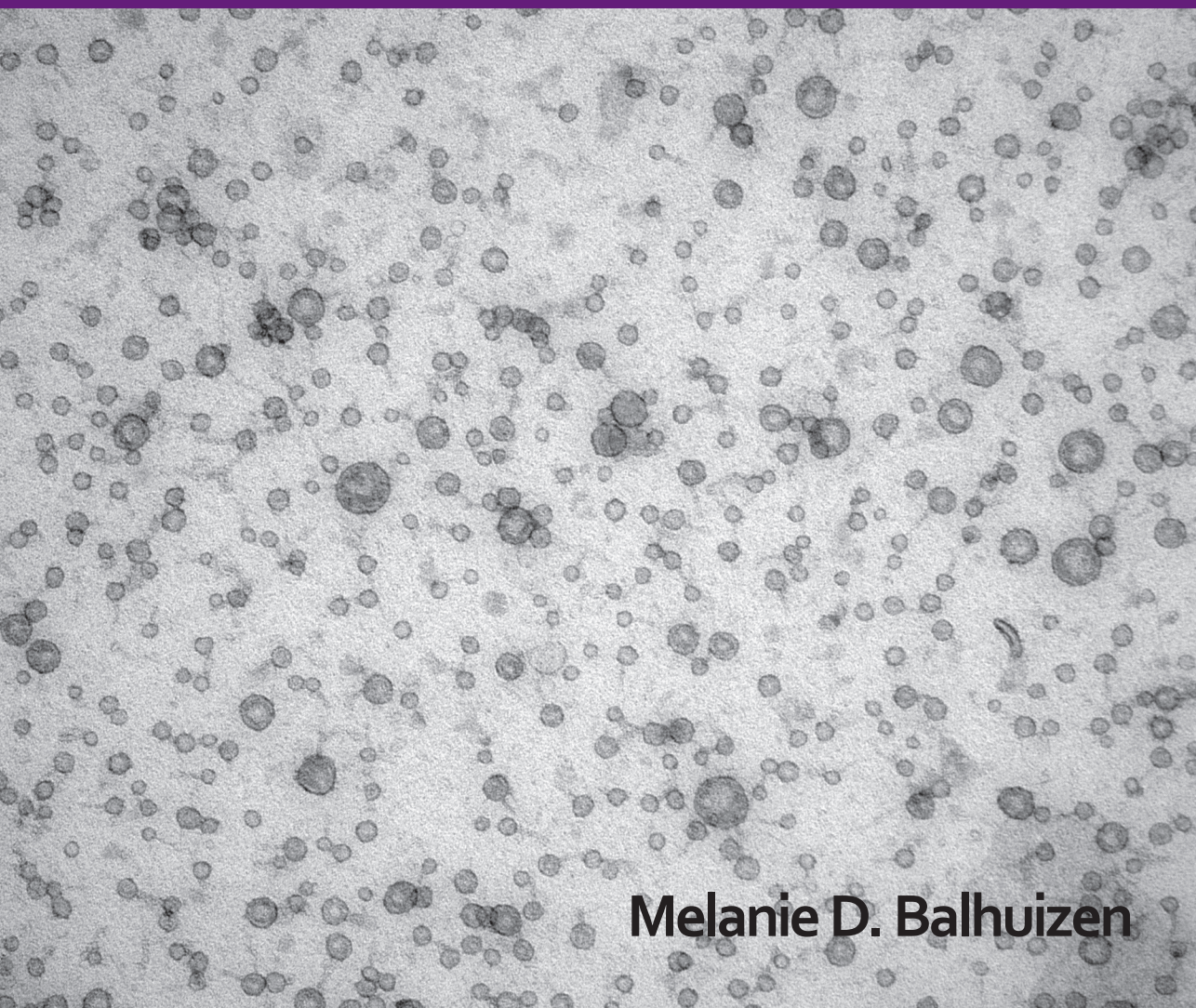
Interplay between Outer Membrane Vesicles and Host Defense Peptides

Bacterial shields versus Host swords



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Bacterial shields versus Host swords

Samenspel tussen Buitenmembraanblaasjes en Gastheerverdedigingspeptiden

BACTERIËLE SCHILDEN VERSUS GASTHEER ZWAARDEN

(met een samenvatting in het Nederlands)

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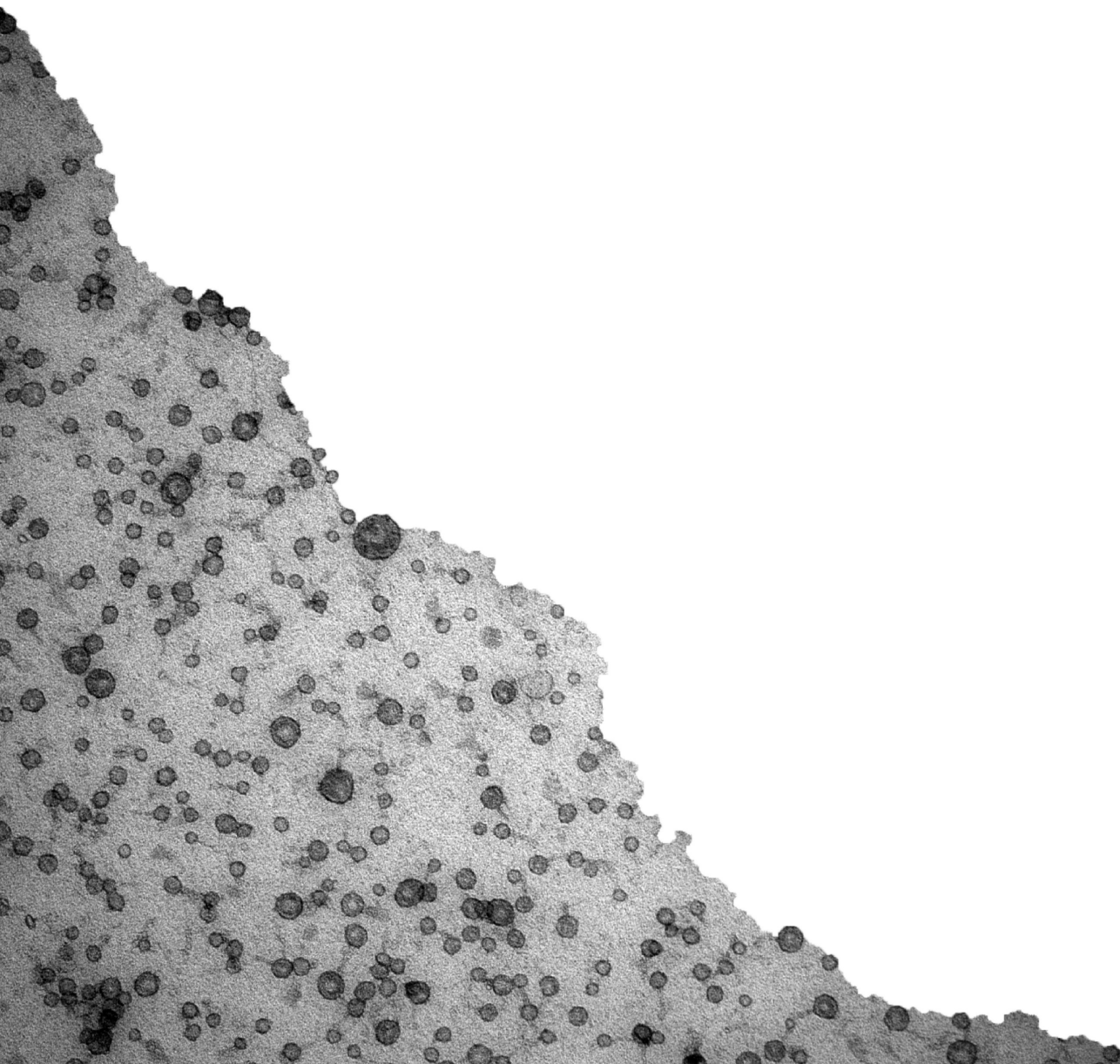

Chapter 1

Outer Membrane Vesicle Induction and Isolation for Vaccine Development

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Abstract

Gram-negative bacteria release vesicular structures from their outer membrane, so called outer membrane vesicles (OMVs). OMVs have a variety of functions, such as waste disposal, communication and antigen or toxin delivery. These vesicles are promising structures for vaccine development since OMVs carry many surface antigens identical to the bacterial surface. However, isolation is often difficult and results in low yields. Several methods to enhance OMV yield exist, but these do affect the resulting OMVs. In this review, our current knowledge about OMVs will be presented. Different methods to induce OMVs will be reviewed and their advantages and disadvantages will be discussed. The effects of the induction and isolation methods used in several immunological studies on OMVs will be compared. Finally, the challenges for OMV-based vaccine development will be examined and one example of a successful OMV-based vaccine will be presented.

Introduction on Outer Membrane Vesicles

Gram-negative bacteria have two membranes, the inner membrane (IM) and the outer membrane (OM) with a network of peptidoglycan (PG) and the periplasmic space in between. Both the IM and OM consist of phospholipids and membrane proteins, with only the outer leaflet of the OM containing lipopolysaccharide (LPS). From the OM, small protrusions can form that pinch off and become extracellular vesicles, called outer membrane vesicles (OMVs) **(Fig. 1)** [1]. Resulting OMVs are between 20 to 300 nm in diameter. They consist of a single lipid bilayer containing LPS, phospholipids and various outer membrane proteins (OMPs) which represents the OM of the originating bacteria. Formation of OMVs has been the subject of much debate, since the driving force of OMV formation was long unknown [2,3]. The formation of OMVs was long thought to be an arbitrary stress response from bacterial cells [4], but OMVs were later proven to have many more functions, which will be discussed below.

Formation of OMVs

For OMV formation it is necessary to detach the OM from the PG layer and the IM. These layers are stably linked by many different lipoproteins. A local decrease in the number of lipoproteins, and therefore the number of crosslinks, has been implicated in OMV formation. For example, deletion of lipoprotein (Lpp) in combination with magnesium starvation or deletion of outer membrane protein A (OmpA) in *Escherichia coli* results in hypervesiculating mutants [5,6]. Similarly, the Tol-Pal system consists of several proteins connecting the IM with the OM and disruption of the Tol-Pal system resulted in hypervesiculation in *Salmonella* and *E. coli* [7,8]. Furthermore, alterations to the PG structure can prevent proper attachment of lipoproteins which in turn decreases the number of crosslinks between the IM and OM. This indirectly causes an increase in OMV formation due to outer-membrane instability. For instance, a PG hydrolase mutant of *E. coli*, defective in peptide crosslinks of the PG, prevented attachment of Lpp in the PG layer [9].

An increase in membrane turgor, the force of internal fluids pressing outward, also results in an increase in OMV release. For example, accumulation of misfolded periplasmic proteins in a periplasmic serine endoprotease (*degP*) mutant, resulting in loss of a periplasmic chaperone, resulted in hypervesiculation [10]. In a mutant defective in PG recycling, PG fragments accumulated and increased membrane turgor, leading to membrane pressure and increased OMV release. It was shown that Lpp-based crosslinks between the PG and IM in this mutant remained at a similar level as in the wildtype strain [9]. This suggests that this mechanism is independent of crosslink formation and therefore increases OMV formation through a distinct mechanism [9,10].

