiratory infection (not tested to date for ESKAPE Gram-negatives) when exogenously administered/artificially over-expressed in transgenic mice (Wu *et al.*, 2010).

(vii) Surfactant-associated proteins. Although SPs do not show synergy with antibiotics (Ferrara, Dos Santos & Lupi, 2001), exogenous administration of human SP-A has been found to increase mouse survival after lung KP infection, from 40% to almost 100% (Thorenoor *et al.*, 2018). Similar results were obtained after the induction of SP-D expression in a rat model of PA respiratory infection (Thacker *et al.*, 2014). The 20-amino-acid fragment derived from the TLR4-interacting region of SP-A (SP-A4) is not directly bactericidal but, when tested in a murine model of respiratory infection, efficiently reduced PA burden, inflammation and tissue damage, suggesting that their action involves opsonization and immunomodulation (Awasthi *et al.*, 2019).

(3) Bacterial envelope-targeting immunity-based treatments under clinical trials

Some envelope-targeting treatments potentially effective against multidrug-resistant Gram-negatives are under clinical trials. Despite preliminary encouraging results against ESKAPE Gram-negatives, most trials [e.g. the BPI derivatives XMP-629 and Neuprex (Koo & Seo, 2019; Mercer *et al.*, 2020) and the histatin-5-derived peptide P-113 (Cheng *et al.*, 2018)] have not involved these pathogens. A 28-residue peptide with antipseudomonal and immunomodulatory activities derived from the human hormone ghrelin, is in phase II trials for the treatment of inflammation in CF patients (Min *et al.*, 2012). The antipseudomonal and immunomodulatory kininogen-derived peptide, DPK-060 (at 0.5 μM eradicated 10^6 PA cells/ml in 2 h) has finished phase II trials for the treatment of otitis/dermatitis (Butler, Blaskovich & Cooper, 2013; Koo & Seo, 2019; Mercer *et al.*, 2020). For LL-37 and human LF1-11, proposed as treatments for chronic leg ulcer and KP infections, respectively, phase II trials have not been completed (van der Does *et al.*, 2012; Koo & Seo, 2019; Mercer *et al.*, 2020). The bovine indolicidin-derived 12-amino-acid peptide, omiganan (formerly MBI226), shows considerable *in vitro* activity against AB, KP, and PA (Sader *et al.*, 2004; Melo, Dugourd & Castanho, 2006; Melo & Castanho, 2007), although low success against biofilms (Jaśkiewicz *et al.*, 2019). *E. cloacae* load was reduced by 3 log units after topical application of omiganan 1% gel in a pig skin colonization model (Rubinchik *et al.*, 2009). Sadly, although phase II–III trials for skin antisepsis and for acne/dermatitis treatment are ongoing, application against ESKAPE Gram-negatives seems far off (Isaacson, 2003; Septimus & Schweizer, 2016; Koo & Seo, 2019; Mercer *et al.*, 2020).

The 17-amino-acid PG-1 analog, iseganan (also known as IB-367), developed for the treatment of oral mucositis/stomatitis and ventilator associated pneumonia (VAP) prevention, initially displayed very interesting antipseudomonal activity *in vitro*: when tested against exponentially growing PA, a reduction in viability similar to that of polymyxin B was reported (IB 367, 1999; Mosca *et al.*, 2000; Bogucka *et al.*, 2004). In a rat model of chronic respiratory infection, iseganan reduced PA load ≈1000-fold (Fricker, 2002), and it displayed a strong anti-endotoxic capacity useful for the treatment of septic shock, and potent activity against KP (MIC ≈5 mg/l) and AB (MIC 2 mg/l) (Chen *et al.*, 2000; Giles *et al.*, 2002; Giacometti *et al.*, 2003a,b; Bogucka *et al.*, 2004). A MIC_{90} of 16 mg/l was later reported for a larger collection of AB, KP, and PA strains, and a reduction of *ca.* 3 log units in PA load was achieved through topical iseganan in murine wound infections. Synergistic treatment with colistin/imipenem reduced bacterial counts by 3 additional log units (Simonetti *et al.*, 2014). Nevertheless, iseganan in oral solution as a VAP prophylaxis was found to provide no improvement compared to non-treated patients (Kollef *et al.*, 2006; Muscedere *et al.*, 2008; Li *et al.*, 2013; Messika, La Combe & Ricard, 2018). Topical/mouthwash use of iseganan for mucositis/stomatitis treatment has shown contradictory results in phase III trials (Hashemi *et al.*, 2015; Saunders *et al.*, 2015), either showing no reduction in risk or clinical symptoms (Giles *et al.*, 2004; Trotti *et al.*, 2004) or reducing stomatitis-associated symptoms and oral microbial load, suggesting its applicability as a prophylaxis (although not against ESKAPE Gram-negatives specifically) (Giles *et al.*, 2004; Elad *et al.*, 2012).

The bacterial envelope-targeting peptides closest to clinical application are probably the protegrin analogs murepavadin (formerly POL7080) and its derivative POL7001. Murepavadin is a 14-amino-acid cyclized analog peptidomimetic obtained from PG-1 through the introduction of beta-

hairpin domains. Both murepavadin and POL7001 are derived from L27-11, which was not a valid therapeutic option because of high susceptibility to proteolysis in human serum (Cigana *et al.*, 2016; Romano *et al.*, 2019). These cyclized compounds display specific anti-PA activity through binding and inhibition of the LPS transport protein LptD, therefore blocking outer envelope biogenesis (Srinivas *et al.*, 2010; Andolina *et al.*, 2018). Although their initial MICs were extremely low (0.06 mg/l), the appearance of resistant mutants (MICs >32 mg/l for murepavadin and 8 mg/l for POL7001) has been reported, selected through non-inactivating mutations in *lptD* with low frequency ($\leq 1 \times 10^{-10}$) (Romano *et al.*, 2019). It also has been shown that PA can acquire resistance to murepavadin and POL7001 through non-inactivating mutations in the two-component sensor gene *pmrB*, which involves cross resistance to colistin and PG-1 mediated by LPS modifications. These mutants display much lower resistance levels (MICs up to 1.6 mg/l) than those with altered LptD (Srinivas *et al.*, 2010; Romano *et al.*, 2019).

Regardless of these resistance-related drawbacks, a study with 1219 PA clinical isolates provided a murepavadin MIC_{90} of 0.12 mg/l, whereas a parallel study, performed using 785 extensively drug resistant isolates (non-susceptible to at least one agent in all but two or fewer antimicrobial categories), found a MIC_{90} of 0.25 mg/l (eightfold higher activity than colistin) (Sader *et al.*, 2018a,b). In another study, murepavadin activity against CF patient-proceeding PA strains was shown to be weaker (MIC_{90}: 2 mg/l), with a higher MIC_{90} (8 mg/l) for small colony variants (slow-growing isolates usually selected during CF, characterized by their excellent adaptation to this niche) (Ekkelenkamp *et al.*, 2020). Analyses of pharmacokinetic/pharmacodynamic (PK/PD) parameters in mice indicate that the concentration–time curve for the drug's unbound fraction was better correlated with murepavadin efficacy (Melchers *et al.*, 2019). Six phase I (POL7080-001, NP29332, NP29333, NP29334, POL7080-005, and POL7080-009) and four phase II trials (POL7080-002, POL7080-003, NCT03582007, and NCT03409679) have been completed for murepavadin, with different goals (Dembowsky *et al.*, 2012; Martin-Loeches *et al.*, 2018; Poulakou *et al.*, 2018; Wach, Dembowsky & Dale, 2018; Horcajada *et al.*, 2019). In patients with VAP, intravenous murepavadin treatment achieved clinical cure in 10 out of 12 participants (Armaganidis, Zakynthinos & Mandragos, 2017), but in individuals with impaired renal function, murepavadin clearance was significantly reduced (Dale *et al.*, 2018; Poulakou *et al.*, 2018). Additionally, pivotal phase III studies, POL7080-002, POL7080-003, NCT03582007, and NCT03409679 have been discontinued because of high rates of kidney failure (Polyphor, 2019; Ekkelenkamp *et al.*, 2020; Jabbour, Sharara & Kanj, 2020; Matos De Opitz & Sass, 2020). Although this nephrotoxicity is a disappointment, the development of inhalation-based formulations to avoid this drawback is ongoing, supported by recent *in vivo* models (Bernardini *et al.*, 2019; Polyphor, 2019; Tümmler, 2019; Ekkelenkamp *et al.*, 2020; Jabbour *et al.*, 2020; Provenzani *et al.*, 2020). Less information is available for POL7001, although *in vitro* activity comparable to murepavadin and therapeutic efficacy in murine models of PA respiratory infection has been demonstrated (Srinivas *et al.*, 2010; Cigana *et al.*, 2016; Romano *et al.*, 2019). Finally, although quite far from the original molecule, some murepavadin-derived compounds (chimeric constructs linked to the peptide macrocycle of polymyxins) have recently been developed and shown to target LPS and the bacterial BamA protein (required for the correct insertion of β-barrel proteins into the outer membrane of Gram-negatives). The *in vitro* activity of some of these membrane biogenesis-targeting constructs (the most successful are compound 3 and 4) is excellent against all ESKAPE Gram-negatives (MICs well below 1 mg/l). Some of these compounds, which also display low eukaryotic toxicity, low propensity for resistance development, satisfactory pharmacokinetics and efficacy in murine infection models, are currently in preclinical toxicology studies, and envisaged as very promising (Luther *et al.*, 2019; McLaughlin & Van Der Donk, 2020).