The most recent hypothesis for OMV formation is the induction of curvature in the OM due to an increase in phospholipid (PL) content. An increase in OMV formation was shown for mutants missing components of the retrograde PL transporter system [11]. This was shown for *Hemophilus influenzae*, *Vibrio cholerae* and *E. coli*, indicating that it is a conserved mechanism in several species. Altogether, many different mechanisms for OMV formation have been described in the past decades and most likely all these mechanisms are simultaneously at play in bacteria [12–15].

Functions of OMVs

OMVs exert many different functions, all beneficial to the bacterium. Mostly, OMVs act as a transportation system for proteins, but also for DNA and RNA [16–18]. Vesicles provide a protected environment for bacterial molecules and delivery by OMVs may act as a long-distance delivery system [19,20]. Additionally, transport by OMVs prevents dilution of cargo. OMV cargo has been shown to be involved in inter-cellular communication. For example, OMVs of *Pseudomonas aeruginosa* contain the pseudomonas quinolone signal (PQS) and removal of OMVs from the bacterial culture inhibits cell-cell communication [21]. Furthermore, antibiotic resistance genes are often transported via OMVs. OMVs from *Neisseria gonorrhoeae* were shown to contain circular DNA and supplementation with these OMVs provided penicillin resistance in susceptible bacterial strains [17]. Additionally, *Acinetobacter baumannii* was shown to transfer carbapenem resistance genes in their OMVs [22]. However, OMVs are not only used for communication within one bacterial species. When *E. coli* or *Salmonella* species were incubated with OMVs derived from *P. aeruginosa* or *Shigella flexneri,* antigens of the latter two were readily detected on the surface of the first two bacterial species, suggesting inter-species communication by OMVs [23]. Furthermore, *E. coli* OMVs were shown to package Shiga toxins [24] and *P. aeruginosa* OMVs were shown to contain PG hydrolases and fuse with *E. coli* and *Staphylococcus aureus* membranes, thereby eradicating competing bacterial species [25].

Besides bacterial interactions, OMVs are involved in pathogen-host interactions [20,26]. OMVs are used by many bacterial species to deliver toxins and other virulence factors [27–31]. For example, *P. aeruginosa* was shown to package small RNAs in OMVs that silenced host RNA involved in the innate immune response [18]. Sorting of OMV cargo must therefore be a selective process and might be regulated by LPS microdomains, but the exact sorting mechanism has yet to be elucidated [3,32].

Furthermore, OMVs are beneficial to bacterial growth in several ways. Despite the fact that OMV release seems to be a one-way process, OMVs also have been shown to fuse with bacterial membranes, for instance to aid in nutrient acquisition. For *Neisseria meningitidis* it was shown that OMVs are enriched in proteins involved in iron and zinc acquisition [33].

Similarly, for *Bordetella pertussis* the process of iron retrieval by OMVs from medium was demonstrated. When OMVs from an iron-rich culture were supplemented to a culture growing in iron-limited conditions, they were able to transfer iron to bacterial cells and boost bacterial growth [29].

Another function related to OMV production is protection, both from exogenous and endogenous molecules. For instance, OMVs are used to dispose of bacterial waste, such as misfolded proteins, to prevent bacteria from collapsing under the pressure [34,35]. This is regulated by stress responses, such as the sigma E pathway [13] or independent of envelope stress responses [4], as a protection mechanism. Many exogenous molecules can also threaten bacteria, such as antimicrobial peptides (AMPs) and bacteriophages. Addition of OMVs to an *E. coli* or *Helicobacter pylori* culture increased bacterial resistance to AMPs and bacteriophages [36–38], presumably by acting as a decoy for these substances to attach, instead of targeting the bacterial membrane.

The functions of OMVs in biofilms have been described in all stages of biofilm formation, being a common component of the biofilm matrix [39]. Addition of OMVs to *H. pylori* cultures was shown to correlate with increased biofilm forming ability [40]. OMVs of *P. aeruginosa* have been shown to aid in attachment and aggregation of bacterial cells in early stages of biofilm formation and carry molecules to protect the biofilm later on, such as β-lactamases [41]. The most well-known functions of OMVs are schematically depicted in **Figure 1**.

Despite the many physiological functions of OMV, their release is often insignificant and insufficient for industrial purposes [42]. Several methods exist to induce OMV release in bacterial cultures and increase OMV yields [43]. However, these induced OMVs may have different properties compared to OMVs that are spontaneously released from bacteria [10,44–46]. In this review, we sought out to describe the different methods used to induce OMVs and to compare the properties of the resulting vesicles. Additionally, a standard nomenclature is introduced to prevent confusion between different types of OMVs. The potential of OMV-based vaccines is illustrated using *N. meningitidis* as an example, since it is the only licensed OMV-based vaccine up to date. Furthermore, we compared immunological properties of differently induced OMVs from *B. pertussis*, a pathogen for which an OMV-based vaccine exhibits great potential. Future challenges for OMV-based vaccines are discussed, as well as different applications for use of OMVs.

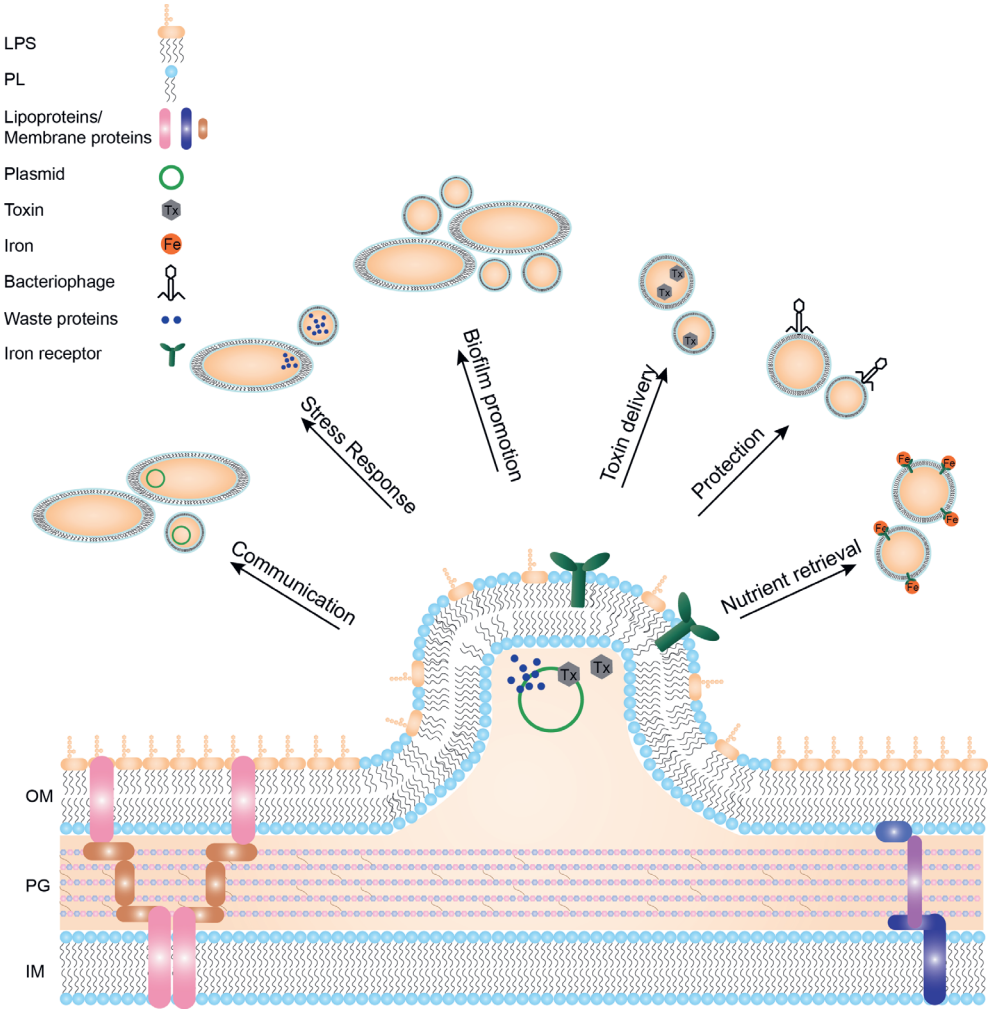


Figure 1: Gram-negative bacterial membrane during OMV formation and functions of resulting OMVs. *OMVs have been implicated in many different processes. Depicted here are the different functions OMVs have been shown to be involved in, such as transport of toxins, waste removal or communication between bacteria. LPS: lipopolysaccharide, PL: phospholipid, OM: outer membrane, PG: peptidoglycan, IM: inner membrane.*

Induction and Isolation of OMVs for Therapeutic Purposes

OMVs have many potential therapeutic applications which will be described later, but often their release is insignificant, resulting in low harvested yields from bacterial cultures. Spontaneous OMVs (sOMVs) are naturally released by Gram-negative bacteria and considered most similar to OMVs formed *in vivo* based on protein and lipid content [47–49]. These OMVs can be obtained by growing bacteria until end-logarithmic phase and harvested without the addition of any foreign molecules. Therefore, all OMVs have been

formed spontaneously and resemble the composition of *in vivo* formed OMVs by unstressed bacteria. The low yield of sOMVs makes them not easily feasible for vaccine production, yet these vesicles are most desirable for vaccine development due to their natural composition resembling the outer membrane of the bacterium.

Induction methods of OMVs

Several methods exist to increase release of OMVs, all with their own advantages and disadvantages, as summarized in **Figure 2**. For instance, vesicles can be induced by disruption of the membrane with either addition of a detergent or by sonication. OMVs can also be induced by an extracting agent such as ethylenediaminetetraacetic acid (EDTA) or with sub-lethal concentrations of antibiotics [50,51]. Furthermore, OMVs might also be induced by genetic modifications, which will be discussed in more detail below. These different methods can all distinctly affect the resulting OMVs in size, proteolytic or thermal stability or composition, which may influence the immune responses evoked by the OMVs [44].

In the next paragraphs, different methods to induce OMVs will be discussed in more detail, starting with genetic modifications that are applied to increase yields of OMVs (gOMVs) [52]. Included in the term gOMVs are generalized modules for membrane antigens (GMMA), since this term likewise refers to OMVs from bacteria in which mutations induce hypervesiculation [53]. These gOMVs can be produced by various mutations, for example by deletion of the *tolR* gene, which is part of the Tol-Pal system discussed above [54]. The Tol-Pal system has often been a target for creation of hypervesiculating mutants [55–57]. Deletion of lipoproteins connecting the OM and the PG layer, such as Lpp for *E. coli*, has been shown to increase OMV production [5]. Another example is the knock-out of chaperones to increase stress due to the presence of misfolded proteins, which in turn increases vesicle formation, as shown for a *degP* mutant of *E. coli* [12]. Deletion of a lytic transglycosylase which resulted in hypervesiculating *N. meningitidis* strain, is another example of a long list of deletion mutants [58,59]. Deletion of genes is not the only modification that resulted in hypervesiculating bacteria. For example, overexpression of the outer membrane protease OmpT resulted in hypervesiculation in *E. coli* [60]. Additionally, expression of the deacylase PagL resulted in hypervesiculation, due to increased curvature of the bacterial outer membrane, caused by an inverted cone-shaped LPS [61]. This list is not exhaustive and research is still performed to identify additional hypervesiculating mutations. These modifications result in spontaneously formed vesicles, but a disadvantage is that these gOMVs can differ from *in vivo* formed vesicles since the bacterium has been genetically altered. For example, cargo in gOMVs resulting from a *degP* mutant is substantially different from cargo in sOMVs, with an increased presence of periplasmic proteins, which are suggested to be misfolded [10]. Analysis of gOMVs

produced by a Δ*tolB* mutant in *Buttiauxella agrestis* even revealed multilamellar vesicles [62] and *E. coli* Δ*tolR* gOMVs were shown to have reduced entry into epithelial Caco-2 cells [63]. Another disadvantage of genetic modification is that one mutation may not work in all Gram-negative bacteria, requiring research to find distinct mutations for different bacteria. To facilitate this, publishing data on genetic mutations not resulting in hypervesiculating bacterial strains would prevent other research groups from trying similar strategies.