(4) Bacterial targets leading to increased susceptibility to envelope-targeting immune components

In this section we discuss the main recently discovered bacterial targets that can lead to sensitization against mammalian envelope-targeting immunity, potentially useful for the development of future anti-virulence therapies (Bergman *et al.*, 2020; Duperthuy, 2020; Rezzoagli *et al.*, 2020). Advances have been made in the identification of bacterial targets leading to a blockade of the capacity for resistance development to these experimental treatments, for example for the chimeric compounds 3 and 4 mentioned above (Luther *et al.*, 2019; Vitale *et al.*, 2020). A set of 13 genes, many of which are involved in LPS modification, have been identified as drivers of PA resistance to these chimeric compounds, and therefore, disabling these genes would allow us to enhance the chances of therapeutic success (Shang *et al.*, 2020; Vitale *et al.*, 2020).

(a) *Peptidoglycan-targeting proteins*

Although bacterial muramidase inhibitors (Ivy and MliC/PliC families) are essential for lysozyme resistance in certain Gram-negatives (Callewaert *et al.*, 2012; Zielke *et al.*, 2018; Torrens *et al.*, 2020), knowledge concerning the ESKAPE representatives is scarce. In PA, the protection exerted by these inhibitors seems expendable (Torrens *et al.*, 2017), and although in KP they may be an advantage for infection, their presence is not uniform in clinical strains (Fialkina *et al.*, 2014). Although basic local alignment search tool (BLAST) or other similar tools show that both AB and ES have these inhibitors encoded in their genomes, no studies to date have analysed their therapeutic potential.

The AB capsule provides outstanding protection against lysozyme: a defective mutant is 137-fold more susceptible than the wild-type (Tipton *et al.*, 2018). PA-secreted

rhamnolipid seemingly participates in a complex strategy contributing to lysozyme degradation by host proteases and PA elastase (Andersen *et al.*, 2017). BamB and FabY proteins have also been shown to be essential for PA protection (*in vitro* and in a murine model); their inactivation likely causes cell envelope perturbation leading to increased lysozyme influx (Lee *et al.*, 2017). Peptidoglycan recycling blockade (through *nagZ* or *ampG* deletion) has been demonstrated to raise PA susceptibility to host immune components targeting this structure (incuding lysozyme and PGLYRPs), after they overcome the permeability barrier in the presence of subinhibitory levels of colistin. The inactivation of Mpl ligase, involved in β-lactam resistance and peptidoglycan recycling, also sensitized PA to lysozyme (Torrens *et al.*, 2017, 2019a). These findings were corroborated *in vivo*: peptidoglycan recycling-defective mutants showed attenuated virulence (increased mouse survival and decreased PA load), whereas subtherapeutic colistin cleared systemic infection by these mutants, likely through synergy with the endogenous peptidoglycan-targeting immunity (Torrens *et al.*, 2019b).