A second method, using detergent for extraction of OMVs (dOMVs), has been used for decades and is a widespread method in industry. *N. meningitidis* OMV vaccines used to be prepared based on detergent extraction [64]. With this method OMVs are induced with detergent-like molecules such as deoxycholate or sodium dodecyl sulfate. These molecules interact with the bacterial membrane to increase vesicle formation and additionally remove LPS from the outer membrane, creating LPS-containing micelles. The resulting dOMVs lack LPS [42], which will decrease the undesired LPS-based innate immune response. However, the loss of LPS results in loss of many antigens which are loosely attached to the membrane. Additionally, the intrinsic adjuvant activity of OMVs is likewise lost upon LPS removal [42]. This shows that there is a fine balance between potential beneficial and detrimental effects of LPS in OMVs.

Furthermore, OMVs can be induced by membrane destabilization using sonication, which does not remove LPS from the membrane [65–67]. These vesicles are prepared by sonication of the bacterial pellet, thereby forming membrane fragments which fuse to form lysis OMVs (lOMVs). These lOMVs are not prepared from the bacterial supernatant, where the sOMVs can be found, and therefore likely contain cargo not natively present in OMVs [66]. So, despite high OMV yields obtained through sonication of bacteria, these vesicles do not represent *in vivo* protein compositions of sOMVs and therefore are not always suitable for vaccine development.

Another method to induce OMVs is the use of extraction molecules, such as EDTA [68]. These extraction molecules aim to destabilize the bacterial membrane similar to the two methods described above (dOMVs, lOMVs) but are relatively mild and thus retain LPS and native cargo in the OMVs [69]. Therefore, they are named native OMVs (nOMVs). One such a molecule is EDTA, which is a chelating agent that removes calcium ions from the environment [70]. Calcium ions stabilize bacterial membranes by neutralization of repelling negative charges of LPS and other anionic lipids [71]. Removal of calcium ions causes the negative charges of LPS to repel each other and thereby it destabilizes the membrane [72]. Therefore, yields of OMVs are increased using EDTA, but LPS remains present. These vesicles are better suited for vaccine development but might be less stable due to the lack of calcium ions.

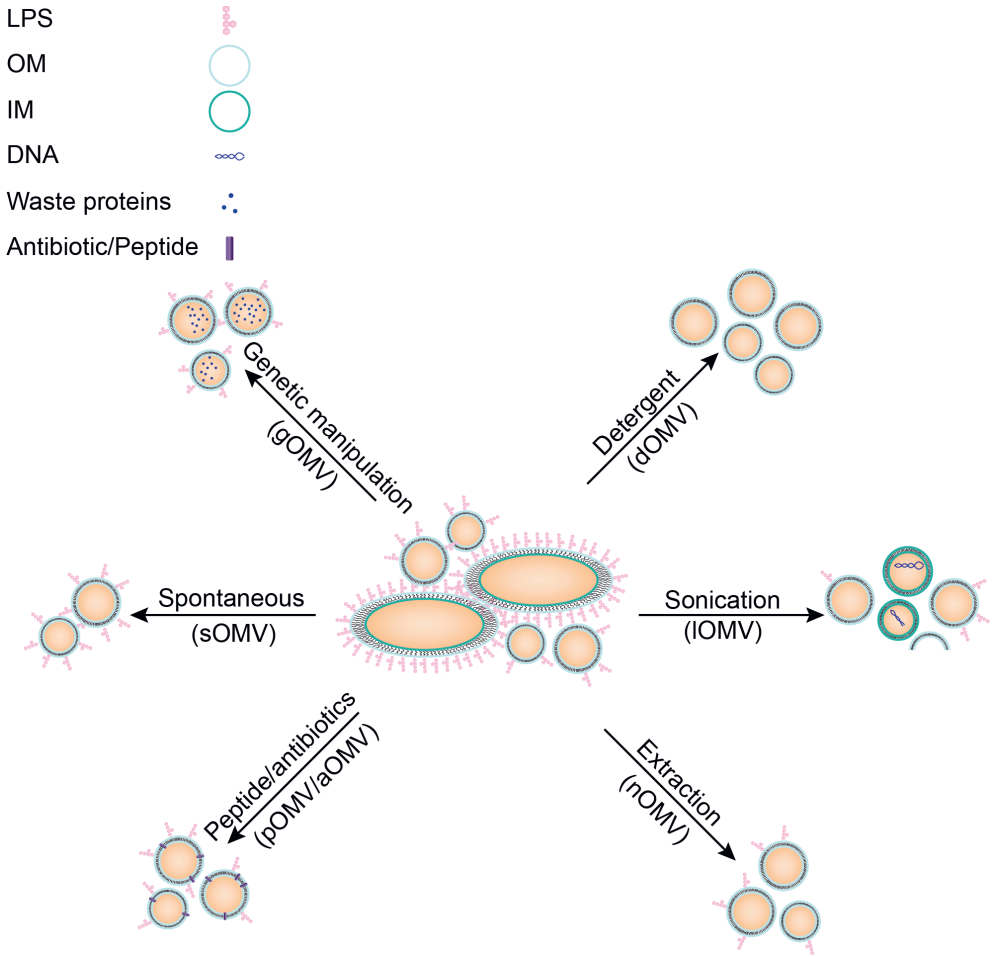


Figure 2: Schematic overview of different induction methods for OMVs, including characteristics of resulting OMVs. *No stimulation: these vesicles are most similar to spontaneous vesicles released in vivo (sOMVs). Genetic manipulation may alter OMV cargo (gOMVs). Detergent isolation of OMVs results in OMVs lacking LPS (dOMVs), an important immunogenic molecule. Sonication of bacteria disrupts the entire membrane, resulting in impurities in the vesicles fraction due to cell lysis (lOMVs). Extraction with membrane destabilizing molecules may alter vesicle composition (nOMVs), but they are more representative of the OM. OMV induction by peptides or antibiotics may alter membrane stability and may result in the peptide or antibiotic being present in the resulting OMV (pOMVs/aOMVs). A new technique researched to induce OMVs is heat-shock, resulting in hOMVs , but this technique is not yet established and therefore not included in this figure.*

Yet another method to increase OMV release is the induction of membrane stress by supplementation of external molecules , as was shown for naturally occurring antimicrobial peptides (AMPs) [36,46]. These OMVs have been named peptide induced OMVs (pOMVs). AMPs are part of the innate immune system and are expressed by different cell types,

Table 1: Summary of used abbreviations for OMVs based on their induction method, as described in the text.

Method	*Abbreviation*	*Yield*	*Remarks*
No induction	sOMV	Low	-
Genetically induced OMVs	gOMV	Variable	Possible change in cargo
Detergent induced OMVs	dOMV	High	Loss of LPS and lipoproteins
Sonication induced OMVs	lOMV	High	Contamination with IM
Extraction molecule induced OMVs	nOMV	High	Potential loss of membrane stability
Peptide induced OMVs	pOMV	Low	Potential loss of membrane stability
Antibiotic induced OMVs	aOMV	Variable	Antibiotic presence or resistance
Heat induced OMVs	hOMV	High	Possible change in lipid composition

such as granulocytes or epithelial cells, in response to bacterial signals, such as LPS, or cytokines, such as interleukin 1-beta (IL-1β) [73–76]. These AMPs often have high affinity for bacterial membranes which is part of their antibacterial mechanism of action [77–80]. As discussed above, increased OMV production may be a means of the bacterium to protect from induced stress. *Bordetella bronchiseptica* pOMVs resulting from induction by the porcine myeloid antimicrobial peptide 36(PMAP-36), were indeed shown to contain PMAP-36 [46]. Furthermore, pOMVs contained relatively more phosphatidylglycerol compared to sOMVs, a negatively charged lipid which might interact with the positively charged AMP. *B. bronchiseptica* pOMVs were also shown to have decreased thermal stability compared to sOMVs, possibly due to the presence of PMAP-36 in the membrane [46]. As for peptide-based antibiotics, such as polymyxin B, also these molecules were shown to induce OMV release [81]. The mechanism of OMV induction might be similar to AMPs mechanism and is based on membrane disruption resulting in stress for the bacterium and subsequent OMV production.

Antibiotics targeting intracellular processes were also shown to induce OMV release. OMV formation might be a response to antibiotics, since in *P. aeruginosa* it was shown that antibiotics induce PQS secretion [82] and PQS was shown to induce OMV formation [83]. However, antibiotic-induced OMVs (aOMVs) are mostly characterized based on protein content [84], so a good comparison to pOMVs cannot be made yet. aOMVs of extra-intestinal pathogenic *E. coli* were characterized after induction by gentamicin and particle sizes were not altered [85]. Remarkably, when the cargo of these aOMVs was assessed

using mass-spectrometry, mostly cytoplasmic and periplasmic proteins were enriched, relative to sOMVs. Likely these are misfolded proteins formed after gentamicin's interference with the ribosome machinery. *E. coli* aOMVs induced by ampicillin were shown to have an increased amount of the OMP Pal, further demonstrating that antibiotics can alter OMV cargo [45]. In another study, *A. baumannii,* was stimulated with tetracycline, imipenem and eravacycline and the resulting OMVs were quantified [86,87]. Whereas tetracycline did not induce OMV release, imipenem did induce release of aOMVs, which showed a relative increase in OMPs and proteases [86]. Eravacycline-induced aOMVs likewise contained relatively more OMPs, but also resistance-associated proteins, such as ATP-binding cassette (ABC) and other transporter proteins [87]. This demonstrates the possible risks of this induction method, as sub-lethal concentrations of antibiotics may result in development of antibiotic resistance.

Since these different methods all result in slightly different vesicles, nomenclature to distinguish between different categories is important. However, the current use of abbreviations in literature is not consistent. Different abbreviations are used in literature for an identical OMV type while, vice versa, one abbreviation is sometimes used for two different OMV types. Different induction methods will result in different OMVs. Therefore, OMVs should be extensively studied before being used in immunization studies. In order to compare results from different studies a common nomenclature is useful. Therefore, a suggested nomenclature is summarized in **Table 1** for all OMV types currently described.