(*b*) *Bacterial membranes interaction-based immune components*

There are many strategies by which bacteria resist the action of membrane-targeting immune components, mainly relying on their surface structures. Among these, LPS modification, usually of transient nature, is one of the most important (Guilhelmelli *et al.*, 2013; Nuri, Shprung & Shai, 2015; Abdi, Mirkalantari & Amirmozafari, 2019; Duperthuy, 2020). For example, the attachment of aminoarabinose to lipid A contributes to PA resistance against hBD-2 and PG-1, and also to KP survival (Moskowitz, Ernst & Miller, 2004; Cheng, Chen & Peng, 2010; Lam *et al.*, 2011; Abdi *et al.*, 2019). Lipid A hepta-acylation (mediated by Lpx acyltransferases) enables AB resistance to certain CAMPs (Boll *et al.*, 2015) and LpxO-dependent hydroxylation protects against hBD-3 (Bartholomew *et al.*, 2019). Lipid A acylation (mediated by LpxM) also protects KP, especially against defensins (Clements *et al.*, 2007). LpxL2 acytransferase also has been shown to participate in KP lipid A acylation, which is necessary for survival in *G. mellonella* (Mills *et al.*, 2017). These modifications are generally governed by two-component systems responding to stress (e.g. PmrAB, PhoPQ), and therefore could represent therapeutic targets (Moskowitz *et al.*, 2004; Gooderham & Hancock, 2009; Beceiro *et al.*, 2011; Bhagirath *et al.*, 2019).

In KP the capsule plays a prominent role in protection against host defences: the majority of clinical strains express a pronounced capsule causing the characteristic mucoid phenotype. This anionic polysaccharide provides a physical barrier against envelope-targeting elements, but also works through the release of fragments that act as decoys (Campos *et al.*, 2004; Llobet, Tomás & Bengoechea, 2008). Capsule-defective mutants display increased susceptibility to defensins, lactoferrin-derived CAMPs, and Hst5 (Campos *et al.*, 2004; Llobet *et al.*, 2008; Du *et al.*, 2017). The KP capsule additionally masks certain epitopes to avoid their detection and reduce the host's expression of antimicrobial peptides and inflammatory mediators (Moranta *et al.*, 2010). Therefore, any participant in capsule production (e.g. the *cps* operon, *dsbC*, *wzm/t*, *ycgE*, etc.) can be seen as a potential anti-KP target (Wyres *et al.*, 2016).

Other surface-related protective strategies include the MprF protein-mediated addition of positively charged L-alanine to membranes, causing repulsion of CAMPs (Klein *et al.*, 2009; Abdi *et al.*, 2019), and the regulator PsrA, which promotes the expression of genes involved in PA membrane impermeability and derived resistance against indolicidin (Gooderham *et al.*, 2008; Abdi *et al.*, 2019). Type IV pili and their correct glycosylation have been shown to contribute to resistance to SPs, likely because these structures contribute to PA membrane integrity (Tan *et al.*, 2015). Also linked to correct envelope structure, *pch* and *ptsP* have been described to participate in PA resistance to SP-A (Zhang *et al.*, 2005). KP OmpA is essential for protection against HNP-1 (Llobet *et al.*, 2009), whereas its AB homolog is key for resistance to LL-37 (Lin *et al.*, 2015b). Finally, KP peptidoglycan-associated and murein lipoproteins are needed for serum resistance and full virulence, likely because they contribute to cell wall integrity (Hsieh *et al.*, 2013).

Biofilm formation is another strategy that protects bacteria against host defences. PA biofilm, mainly made up of alginate, is a paradigmatic example of how this extracellular element acts as a physical barrier to the diffusion of immune mediators/antibiotics (Thi, Wibowo & Rehm, 2020). Alginate induces conformational changes in CAMPs similar to those occurring when they insert into membranes, thereby preventing them from interacting with the PA surface. Alginate also induces aggregation of CAMPs, hampering their diffusion (Chan, Burrows & Deber, 2005; Benincasa *et al.*, 2009; Foschiatti *et al.*, 2009; Guilhelmelli *et al.*, 2013). Thus, any participant in the complex pathways responsible for alginate production (and other polymers produced by PA) and biofilm formation is a potential antipseudomonal target (Thi *et al.*, 2020).