Isolation of secreted OMVs

Isolation of OMVs is independent of the induction method used and literature shows very similar procedures with small differences between studies. First, OMVs are separated from bacteria by centrifugation [43]. Next, contaminations are removed by filtration. In literature, the use of both 0.22 µm and 0.45 µm filters have been described, being the first discrepancy between methods. The use of a 0.22 µm filter could decrease yields by preventing passage of the larger vesicles, since vesicle sizes range between 20 nm and 300 nm [13,15]. After filtration, OMVs can be concentrated by precipitation or ultrafiltration [43]. Vesicles are eventually collected by ultracentrifugation, ranging from 40,000 x g up to 175,000 x g, depending on the bacterial species studied. Unfortunately, rotor type and centrifugation times are not specified in most papers, although these parameters are critical for yields of OMVs [88]. Furthermore, ultracentrifugation alone may leave contaminants still present in the isolated OMV fraction. Sucrose density gradient ultracentrifugation will result in the purest fraction of OMVs and therefore also in the most consistent results between labs [89]. When isolation methods are not described in detail, results obtained in immunization studies are not relevant for industrial application. To ensure possibilities to replicate experiments, transparency and detailed description of

methods is critical. This will aid the scientific community and increase the relevance and comparability of described results, which could eventually accelerate OMV-based therapeutic applications.

Applications of OMVs

The use of OMVs as a vaccine for their originating bacterium will be elaborated on below, but OMVs have many more therapeutic purposes. For instance, OMVs could also be suitable as a carrier system for proteins, glycans and other molecules [90,91]. OMVs may be decorated with proteins, for instance, by coupling heterologous antigens to endogenous autotransporters in a hypervesiculating bacterial strain. This technique is developed for the hemoglobin-binding protease (Hbp) of *E. coli* in a hypervesiculating *Salmonella enterica* serovar typhimurium SL3261, using not only genetic engineering but also click chemistry to ensure display of larger antigens [92,93]. This technique can provide a robust system using well-defined OMVs as carrier, that can be decorated with antigens of any bacterium of interest. The principle was demonstrated for antigens of *Mycobacterium tuberculosis* and *Chlamydia trachomatis*, where the antigens were shown to be processed and recognized [94]. Not only can protein antigens be displayed on the OMV surface, also heterologous glycans can be displayed. Delivery of *Salmonella* O-antigen by gOMVs induced high levels of IgG antibodies in mice [95]. Glycosylated OMVs have also been proven to protect against subsequent bacterial challenges and may be another route of immunization with the use of OMVs [90]. Thus, OMVs are useful as carrier system, and they also have useful intrinsic adjuvant properties [96]. The presence of LPS can activate the innate immune system, thereby enhancing a subsequent immune response.

Besides using OMVs as carrier for the delivery of antigens, they could also be loaded with therapeutic molecules. *E. coli* OMVs decorated with human epidermal growth factor receptor 2 (HER2) specific antibodies and loaded with siRNAs were shown to target HER2-tumor cells and exert cytotoxic effects [97]. The advantage of using natural OMVs over synthetic liposomes is their enhanced fusion capability with target cells [98]. These examples altogether show the versatile applications of OMVs and the exciting progress made over the last decades.

OMVs in vaccination

OMVs have been implicated in many different carrier functions, as described above. However, OMVs also have a great potential as endogenous vaccine. The presence of several antigens on OMVs limits the possibilities for pathogens to mutate all the target antigens present in the vaccine and thereby limits the possibility to generate vaccine escape variants. Furthermore, OMV isolation is relatively low-cost, compared to

manufacturing of synthetic molecules for instance. This altogether makes OMVs of great interest for vaccine development [42].

In vivo, OMVs have a wide variety of interactions with immune cells showing their potential to be used for immunization [99,100]. The first studies into immune responses evoked by OMVs already showed promising inductions of cytokines and chemokines in macrophages and other cell types. sOMVs isolated from *Brucella melitensis* were used to stimulate bone marrow-derived macrophages and showed induction of interleukin (IL)-6, IL-10, IL-12 or tumor necrosis factor (TNF)α, depending on the LPS structure of the strain used [101]. sOMVs from *E. coli* were shown to induce CXCL1 expression in mouse endothelia, leading to an increased influx of neutrophils [102]. *E. coli* gOMVs, loaded with a *Chlamydia muridarum* antigen, elicited a neutralizing antibody response, in contrast to recombinant antigen [103]. This was confirmed for several other heterologous antigens loaded in *E. coli* gOMVs [104], showing the benefit of retaining native conformation of antigens in OMVs.

For some bacteria, studies on immunization with OMVs in mice have been performed and showed protection against subsequent infection. For example, immunization with sOMVs from *V. cholerae* in mice induced immunoglobulin production and demonstrated a protective effect towards this bacterium in their offspring [105]. Studies on *E. coli* sOMVs in mice revealed that immunization with sOMVs protected against sepsis and mainly induced the protective effect via T cell immunity [106]. For *Shigella flexneri* merged sOMVs were used to immunize mice and also this provided protection against a subsequent lethal bacterial *Shigella* challenge [107]. An sOMV-based vaccine against *Burkholderia pseudomallei* provided protection in a mouse model and even induced humoral immunity in a nonhuman primate immunization model [108]. In chicken, a sOMV-based vaccine against *S. enterica* protected against a subsequent challenge and induced high expression of interferon γ [109]. All together the potential of OMVs for the use as a vaccine component seems promising. Induction and isolation methods will have consequences for immune properties of OMVs, which was shown for *A. baumannii*. sOMVs and two types of vesicular structures prepared from the bacterial pellet were tested and while immunization with both types elicited protection against subsequent challenge, antibody profiles differed substantially [110]. However, two types of OMV-based vaccine against *N. meningitidis* are currently the only OMV-based vaccines licensed, MeNZB and Bexsero, and research into these will be discussed in more detail below [111–113].

The success story of Neisseria meningitidis

One Gram-negative bacterium for which a safe and effective OMV-based vaccine has been in use since 1990 is *N. meningitidis* [114]. This capsule forming bacterium has several serogroups and for most serogroups vaccines have been developed, except for serogroup

B, which is estimated to be the cause of 65% of all meningitis cases in children under 5 years of age in the United States and 51% of total cases in Europe [115,116]. The vaccines for other *N. meningitidis* serogroups rely on recombinant capsular proteins, but for serogroup B the capsular protein resembles a molecule in the human brain [117]. This provokes the risk of auto-immunity when used in a vaccine and therefore a different vaccine approach was necessary.

The vaccine approach for serogroup B was focused on OM proteins. To maintain stability and native fold of OM proteins, it is essential to utilize them in a membranous environment and therefore OMVs were considered most promising for this approach. The most abundant OM protein in *N. meningitidis* OMVs was shown to be the porin protein PorA which is also the most immunogenic protein [118]. Unfortunately, variation in PorA is substantial among various serogroup B strains and little cross-protection is observed [119]. It was suggested that more than twenty different PorA molecules should be included in the vaccine to cover all *N. meningitidis* strains circulating worldwide [119]. Therefore, no worldwide vaccine has been developed yet. However, OMV-based vaccines have proven to be very effective to control clonal outbreaks. Several outbreaks have occurred in the past, including in Cuba, Norway [120], New Zealand [114] and Normandy [121]. Because these outbreaks were caused by a single *N. meningitidis* serogroup B strain, a dOMV vaccine was employed to prevent further spread and causalities. Analysis of the immune responses elicited by the OMV-based vaccine in Normandy demonstrated that it indeed elicited short-lasting responses, but it also elicited larger strain coverage than expected [121]. Effectiveness of OMV-based vaccines was determined to be 87% after ten months for the vaccines used in Cuba and Norway [120], and around 80% in New Zealand [114]. However, these numbers are not based on clinical efficacy trials and therefore have to be assessed critically. Nevertheless, OMV-based vaccines are a safe and effective measure to control clonal epidemics of *N. meningitidis* and might even show cross-protection [122].

OMV-based vaccines for *N. meningitidis* used in clonal outbreaks were prepared using detergent extraction, and thus removal of large amounts of LPS, decreasing the reactogenicity of the vaccine and increasing the necessity of an external adjuvant. Currently, detergent free nOMVs from *Neisseria* are being developed, using EDTA [68,123]. Additionally, OMV yields have been improved by deletion of the *rmpM* gene in the bacterium [68]. This gene codes for a peptidoglycan-binding outer membrane protein. Removal of the *rmpM* gene results in decreased attachment between the PG and OM, and thereby an increased formation of vesicles. Since OMVs were purified without detergents, no LPS was removed. To decrease toxicity of LPS, a second genetic modification has been implemented, by generating a knock-out of the *lpxL1* gene [124]. Mutants lacking the acyltransferase lpxL1 produce LPS containing five acyl chains as opposed to the regular six. This altered LPS results in decreased activation of toll like receptor 4 (TLR4) and is

therefore less reactogenic, but it does not affect bacterial growth [124,125]. This ngOMV-based vaccine of *Neisseria* has shown promising results in clinical trials and no severe adverse effects have been observed [126]. Research has even shown the possibility of a continuous production of *N. meningitidis* gOMVs, without the use of EDTA [127]. This example shows how OMV-based vaccines could be a promising strategy for combatting diseases caused by Gram-negative bacteria. The different types of OMVs studied for *N. meningitidis* are summarized in **Table 2**.

OMV-vaccine candidate: Bordetella pertussis

B. pertussis is a Gram-negative bacterium for which an OMV-based vaccine might be the optimal strategy for disease prevention. The bacterium is the causative agent for pertussis, or whooping cough, a disease most dangerous for infants [128]. Upon inhalation or ingestion of the bacterium, it adheres to ciliated cells and invades the lungs [129]. Because *B. pertussis* attaches to and immobilizes the cilia, the infected individual cannot clear debris from the lungs and develops coughing fits. This results in the risk of suffocation, particularly in infants [130].

Due to the severity of *B. pertussis* infection and the mortality caused in infants, vaccines were developed as soon as the causative agent of pertussis was identified in 1906 by Jules Bordet and Octave Gengou. The first pertussis vaccine was licensed in 1914 and consisted of whole-cell inactivated bacteria [131]. This whole-cell pertussis vaccine (wPv) provided satisfactory efficacy but due to adverse effects of the vaccine, like systemic fever,

Table 2: Overview of tested OMV types for N. meningitidis and B. pertussis and their results. *N. meningitidis OMVs results are obtained in humans* [114,120,126]*, B. pertussis OMV results are obtained from mice experiments* [65,66,132].

Bacterium	***OMV type***	***Modifications***	***Results***	***Remarks***
Neisseria meningitidis	dOMVs	none	80-87% effectiveness	Clonal outbreaks
	ngOMVs	ΔrmpM, ΔlpxL1	79% effectiveness	41-82% cross-reactivity
Bordetella pertussis	lgOMVs	PagL	5-fold decrease in bacterial colonization	Compared to naïve mice
	nOMVs	None	5-fold decrease in bacterial colonization	Compared to naïve mice
	sOMVs	None	5-fold decrease in bacterial colonization	Compared to naïve mice at day 63

convulsions and even acute encephalopathy, most countries switched in the 1990's to an acellular pertussis vaccine (aPv). aPv contains three to five purified *B. pertussis* proteins and does not elicit adverse effects. However, aPv has shown waning immunity, partly because *B. pertussis* mutates vaccine antigens, such as pertactin [133,134]. Additionally, the aPV does not evoke the effective T helper 1 cell (Th1)/T helper 17 cell (Th17) response that a natural infection evokes in humans, but a T helper 2 cell (Th2) response [135]. Furthermore, the current vaccine can prevent disease but not transmission as shown by studies in a baboon model [136]. By this, *B. pertussis* can maintain itself in a population, causing disease in non-vaccinated individuals, such as infants.

The incidences of *B. pertussis* infections are increasing, despite a high vaccination coverage. Worldwide approximately 140,000 cases were reported in 2016, despite a vaccination coverage of approximately 90% [137,138]. This increase in the number of cases was observed around the same time the vaccination program for *B. pertussis* was changed in the 1990's. Therefore, development of an increased immunogenic *B. pertussis* vaccine that can elicit the right immunological response and maintain increased immunological memory has become a priority [139–141]. Recently, the optimal administration of a *B. pertussis* vaccine was investigated in mouse experiments, and was found to be intranasal, which might increase effectiveness of new vaccines [142]. However, experiments in baboons will give more relevant information, since their immune system is more representative of a human immune system.

B. pertussis OMVs have been extensively studied as an alternative strategy for vaccine development, since wPv has shown adverse effects and aPv has shown waning immunity [143,144]. *B. pertussis* lOMVs have been studied first, induced using sonication methods. Additionally, the *pagL* gene was introduced in this bacterial strain, which removes one acyl chain of the LPS, to decrease LPS toxicity (therefore resulting in lgOMVs) [65]. Immunization with these vesicles showed faster clearance of bacteria in the lungs of infected mice compared to non-immunized mice. Furthermore, immunization of mice with lgOMVs showed decreased gene expression of inflammatory cytokines compared to immunization with lOMVs. Previous attempts to detoxify LPS by genetic removal of acyl chains did not always lead to these results, sometimes endotoxic effects were even increased [145]. This is probably due to an increased LPS release upon modification which resulted in increased TLR4 activation [145]. Recently, the immune response evoked by *B. pertussis* lOMVs was studied further and revealed to activate the inflammasome in mice and human macrophages [146]. However, since the lOMVs or lgOMVs were extracted using sonication, which disrupts the entire bacterial membrane, contamination of the OMV sample by other bacterial products could have occurred, or the loss of natural cargo, making the studied immune responses not relevant to *in vivo* produced sOMVs [65].

In later studies, *B. pertussis* nOMVs have also been used in *in vivo* mice experiments [66]. Immunization with *B. pertussis* nOMVs, extracted by EDTA, resulted in a rapid clearance of bacteria after challenge, similar to immunization with killed whole-cell *B. pertussis*. Characterization of *B. pertussis* nOMVs revealed that the presence of pertussis toxin and pertactin in the nOMVs is essential for evoking an effective immune response [147]. *B. pertussis* nOMVs elicited a long-lasting protection, for up to nine months in mice [148]. However, it is unsure how this can be translated to humans.

More recently, *B. pertussis* sOMVs have been used to study the immune response. The adaptive immune responses evoked by these sOMVs have been characterized extensively in mice. Both immunization with sOMVs and heat-killed whole-cell *B. pertussis* evoked mixed Th1/Th2/Th17 responses but the sOMV-based vaccine seems to induce a different antibody response. After booster immunization, the antibody profile was dominated by IgG3 for the sOMV-based vaccine and IgG1 for the whole-cell based vaccine [132]. The most prominent antibody response was shown to be directed against BrkA, Vag8 and LOS, all outer membrane components [149]. Most importantly, the sOMV-based vaccine showed less pro-inflammatory cytokine production compared to the whole-cell vaccine [132]. This suggests that an sOMV-based vaccine could resolve any reactogenicity problems encountered by the whole-cell vaccine. All types of studied *B. pertussis* OMVs are summarized in **Table 2**.

Future prospects

OMV-based vaccines have great potential for next generation vaccine development. Several challenges remain, such as yields of OMVs after isolation and the composition and thereby immunogenicity and toxicity of the vesicles [42]. While OMVs are a natural product and beneficial to the bacterium, no large quantities are produced during bacterial growth but there might be a rather simple solution to increase OMV yields. OMV release has been shown to increase upon stress, as described above. The most trivial stress a bacterium could experience is environmental stress, for instance nutrient depletion, pressure or temperature stress [150]. In *Pseudomonas putida* it was shown that a heat shock of 55°C increased OMV release [151]. Similarly, after treatment with higher temperatures B-band LPS export in OMVs was increased in *P. aeruginosa* [152]. Recently it was shown that heat treatment also increased OMV production in *B. pertussis* [153]. These heat-induced OMVs (hOMVs) were shown to still contain important antigens, which could be detected with antibodies. Furthermore, the same treatment was applied to *B. bronchiseptica* and the resulting OMVs were further characterized to ensure quality of the vesicles. hOMVs were stable up to 40°C and sOMVs even up to 50°C. Additionally, hOMVs had a large increase in the amount of lysophospholipids, as was shown by lipidomic analysis. Despite these differences, hOMVs evoked a comparable immune response to spontaneous OMVs *in vitro*

[46]. However, the quantities of LPS might still pose a problem and molecules to modulate the resulting immune response are needed.

AMPs were originally known for their antimicrobial function, but recently immunomodulatory functions have been described for these peptides as well [74–76,154,155]. For example the human cathelicidin antimicrobial peptide LL-37 has been shown to direct dendritic cell (DC) differentiation to promote a Th1 response [156]. This could be employed in vaccine development by steering the immune response to a desired Th1/Th17 response. Furthermore, the chicken cathelicidin 2 (CATH-2) was shown to induce several chemokines, suggesting that immunomodulatory mechanisms might be conserved among species [74,157]. On the other hand, LL-37 has also been shown to inhibit TLR4 activation on DCs by agonists such as LPS [158]. Likewise, CATH-2 was shown to neutralize LPS-induced TLR4 activation by interacting with LPS. This was shown in the context of non-viable bacteria, possibly as a mechanism to prevent an unnecessary immune response [76,159]. Therefore, AMPs could decrease LPS-induced TLR4 activation in an OMV-based vaccine, as was recently been shown for *B. bronchiseptica* OMVs. When the porcine AMP, PMAP-36, was supplemented to isolated sOMVs and subsequently used to stimulate macrophages, cytokine secretion decreased [46]. Furthermore, a synthetic anti-endotoxin (non-AMP) peptide was also shown to decrease *E. coli* OMV-induced activation of human macrophages [160]. These results indicate that AMPs are promising molecules for tailoring immune responses in vaccines, however studies on other pathogens should reveal whether this mechanism is broadly applicable. Furthermore, tailor-made AMPs could be synthesized with desired immune modulating properties [161–163].

Concluding remarks

OMVs are a promising tool for vaccine development, especially compared to acellular vaccines. The immunogenicity of OMV based vaccines is increased compared to acellular vaccines and the risk of evolutionary escape pathogens is almost diminished compared to using an acellular vaccine. Especially in cases where whole-cell approaches are not applicable, OMV-based vaccines pose a potential solution. However, some challenges lie ahead of the OMV-based vaccine field, such as low yields and endotoxic effects due to the presence of LPS. Many solutions have been created, such as extraction to increase vesicle yields or genetic modifications to both increase yields and decrease endotoxicity. However, these solutions often alter vesicles as such that their representation of the originating bacterium is no longer optimal. The use of spontaneous OMVs would circumvent this. To increase yields of sOMVs, a simple solution seems to be optimal; heat induction. To reduce LPS endotoxicity, host defense peptides show great potential. These peptides are known for their antimicrobial activity but additionally have shown to exhibit immunomodulatory activities, such as the neutralization of LPS induced TLR4 activation.

Furthermore, they can steer immune responses, possibly into an ideal Th1/Th17 response. Concluding, induced OMVs are a promising future for bacterial vaccine development, with AMPs being a potential solution to the challenges that lie ahead.

References

[1] T.J. Beveridge, Structures of Gram-Negative Cell Walls and Their Derived Membrane Vesicles, J. Bacteriol. 181 (1999) 4725–4733. doi:10.1128/JB.181.16.4725-4733.1999.

[2] L. Zhou, R. Srisatjaluk, D.. Justus, R.. Doyle, On the origin of membrane vesicles in Gram-negative bacteria, FEMS Microbiol. Lett. 163 (1998) 223–228. doi:10.1111/j.1574-6968.1998.tb13049.x.

[3] M.F. Haurat, J. Aduse-Opoku, M. Rangarajan, L. Dorobantu, M.R. Gray, M.A. Curtis, M.F. Feldman, Selective sorting of cargo proteins into bacterial membrane vesicles, J. Biol. Chem. 286 (2011) 1269–1276. doi:10.1074/jbc.M110.185744.

[4] A.J. McBroom, M.J. Kuehn, Release of outer membrane vesicles by Gram-negative bacteria is a novel envelope stress response, Mol. Microbiol. 63 (2007) 545–558. doi:10.1111/j.1365-2958.2006.05522.x.

[5] I. Sonntag, H. Schwarz, Y. Hirota, U. Henning, Cell envelope and shape of Escherichia coli: multiple mutants missing the outer membrane lipoprotein and other major outer membrane proteins, J. Bacteriol. 136 (1978) 280–285. doi:10.1128/jb.136.1.280-285.1978.

[6] D.W. Yem, H.C. Wu, Physiological characterization of an Escherichia coli mutant altered in the structure of murein lipoprotein, J. Bacteriol. 133 (1978) 1419–1426. doi:10.1128/jb.133.3.1419-1426.1978.

[7] B.L. Deatherage, J.C. Lara, T. Bergsbaken, S.L.R. Barrett, S. Lara, B.T. Cookson, Biogenesis of bacterial membrane vesicles, Mol. Microbiol. 72 (2009) 1395–1407. doi:10.1111/j.1365-2958.2009.06731.x.

[8] A. Bernadac, M. Gavioli, J.C. Lazzaroni, S. Raina, R. Lloubès, Escherichia coli tol-pal mutants form outer membrane vesicles, J. Bacteriol. 180 (1998) 4872–4878. doi:10.1128/jb.180.18.4872-4878.1998.

[9] C. Schwechheimer, A. Kulp, M.J. Kuehn, Modulation of bacterial outer membrane vesicle production by envelope structure and content, BMC Microbiol. 14 (2014) 1–12. doi:10.1186/s12866-014-0324-1.

[10] C. Schwechheimer, M.J. Kuehn, Synthetic effect between envelope stress and lack of outer membrane vesicle production in escherichia coli, J. Bacteriol. 195 (2013) 4161–4173. doi:10.1128/JB.02192-12.

[11] S. Roier, F.G. Zingl, F. Cakar, S. Durakovic, P. Kohl, T.O. Eichmann, L. Klug, B. Gadermaier, K. Weinzerl, R. Prassl, A. Lass, G. Daum, J. Reidl, M.F. Feldman, S. Schild, A novel mechanism for the biogenesis of outer membrane vesicles in Gram-negative bacteria, Microb. Cell. 3 (2016) 257–259.

doi:10.1038/ncomms10515.

[12] A.J. McBroom, A.P. Johnson, S. Vemulapalli, M.J. Kuehn, Outer membrane vesicle production by Escherichia coli is independent of membrane instability, J. Bacteriol. 188 (2006) 5385–5392. doi:10.1128/JB.00498-06.

[13] A. Kulp, M.J. Kuehn, Biological Functions and Biogenesis of Secreted Bacterial Outer Membrane Vesicles, Annu. Rev. Microbiol. 64 (2010) 163–184. doi:10.1146/annurev.micro.091208.073413.