Production of proteases is another strategy studied in detail in PA: LasB degrades LL-37 in human wounds (Schmidtchen *et al.*, 2002), as well as SP-A/–D (Alcorn & Wright, 2004; Kuang *et al.*, 2011b). These proteins are also degraded by PA protease IV, which impairs pulmonary surfactant function (Malloy *et al.*, 2005). Interestingly, PA flagellum-defective mutants produce an insufficient quantity of exoproteases to degrade SP-A, leading to their increased clearance in mouse lungs. This defective protease production apparently also renders PA unable to degrade lysozyme (Kuang *et al.*, 2011a). The other ESKAPE Gram-negatives are not known to produce this type of proteases (Lindsay, Oates & Bourdillon, 2017), a topic worthy to investigate further to unveil potential bacterial weak points. Some studies showed the production of proteases by AB that lead to immunoglobulin A (IgA) degradation (Diebel *et al.*, 2009), suggesting that AB and other ESKAPE Gram-negatives may produce mediators that contribute to the inactivation of envelope-targeting immunity.

Finally, unlike AB, ES, or PA (Rieg *et al.*, 2009), in which there is little extrusion of immune compounds, efflux enables KP resistance against certain CAMPs. More specifically, the AcrAB-TolC system contributes to resistance against HNP-1, hBD-1, and hBD-2, with the expression of this pump essential for full virulence in a murine model (Padilla *et al.*, 2010; Veleba *et al.*, 2012). The well-known role of efflux pumps in antibiotic resistance adds interest to their use as anti-virulence targets, which would not only boost the power of CAMPs, but also enable the re-use of classic antibiotics. In this regard, it has been proposed that bacteria may display increased susceptibility to envelope-targeting immunity when they exert responses to antibiotic challenge (or *vice versa*). Besides the examples cited throughout this review, other studies have demonstrated clear synergy for quinolones plus hBD-1 or HNP-1 against KP and PA (Campos *et al.*, 2006), and enhanced PA susceptibility to ciprofloxacin promoted by HNP-3, likely because these CAMPs activate a massive ATP efflux reducing drug expulsion in exchange (Wang, Dong & Lou, 2011a). A similar pattern is seen for indolicidin derivatives, based on the fact that these peptides inhibit phosphotransferase/acetyltransferase activities, mechanisms on which aminoglycoside resistance strongly depends (Boehr *et al.*, 2003; Ruden *et al.*, 2019). Finally, avibactam treatment has been shown to sensitize KP against LL-37, probably because this β-lactamase inhibitor alters cell wall mechanics in this species (Ulloa *et al.*, 2019).

(5) Host-related targets that improve the performance of bacterial envelope-targeting immune components

Besides bacterial targets that could reduce pathogen resistance to envelope-targeting immunity, other host-related alternatives might improve its performance. In this regard, PA stimulates the production of host cathepsins B, L, and S, which are proteases affecting hBD-2/-3 (Taggart *et al.*, 2003). Besides acting in the direct degradation of host immune compounds, the PA proteases AprA, LasA, and LasB also affect certain host proteins such as decorin, an extracellular matrix constituent. Decorin degradation releases dermatan sulfate that binds to α-defensins, abrogating their bactericidal activity (Schmidtchen, Frick & Björck, 2001). Similarly, the degradation of heparan sulfate proteoglycans by LasA causes the release of free syndecan-1 that neutralizes several CAMPs (Park *et al.*, 2001). More recently, PA rhamnolipid has been shown to interfere with the host's protein kinase C pathways, leading to a decrease in hBD-2 and inflammatory mediator production (Dössel *et al.*, 2012). Therefore, any treatment interfering with these mechanisms could trigger increased bacterial envelope-targeting immune effectiveness, a field worthy of further development.