[14] H.M. Kulkarni, M. V. Jagannadham, Biogenesis and multifaceted roles of outer membrane vesicles from Gram-negative bacteria, Microbiology. 160 (2014) 2109–2121. doi:10.1099/mic.0.079400-0.

[15] C. Schwechheimer, M.J. Kuehn, Outer-membrane vesicles from Gram-negative bacteria: Biogenesis and functions, Nat. Rev. Microbiol. 13 (2015) 605–619. doi:10.1038/nrmicro3525.

[16] N.J. Bitto, R. Chapman, S. Pidot, A. Costin, C. Lo, J. Choi, T. D'Cruze, E.C. Reynolds, S.G. Dashper, L. Turnbull, C.B. Whitchurch, T.P. Stinear, K.J. Stacey, R.L. Ferrero, Bacterial membrane vesicles transport their DNA cargo into host cells, Sci. Rep. 7 (2017) 1–11. doi:10.1038/s41598-017-07288-4.

[17] D.W. Dorward, C.F. Garon, R.C. Judd, Export and intercellular transfer of DNA via membrane blebs of Neisseria gonorrhoeae, J. Bacteriol. 171 (1989) 2499–2505. doi:10.1128/jb.171.5.2499-2505.1989.

[18] K. Koeppen, T.H. Hampton, M. Jarek, M. Scharfe, S.A. Gerber, D.W. Mielcarz, E.G. Demers, E.L. Dolben, J.H. Hammond, D.A. Hogan, B.A. Stanton, A Novel Mechanism of Host-Pathogen Interaction through sRNA in Bacterial Outer Membrane Vesicles, PLoS Pathog. 12 (2016) 1–22. doi:10.1371/journal.ppat.1005672.

[19] J.M. Bomberger, D.P. MacEachran, B.A. Coutermarsh, S. Ye, G.A. O'Toole, B.A. Stanton, Long-distance delivery of bacterial virulence factors by pseudomonas aeruginosa outer membrane vesicles, PLoS Pathog. 5 (2009) 1000382. doi:10.1371/journal.ppat.1000382.

[20] E.J. Jones, C. Booth, S. Fonseca, A. Parker, K. Cross, A. Miquel-Clopés, I. Hautefort, U. Mayer, T. Wileman, R. Stentz, S.R. Carding, The Uptake, Trafficking, and Biodistribution of Bacteroides thetaiotaomicron Generated Outer Membrane Vesicles, Front. Microbiol. 11 (2020) 1–14. doi:10.3389/fmicb.2020.00057.

[21] L.M. Mashburn, M. Whiteley, Membrane vesicles traffic signals and facilitate group activities in a prokaryote, Nature. 437 (2005) 422–425. doi:10.1038/nature03925.

[22] C. Rumbo, E. Fernández-Moreira, M. Merino, M. Poza, J.A. Mendez, N.C. Soares, A. Mosquera, F. Chaves, G. Bou, Horizontal transfer of the OXA-24 carbapenemase gene via outer membrane vesicles: A new mechanism of dissemination of carbapenem resistance genes in Acinetobacter baumannii, Antimicrob. Agents Chemother. 55 (2011) 3084–3090. doi:10.1128/AAC.00929-10.

[23] J.L. Kadurugamuwat, T.J. Beveridge, Membrane vesicles derived f rom Pseudomonas aeruginosa and Shigella flexneri can be integrated into the surfaces of other Gram-negative bacteria, Microbiology. 145 (1999) 2051–2060. doi:10.1099/13500872-145-8-2051.

[24] G.L. Kolling, K.R. Matthews, Export of Virulence Genes and Shiga Toxin by Membrane Vesicles of Escherichia coli O157:H7, Appl. Environ. Microbiol. 65 (1999) 1843–1848. doi:10.1128/AEM.65.5.1843-1848.1999.

[25] J.L. Kadurugamuwa, T.J. Beveridge, Bacteriolytic effect of membrane vesicles from Pseudomonas aeruginosa on other bacteria including pathogens: conceptually new antibiotics., J. Bacteriol. 178 (1996) 2767–2774. doi:10.1128/JB.178.10.2767-2774.1996.

[26] F.G. Zingl, P. Kohl, F. Cakar, D.R. Leitner, F. Mitterer, K.E. Bonnington, G.N. Rechberger, M.J. Kuehn, Z. Guan, J. Reidl, S. Schild, Outer Membrane Vesiculation Facilitates Surface Exchange and In Vivo Adaptation of Vibrio cholerae, Cell Host Microbe. 27 (2020) 225-237.e8. doi:10.1016/j.chom.2019.12.002.

[27] J.L. Kadurugamuwa, T.J. Beveridge, Virulence factors are released from Pseudomonas aeruginosa in association with membrane vesicles during normal growth and exposure to gentamicin: A novel mechanism of enzyme secretion, J. Bacteriol. 177 (1995) 3998–4008. doi:10.1128/jb.177.14.3998-4008.1995.

[28] T.N. Ellis, M.J. Kuehn, Virulence and Immunomodulatory Roles of Bacterial Outer Membrane Vesicles, Microbiol. Mol. Biol. Rev. 74 (2010) 81–94. doi:10.1128/MMBR.00031-09.

[29] G. Gasperini, V. Arato, M. Pizza, B. Aricò, R. Leuzzi, Physiopathological roles of spontaneously released outer membrane vesicles of Bordetella pertussis, Future Microbiol. 12 (2017) 1247–1259. doi:10.2217/fmb-2017-0064.

[30] C. Jha, S. Ghosh, V. Gautam, P. Malhotra, P. Ray, In vitro study of virulence potential of Acinetobacter baumannii outer membrane vesicles, Microb. Pathog. 111 (2017) 218–224. doi:10.1016/j.micpath.2017.08.048.

[31] H. Yoon, Bacterial Outer Membrane Vesicles as a Delivery System for Virulence Regulation, J. Microbioly Biotechnol. 26 (2016) 1343–1347. doi:10.4014/jmb.1604.04080.

[32] K.E. Bonnington, M.J. Kuehn, Protein selection and export via outer membrane vesicles, Biochim. Biophys. Acta. 1843 (2014) 1612–1619. doi:10.1016/j.bbamcr.2013.12.011.

[33] M. Lappann, A. Otto, D. Becher, U. Vogel, Comparative proteome analysis of spontaneous outer membrane vesicles and purified outer membranes of neisseria meningitidis, J. Bacteriol. 195 (2013) 4425–4435. doi:10.1128/JB.00625-13.

[34] C. Schwechheimer, C.J. Sullivan, M.J. Kuehn, Envelope control of outer membrane vesicle production in Gram-negative bacteria, Biochemistry. 52 (2013) 3031–3040. doi:10.1021/bi400164t.

[35] A.J. Manning, M.J. Kuehn, Functional advantages conferred by extracellular prokaryotic membrane vesicles, J. Mol. Microbiol. Biotechnol. 23 (2013) 131–141. doi:10.1159/000346548.

[36] A.J. Manning, M.J. Kuehn, Contribution of bacterial outer membrane vesicles to innate bacterial defense, BMC Microbiol. 11 (2011) 1–14. doi:10.1186/1471-2180-11-258.

[37] J. Roszkowiak, P. Jajor, G. Guła, J. Gubernator, A. Żak, Z. Drulis-Kawa, D. Augustyniak, Interspecies outer membrane vesicles (OMVs) modulate the sensitivity of pathogenic bacteria and pathogenic yeasts to cationic peptides and serum complement, Int. J. Mol. Sci. 20 (2019) 1–21. doi:10.3390/ijms20225577.

[38] B.O. Murray, R.A. Dawson, L.M. Alsharaf, J. Anne Winter, Protective effects of Helicobacter pylori membrane vesicles against stress and antimicrobial agents, Microbiology. 166 (2020) 751–758. doi:10.1099/mic.0.000934.

[39] S.R. Schooling, T.J. Beveridge, Membrane vesicles: An overlooked component of the matrices of biofilms, J. Bacteriol. 188 (2006) 5945–5957. doi:10.1128/JB.00257-06.

[40] H. Yonezawa, T. Osaki, S. Kurata, M. Fukuda, H. Kawakami, K. Ochiai, T. Hanawa, S. Kamiya, Outer membrane vesicles of helicobacter pylori TK1402 are involved in biofilm formation, BMC Microbiol. 9 (2009) 1–12. doi:10.1186/1471-2180-9-197.

[41] O. Ciofu, Chromosomal beta-lactamase is packaged into membrane vesicles and secreted from Pseudomonas aeruginosa, J. Antimicrob. Chemother. 45 (2000) 9–13. doi:10.1093/jac/45.1.9.

[42] L. van der Pol, M. Stork, P. van der Ley, Outer membrane vesicles as platform vaccine technology, Biotechnol. J. 10 (2015) 1689–1706. doi:10.1002/biot.201400395.

[43] J. Klimentová, J. Stulík, Methods of isolation and purification of outer membrane vesicles from gram-negative bacteria, Microbiol. Res. 170 (2015) 1–9.

doi:10.1016/j.micres.2014.09.006.

[44] B.S. Collins, Gram-negative outer membrane vesicles in vaccine development., Discov. Med. 12 (2011) 7–15.

[45] L.V. Michel, L. Gallardo, A. Konovalova, M. Bauer, N. Jackson, M. Zavorin, C. McNamara, J. Pierce, S. Cheng, E. Snyder, J. Hellman, M.E. Pichichero, Ampicillin triggers the release of Pal in toxic vesicles from Escherichia coli, Int. J. Antimicrob. Agents. 56 (2020) 106163. doi:10.1016/j.ijantimicag.2020.106163.

[46] M.D. Balhuizen, C.M. Versluis, R.M. van Harten, E.F. de Jonge, J.F. Brouwers, C.H.A. van de Lest, E.J.A. Veldhuizen, J. Tommassen, H.P. Haagsman, PMAP-36 reduces the innate immune response induced by Bordetella bronchiseptica-derived outer membrane vesicles, Curr. Res. Microb. Sci. 2 (2021) 100010. doi:10.1016/j.crmicr.2020.100010.

[47] E. Valguarnera, M.F. Feldman, Glycoengineered Outer Membrane Vesicles as a Platform for Vaccine Development, in: Methods Enzymol., 1st ed., Elsevier Inc., 2017: pp. 285–310. doi:10.1016/bs.mie.2017.06.032.

[48] G. Gasperini, V. Arato, M. Pizza, B. Aricò, R. Leuzzi, Physiopathological roles of spontaneously released outer membrane vesicles of Bordetella pertussis, Future Microbiol. 12 (2017) 1247–1259. doi:10.2217/fmb-2017-0064.

[49] G. Gasperini, M. Biagini, V. Arato, C. Gianfaldoni, A. Vadi, N. Norais, G. Bensi, I. Delany, M. Pizza, B. Aricò, R. Leuzzi, Outer Membrane Vesicles (OMV)-based and Proteomics-driven Antigen Selection Identifies Novel Factors Contributing to Bordetella pertussis Adhesion to Epithelial Cells, Mol. Cell. Proteomics. 17 (2018) 205–215. doi:10.1074/mcp.RA117.000045.

[50] R. Maredia, N. Devineni, P. Lentz, S.F. Dallo, J. Yu, N. Guentzel, J. Chambers, B. Arulanandam, W.E. Haskins, T. Weitao, Vesiculation from Pseudomonas aeruginosa under SOS, Sci. World J. 2012 (2012) 1–18. doi:10.1100/2012/402919.

[51] M. Duperthuy, A.E. Sjöström, D. Sabharwal, F. Damghani, B.E. Uhlin, S.N. Wai, Role of the Vibrio cholerae Matrix Protein Bap1 in Cross-Resistance to Antimicrobial Peptides, PLoS Pathog. 9 (2013) 1–12. doi:10.1371/journal.ppat.1003620.

[52] Y. Ojima, T. Sawabe, K. Konami, M. Azuma, Construction of hypervesiculation *Escherichia coli* strains and application for secretory protein production, Biotechnol. Bioeng. 117 (2020) 701–709. doi:10.1002/bit.27239.

[53] C. Gerke, A.M. Colucci, C. Giannelli, S. Sanzone, C.G. Vitali, L. Sollai, O. Rossi, L.B. Martin, J. Auerbach, V. Di Cioccio, A. Saul, Production of a Shigella sonnei vaccine based on generalized modules for membrane antigens (GMMA), 1790GAHB, PLoS One. 10 (2015). doi:10.1371/journal.pone.0134478.

[54] F. Micoli, R. Alfini, R. Di Benedetto, F. Necchi, F. Schiavo, F. Mancini, M. Carducci, E. Palmieri, C. Balocchi, G. Gasperini, B. Brunelli, P. Costantino, R. Adamo, D. Piccioli, A. Saul, GMMA is a versatile platform to design effective multivalent combination vaccines, Vaccines. 8 (2020) 1–16. doi:10.3390/vaccines8030540.

[55] L. Chen, J.L. Valentine, C.J. Huang, C.E. Endicott, T.D. Moeller, J.A. Rasmussen, J.R. Fletcher, J.M. Boll, J.A. Rosenthal, J. Dobruchowska, Z. Wang, C. Heiss, P. Azadi, D. Putnam, M.S. Trent, B.D. Jonesb, M.P. DeLisa, Outer membrane vesicles displaying engineered glycotopes elicit protective antibodies, Proc. Natl. Acad. Sci. U. S. A. 113 (2016) E3609–E3618. doi:10.1073/pnas.1518311113.

[56] T.C. Stevenson, C. Cywes-Bentley, T.D. Moeller, K.B. Weyant, D. Putnam, Y.F. Chang, B.D. Jones, G.B. Pier, M.P. DeLisa, Immunization with outer membrane vesicles displaying conserved surface polysaccharide antigen elicits broadly antimicrobial antibodies, Proc. Natl. Acad. Sci. U. S. A. 115 (2018) E3106–E3115. doi:10.1073/pnas.1718341115.

[57] T. Henry, S. Pommier, L. Journet, A. Bernadac, J.P. Gorvel, R. Lloubès, Improved methods for producing outer membrane vesicles in Gram-negative bacteria, Res. Microbiol. 155 (2004) 437–446. doi:10.1016/j.resmic.2004.04.007.

[58] J. Adu-Bobie, P. Lupetti, B. Brunelli, D. Granoff, N. Norais, G. Ferrari, G. Grandi, R. Rappuoli, M. Pizza, GNA33 of Neisseria meningitidis Is A Lipoprotein Required for Cell Separation, Membrane Architecture, and Virulence, Infect. Immun. 72 (2004) 1914–1919. doi:10.1128/IAI.72.4.1914-1919.2004.

[59] G. Ferrari, I. Garaguso, J. Adu-Bobie, F. Doro, A.R. Taddei, A. Biolchi, B. Brunelli, M.M. Giuliani, M. Pizza, N. Norais, G. Grandi, Outer membrane vesicles from group B Neisseria meningitidis Δgna33 mutant: Proteomic and immunological comparison with detergent-derived outer membrane vesicles, Proteomics. 6 (2006) 1856–1866. doi:10.1002/pmic.200500164.

[60] V. Premjani, D. Tilley, S. Gruenheid, H. Le Moual, J.A. Samis, Enterohemorrhagic Escherichia coli OmpT regulates outer membrane vesicle biogenesis, FEMS Microbiol. Lett. 355 (2014) 185–192. doi:10.1111/1574-6968.12463.

[61] W. Elhenawy, M. Bording-Jorgensen, E. Valguarnera, M.F. Haurat, E. Wine, M.F. Feldman, LPS remodeling triggers formation of outer membrane vesicles in salmonella, MBio. 7 (2016). doi:10.1128/mBio.00940-16.

[62] K. Takaki, Y.O. Tahara, N. Nakamichi, Y. Hasegawa, M. Shintani, M. Ohkuma, M. Miyata, H. Futamata, Y. Tashiroa, Multilamellar and Multivesicular Outer Membrane Vesicles Produced by a Buttiauxella agrestis tolB Mutant, Appl. Environ. Microbiol. 86 (2020) 1–15. doi:10.1128/AEM.01131-20.

[63] C. Pérez-Cruz, M.-A. Cañas, R. Giménez, J. Badia, E. Mercade, L. Baldomà, L.

Aguilera, Membrane Vesicles Released by a hypervesiculating Escherichia coli Nissle 1917 tolR Mutant Are Highly Heterogeneous and Show Reduced Capacity for Epithelial Cell Interaction and Entry, PLoS One. 11 (2016) e0169186. doi:10.1371/journal.pone.0169186.

[64] R. Acevedo, S. Fernández, C. Zayas, A. Acosta, M.E. Sarmiento, V.A. Ferro, E. Rosenqvist, C. Campa, D. Cardoso, L. Garcia, J.L. Perez, Bacterial outer membrane vesicles and vaccine applications, Front. Immunol. 5 (2014) 1–6. doi:10.3389/fimmu.2014.00121.

[65] C.J.A. Asensio, M.E. Gaillard, G. Moreno, D. Bottero, E. Zurita, M. Rumbo, P. van der Ley, A. van der Ark, D. Hozbor, Outer membrane vesicles obtained from Bordetella pertussis Tohama expressing the lipid a deacylase PagL as a novel acellular vaccine candidate, Vaccine. 29 (2011) 1649–1656. doi:10.1016/j.vaccine.2010.12.068.

[66] R. Roberts, G. Moreno, D. Bottero, M.E. Gaillard, M. Fingermann, A. Graieb, M. Rumbo, D. Hozbor, Outer membrane vesicles as acellular vaccine against pertussis, Vaccine. 26 (2008) 4639–4646. doi:10.1016/j.vaccine.2008.07.004.

[67] D. Bottero, M.E. Zurita, M.E. Gaillard, E. Bartel, C. Vercellini, D. Hozbor, Membrane vesicles derived from Bordetella bronchiseptica: Active constituent of a new vaccine against infections caused by this pathogen, Appl. Environ. Microbiol. 84 (2018) 1–12. doi:10.1128/AEM.01877-17.

[68] B. van de Waterbeemd, M. Streefland, P. van der Ley, B. Zomer, H. van Dijken, D. Martens, R. Wijffels, L. van der Pol, Improved OMV vaccine against Neisseria meningitidis using genetically engineered strains and a detergent-free purification process, Vaccine. 28 (2010) 4810–4816. doi:10.1016/j.vaccine.2010.04.082.

[69] B. van de Waterbeemd, G. Zomer, P. Kaaijk, N. Ruiterkamp, R.H. Wijffels, G.P.J.M. van den Dobbelsteen, L.A. van der Pol, Improved Production Process for Native Outer Membrane Vesicle Vaccine against Neisseria meningitidis, PLoS One. 8 (2013) 1–11. doi:10.1371/journal.pone.0065157.

[70] J.R. Hart, Ethylenediaminetetraacetic Acid and Related Chelating Agents, in: Ullmann's Encycl. Ind. Chem., Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, 2000. doi:10.1002/14356007.a10_095.

[71] K.J. Thomas, C. V. Rice, Revised model of calcium and magnesium binding to the bacterial cell wall, BioMetals. 27 (2014) 1361–1370. doi:10.1007/s10534-014-9797-5.

[72] L.A. Clifton, M.W.A. Skoda, A.P. Le Brun, F. Ciesielski, I. Kuzmenko, S.A. Holt, J.H. Lakey, Effect of divalent cation removal on the structure of gram-negative bacterial outer membrane models, Langmuir. 31 (2015) 404–412.

doi:10.1021/la504407v.

[73] M. Zasloff, Antimicrobial peptides of multicellular organisms, Nature. 415 (2002) 389–395. doi:10.1038/415389a.

[74] R. van Harten, E. van Woudenbergh, A. van Dijk, H. Haagsman, Cathelicidins: Immunomodulatory Antimicrobials, Vaccines. 6 (2018) 63. doi:10.3390/vaccines6030063.

[75] N. Mookherjee, M.A. Anderson, H.P. Haagsman, D.J. Davidson, Antimicrobial host defence peptides: functions and clinical potential, Nat. Rev. Drug Discov. 19 (2020) 311–332. doi:10.1038/s41573-019-0058-8.

[76] M.R. Scheenstra, R.M. van Harten, E.J.A. Veldhuizen, H.P. Haagsman, M. Coorens, Cathelicidins Modulate TLR-Activation and Inflammation, Front. Immunol. 11 (2020) 1137. doi:10.3389/fimmu.2020.01137.

[77] L. Hazlett, M. Wu, Defensins in innate immunity, Cell Tissue Res. 343 (2011) 175–188. doi:10.1007/s00441-010-1022-4.

[78] M. Zanetti, Cathelicidins, multifunctional peptides of the innate immunity, J. Leukoc. Biol. 75 (2004) 39–48. doi:10.1189/jlb.0403147.

[79] V.A.F. Schneider, M. Coorens, J.L.M. Tjeerdsma-van Bokhoven, G. Posthuma, A. van Dijk, E.J.A. Veldhuizen, H.P. Haagsman, Imaging the Antistaphylococcal Activity of CATH-2: Mechanism of Attack and Regulation of Inflammatory Response, MSphere. 2 (2017) e00370-17. doi:10.1128/mSphere.00370-17.

[80] V.A.F. Schneider, M. Coorens, S.R. Ordonez, J.L.M. Tjeerdsma-Van Bokhoven, G. Posthuma, A. Van Dijk, H.P. Haagsman, E.J.A. Veldhuizen, Imaging the antimicrobial mechanism(s) of cathelicidin-2, Sci. Rep. 6 (2016) 1–11. doi:10.1038/srep32948.

[81] H.M. Kulkarni, R. Nagaraj, M. V Jagannadham, Protective role of E. coli outer membrane vesicles against antibiotics, Microbiol. Res. 181 (2015) 1–7. doi:10.1016/j.micres.2015.07.008.

[82] J.L. Bru, B. Rawson, C. Trinh, K. Whiteson, N.M. Høyland-Kroghsbo, A. Siryaporn, PQS produced by the pseudomonas aeruginosa stress response repels swarms away from bacteriophage and antibiotics, J. Bacteriol. 201 (2019). doi:10.1128/JB.00383-19.

[83] J.W. Schertzer, M. Whiteley, A bilayer-couple model of bacterial outer membrane vesicle biogenesis, MBio. 3 (2012). doi:10.1128/mBio.00297-11.

[84] R. Godlewska, Influence of Environmental and Genetic Factors on Proteomic Profiling of Outer Membrane Vesicles from Campylobacter jejuni, Polish J.

Microbiol. 68 (2019) 255–261. doi:10.33073/pjm-2019-027.