Other host elements may also be considered as direct therapeutic allies to improve immune performance. For instance, boosting the production of specific immune components such as β-defensin-2, which is below the normal range in COPD patients (Arnason *et al.*, 2017), could help limit the spread of infection in this context but possibly also in other situations. Moreover, it may also be possible to harness the immunomodulatory power of other immune resources. For instance, PGLYRP2 has been shown to increase inflammation and reduce the expression of CAMPs during infection, thus silencing its expression could potentially improve the clinical outcome in specific cases (Gowda *et al.*, 2015). Conversely, lysozyme displays an anti-inflammatory capacity in certain contexts; therefore, increasing its expression could provide benefits to the host and in bactericidal activity (Ganz *et al.*, 2003). Minimizing the expression of certain host proteases/peptidases such as those discussed above could increase the half-life of endogenous or exogenously administered immune elements (Mardirossian *et al.*, 2016). Additionally, modulating some features in specific patient profiles could improve immune performance, for example: overproduction of cathepsins (mainly in CF and/or COPD) (Taggart *et al.*, 2003; Dalcin & Ulanova, 2013); docosahexaenoic acid levels below normal values in CF, which impairs lysozyme performance (Martinez *et al.*, 2009); expression of autoantibodies in CF, which bind to BPI and dramatically reduce its activity (Carlsson *et al.*, 2011); host expression of dipeptidyl peptidase I, shown to degrade SP-D, which in turn favours KP survival *in vivo* (Sutherland *et al.*, 2014); and modulation of oxidative stress in CF patients that dampens SP-A/-D natural defences (Kuzmenko *et al.*, 2005; Starosta & Griese, 2006). Finally, in a step closer to therapeutic applications, experimental treatments disabling the neutrophil elastase-mediated degradation of SPLUNC1 in the lungs have shown positive effects in alleviating PA infection severity in mice (Nichols *et al.*, 2015).

IV. RESOURCES YET TO BE EXPLOITED: NOVEL HUMAN ANTIMICROBIAL PEPTIDES TARGETING THE GRAM-NEGATIVE ENVELOPE

Some inconspicuous peptides (known as 'cryptides') released through the action of bacterial/host proteases have recently been identified within larger human non-immune proteins, often displaying membrane disruption-based antimicrobial activity (Pane *et al.*, 2017, 2016). Three cryptides have been found within pepsinogen A3 (PAP-A3, IMY25 and FLK22) that show activity against AB, KP, and PA (Pane *et al.*, 2018). PAP-A3 stood out in reducing the PA burden (by 4 log units) in a murine skin abscess model, whereas IMY25 showed the greatest antibiofilm capacity, likely because alginate does not significantly sequester it (Pane *et al.*, 2018). Human apolipoproteins are also a source of cryptides [e.g. r(P)ApoBL, r(P)ApoBS, etc.] that display non-negligible anti-inflammatory and anti-KP or anti-PA activities *in vitro* (Pane *et al.*, 2016; Zanfardino *et al.*, 2018; Gaglione *et al.*, 2020).

Some cryptides have been identified in coagulation-related proteins, e.g. GKY25, released from pro-thrombin/

thrombin and found in wounds (Van Der Plas *et al.*, 2016; Saravanan *et al.*, 2018). Although GKY25 displays limited bactericidal capacity, when tested in murine models of PA systemic infection or LPS-induced shock, large increases in survival were reported (from 0 to 60% and 0 to 90%, respectively) (Papareddy *et al.*, 2010a; Merza *et al.*, 2014), likely due to its capacity to modulate monocyte/macrophage responses and neutrophil rolling/migration (Hansen *et al.*, 2017; Lim *et al.*, 2017). Its shorter derivative, GKY20, whose carpet-like mechanism is well understood, shows similar features with reduced toxicity (Oliva *et al.*, 2019). The thrombin-derived fragment TCP96 molecules bind to each other to form aggregates with LPS, obviously with an anti-endotoxin role, and also with entire bacteria, boosting phagocytosis. This has been proved in murine models of subcutaneous infection and LPS challenge, in which PA load and inflammation were significantly decreased (Petrlova *et al.*, 2020). Some peptides derived from anti-thrombin III (e.g. FFF21) killed PA *in vitro* and reduced infection burden (by 5 log units) in a murine model of peritoneal infection, but oddly, did not increase mouse survival (Papareddy *et al.*, 2014). The heparin cofactor II-derived peptide KYE28 displays significant LPS-neutralizing capacity *in vitro*/*ex vivo*, reproduced in murine models of LPS-induced shock and sepsis, increasing mouse survival (by more than 70%) and reducing PA load (Kalle *et al.*, 2014).

Other cryptides with comparable activity to LL-37 have been identified, such as GVF27 (released from 11-hydroxysteroid dehydrogenase-1β) (Bosso *et al.*, 2016); RQA21, corresponding to the C-terminus of extracellular superoxide dismutase (Pasupuleti *et al.*, 2009a); and MAN28, KKR20, and LVL20 [all released from the human cellular prion protein PrP(c)] (Pasupuleti *et al.*, 2009b). The *in vitro* antimicrobial power of the anti-endotoxin peptide YR26, derived from the cationic region of furin, is substantial, with MICs ≈4 μM for KP and PA (Sinha *et al.*, 2018).

Hornerin is one of the main protein components of epidermal cornified cell envelopes, and its derived peptides released by the action of skin proteases and generically known as cationic intrinsically disordered antimicrobial peptides (CIDAMPs) have exhibited excellent antipseudomonal activity, with MICs ≈ 0.3 mg/l (Latendorf *et al.*, 2019). Their artificial derivatives obtained by palmitoylation display even greater anti-KP and anti-PA power, relying on a strong membrane-disruption capacity in addition to their constitutive ribosome-targeting activity (Latendorf *et al.*, 2019). The peptides GGL27 and EDC34, derived from the tissue factor pathway inhibitors TFPI-1 and TFP-2, share similar antipseudomonal activities *in vitro*, with some differences (Papareddy *et al.*, 2010b, 2012, 2013): GGL27 treatment barely increased mouse survival after PA systemic infection, whereas in a LPS-induced shock model, survival rose from 0 to 50% Papareddy *et al.*, 2010b). Conversely, EDC34 was incapable of neutralizing LPS-linked inflammation, but in a PA sepsis model increased mouse survival to 70% (Papareddy *et al.*, 2013). Activation of the complement cascade and immunoglobulin binding were proposed to contribute to this activity (Ali *et al.*, 2018).

V. CONCLUSIONS

(1) There is a large body of research showing that mammalian bacterial envelope-targeting immunity has important therapeutic potential against ESKAPE Gram-negatives, notably yet to be explored/exploited, and with variable chances of success depending on pathogen species. It is noteworthy that much of the information published refers to PA, AB, and KP, but there is an important gap regarding our knowledge on ES that must be filled.

(2) The large number of studies showing at least partially contradictory results regarding the *in vitro* activity of a given immune component and the discrepancies between *in vitro*/*in vivo* results in some cases, raise a number of unsolved questions that must be addressed to enable the successful development of therapies.

(3) There is limited information on PK/PD parameters or resistance development dynamics regarding bacterial envelope-targeting immunity-based treatments. Such knowledge will be essential for designing robust therapeutic options. Proteolytic degradation, sequestration by host tissues, toxicity at high doses, and the need for a vehicle to allow suitably high concentrations at infection sites are also critical questions to be studied in more detail.

(4) Although some encouraging results have been found *in vitro* and/or *in vivo* for certain treatments (histatin derivatives, tritrpticin, BPI and SPLUCN1 derivatives, KDAMPs, GIIA-sPLA2, etc.), many research lines related to these findings have been discontinued. To extend these studies is essential, as is the initiation of new clinical trials for the most promising treatments (e.g. iseganan or murepavadin and its derivatives).

(5) Although preliminary *in vitro* and *in vivo* models showed promising results against ESKAPE Gram-negatives for some experimental treatments, their intended use in clinical trials is often reduced to mild infections and/or for other species. Research projects for their application against ESKAPE Gram-negatives and/or severe infections must be restored.

(6) There are likely to be additional antimicrobial peptides discovered in other mammals or even in our own species, hidden in the sequences of larger proteins (cryptides) and whose anti-ESKAPE Gram-negatives potential must be studied.

(7) Although less studied and therefore still far from entry into clinical practice, there are other potential targets in the biology of ESKAPE Gram-negatives and in the host that could be investigated with respect to

developing novel anti-virulence treatments, reducing pathogen resistance and improving the performance of our bacterial envelope-targeting immunity. This new concept for therapy development is a promising option that has been barely explored to date.

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