[85] K.W. Chan, C. Shone, J.R. Hesp, Antibiotics and iron-limiting conditions and their effect on the production and composition of outer membrane vesicles secreted from clinical isolates of extraintestinal pathogenic E. coli, Proteomics - Clin. Appl. 11 (2017) 1600091. doi:10.1002/prca.201600091.

[86] S.H. Yun, E.C. Park, S.Y. Lee, H. Lee, C.W. Choi, Y.S. Yi, H.J. Ro, J.C. Lee, S. Jun, H.Y. Kim, G.H. Kim, S. Il Kim, Antibiotic treatment modulates protein components of cytotoxic outer membrane vesicles of multidrug-resistant clinical strain, Acinetobacter baumannii DU202, Clin. Proteomics. 15 (2018) 28. doi:10.1186/s12014-018-9204-2.

[87] D. Kesavan, A. Vasudevan, L. Wu, J. Chen, Z. Su, S. Wang, H. Xu, Integrative analysis of outer membrane vesicles proteomics and whole-cell transcriptome analysis of eravacycline induced Acinetobacter baumannii strains, BMC Microbiol. 20 (2020). doi:10.1186/s12866-020-1722-1.

[88] P. Kohl, F.G. Zingl, T.O. Eichmann, S. Schild, Isolation of Outer Membrane Vesicles Including Their Quantitative and Qualitative Analyses, in: Methods Mol. Biol., Humana Press, New York, NY, 2018: pp. 117–134. doi:10.1007/978-1-4939-8685-9_11.

[89] J. Lee, O.Y. Kim, Y.S. Gho, Proteomic profiling of Gram-negative bacterial outer membrane vesicles: Current perspectives, Proteomics - Clin. Appl. 10 (2016) 897–909. doi:10.1002/prca.201600032.

[90] Y.M.D. Gnopo, H.C. Watkins, T.C. Stevenson, M.P. DeLisa, D. Putnam, Designer outer membrane vesicles as immunomodulatory systems – Reprogramming bacteria for vaccine delivery, Adv. Drug Deliv. Rev. 114 (2017) 132–142. doi:10.1016/j.addr.2017.05.003.

[91] M.J.H. Gerritzen, D.E. Martens, R.H. Wijffels, L. van der Pol, M. Stork, Bioengineering bacterial outer membrane vesicles as vaccine platform, Biotechnol. Adv. 35 (2017) 565–574. doi:10.1016/j.biotechadv.2017.05.003.

[92] P. Van Ulsen, K.M. Zinner, W.S.P. Jong, J. Luirink, On display: Autotransporter secretion and application, FEMS Microbiol. Lett. 365 (2018) 165. doi:10.1093/femsle/fny165.

[93] W.S. Jong, M.H. Daleke-Schermerhorn, D. Vikström, C.M. ten Hagen-Jongman, K. de Punder, N.N. van der Wel, C.E. van de Sandt, G.F. Rimmelzwaan, F. Follmann, E.M. Agger, P. Andersen, J.-W. de Gier, J. Luirink, An autotransporter display platform for the development of multivalent recombinant bacterial vector vaccines, Microb. Cell Fact. 13 (2014) 162. doi:10.1186/s12934-014-0162-8.

[94] M.H. Daleke-Schermerhorn, T. Felix, Z. Soprova, C.M. ten Hagen-Jongman, D.

Vikström, L. Majlessi, J. Beskers, F. Follmann, K. de Punder, N.N. van der Wel, T. Baumgarten, T. V. Pham, S.R. Piersma, C.R. Jiménez, P. van Ulsen, J.W. de Gier, C. Leclerc, W.S.P. Jong, J. Luirinka, Decoration of outer membrane vesicles with multiple antigens by using an autotransporter approach, Appl. Environ. Microbiol. 80 (2014) 5854–5865. doi:10.1128/AEM.01941-14.

[95] G. De Benedetto, R. Alfini, P. Cescutti, M. Caboni, L. Lanzilao, F. Necchi, A. Saul, C.A. MacLennan, S. Rondini, F. Micoli, Characterization of O-antigen delivered by Generalized Modules for Membrane Antigens (GMMA) vaccine candidates against nontyphoidal Salmonella, Vaccine. 35 (2017) 419–426. doi:10.1016/j.vaccine.2016.11.089.

[96] K. Tan, R. Li, X. Huang, Q. Liu, Outer membrane vesicles: Current status and future direction of these novel vaccine adjuvants, Front. Microbiol. 9 (2018) 783. doi:10.3389/fmicb.2018.00783.

[97] V. Gujrati, S. Kim, S.H. Kim, J.J. Min, H.E. Choy, S.C. Kim, S. Jon, Bioengineered bacterial outer membrane vesicles as cell-specific drug-delivery vehicles for cancer therapy, ACS Nano. 8 (2014) 1525–1537. doi:10.1021/nn405724x.

[98] S. Wang, J. Gao, Z. Wang, Outer membrane vesicles for vaccination and targeted drug delivery, WIREs Nanomedicine and Nanobiotechnology. 11 (2019) e1523. doi:10.1002/wnan.1523.

[99] M. Kaparakis-Liaskos, R.L. Ferrero, Immune modulation by bacterial outer membrane vesicles, Nat. Rev. Immunol. 15 (2015) 375–387. doi:10.1038/nri3837.

[100] W. Cai, D.K. Kesavan, J. Wan, M.H. Abdelaziz, Z. Su, H. Xu, Bacterial outer membrane vesicles, a potential vaccine candidate in interactions with host cells based, Diagn. Pathol. 13 (2018) 1–12. doi:10.1186/s13000-018-0768-y.

[101] E.D. Avila-Calderón, A. Lopez-Merino, N. Jain, H. Peralta, E.O. López-Villegas, N. Sriranganathan, S.M. Boyle, S. Witonsky, A. Contreras-Rodríguez, Characterization of Outer Membrane Vesicles from Brucella melitensis and Protection Induced in Mice, Clin. Dev. Immunol. 2012 (2012) 1–13. doi:10.1155/2012/352493.

[102] J. Lee, Y.J. Yoon, J.H. Kim, N.T.H. Dinh, G. Go, S. Tae, K.-S. Park, H.T. Park, C. Lee, T.-Y. Roh, D. Di Vizio, Y.S. Gho, Outer Membrane Vesicles Derived From Escherichia coli Regulate Neutrophil Migration by Induction of Endothelial IL-8, Front. Microbiol. 9 (2018). doi:10.3389/fmicb.2018.02268.

[103] E. Bartolini, E. Ianni, E. Frigimelica, R. Petracca, G. Galli, F.B. Scorza, N. Norais, D. Laera, F. Giusti, A. Pierleoni, M. Donati, R. Cevenini, O. Finco, G. Grandi, R. Grifantini, Recombinant outer membrane vesicles carrying Chlamydia muridarum HtrA induce antibodies that neutralize chlamydial infection in vitro, J. Extracell.

Vesicles. 2 (2013) 20181. doi:10.3402/jev.v2i0.20181.

[104] L. Fantappiè, M. de Santis, E. Chiarot, F. Carboni, G. Bensi, O. Jousson, I. Margarit, G. Grandi, Antibody-mediated immunity induced by engineered *Escherichia coli* OMVs carrying heterologous antigens in their lumen, J. Extracell. Vesicles. 3 (2014) 24015. doi:10.3402/jev.v3.24015.

[105] S. Schild, E.J. Nelson, A. Camilli, Immunization with Vibrio cholerae outer membrane vesicles induces protective immunity in mice, Infect. Immun. 76 (2008) 4554–4563. doi:10.1128/IAI.00532-08.

[106] O.Y. Kim, B.S. Hong, K.-S. Park, Y.J. Yoon, S.J. Choi, W.H. Lee, T.-Y. Roh, J. Lötvall, Y.-K. Kim, Y.S. Gho, Immunization with Escherichia coli Outer Membrane Vesicles Protects Bacteria - Induced Lethality via Th1 and Th17 Cell Responses , J. Immunol. 190 (2013) 4092–4102. doi:10.4049/jimmunol.1200742.

[107] A.I. Camacho, J. De Souza, S. Sánchez-Gómez, M. Pardo-Ros, J.M. Irache, C. Gamazo, Mucosal immunization with Shigella flexneri outer membrane vesicles induced protection in mice, Vaccine. 29 (2011) 8222–8229. doi:10.1016/j.vaccine.2011.08.121.

[108] H. Petersen, W. Nieves, K. Russell-Lodrigue, C.J. Roy, L.A. Morici, Evaluation of a Burkholderia Pseudomallei Outer Membrane Vesicle Vaccine in Nonhuman Primates, Procedia Vaccinol. 8 (2014) 38–42. doi:10.1016/j.provac.2014.07.007.

[109] Q. Li, J. Ren, H. Xian, C. Yin, Y. Yuan, Y. Li, R. Ji, C. Chu, Z. Qiao, X. Jiao, rOmpF and OMVs as efficient subunit vaccines against Salmonella enterica serovar Enteritidis infections in poultry farms, Vaccine. 38 (2020) 7094–7099. doi:10.1016/j.vaccine.2020.08.074.

[110] S. Li, D.-Q. Chen, L. Ji, S. Sun, Z. Jin, Z.-L. Jin, H.-W. Sun, H. Zeng, W.-J. Zhang, D.-S. Lu, P. Luo, A.-N. Zhao, J. Luo, Q.-M. Zou, H.-B. Li, Development of Different Methods for Preparing Acinetobacter baumannii Outer Membrane Vesicles Vaccine: Impact of Preparation Method on Protective Efficacy, Front. Immunol. 11 (2020) 1069. doi:10.3389/fimmu.2020.01069.

[111] R. Acevedo, S. Fernández, C. Zayas, A. Acosta, M.E. Sarmiento, V.A. Ferro, E. Rosenqvist, C. Campa, D. Cardoso, L. Garcia, J.L. Perez, Bacterial outer membrane vesicles and vaccine applications, Front. Immunol. 5 (2014) 1–6. doi:10.3389/fimmu.2014.00121.

[112] P. Oster, D. Lennon, J. O'Hallahan, K. Mulholland, S. Reid, D. Martin, MeNZB™: A safe and highly immunogenic tailor-made vaccine against the New Zealand Neisseria meningitidis serogroup B disease epidemic strain, Vaccine. 23 (2005) 2191–2196. doi:10.1016/j.vaccine.2005.01.063.

[113] FDA, FDA approves a second vaccine to prevent serogroup B meningococcal

disease, U.S. Food Drug Adm. (2015).

[114] J. Holst, P. Oster, R. Arnold, M. Tatley, L. Næss, I. Aaberge, Y. Galloway, A. McNicholas, J. O'Hallahan, E. Rosenqvist, S. Black, Vaccines against meningococcal serogroup B disease containing outer membrane vesicles (OMV): Lessons from past programs and implications for the future, Hum. Vaccin. Immunother. 9 (2013) 1241–1253. doi:10.4161/hv.24129.

[115] Centers for Disease Control and Prevention, Meningococcal | Surveillance | CDC, (2017). https://www.cdc.gov/meningococcal/surveillance/index.html (accessed January 5, 2021).

[116] European Centre for Disease Prevention and Control, Invasive meningococcal disease annual epidemiological report for 2017, Annu. Epidemiol. Rep. Commun. Dis. Eur. (2019). http://ecdc.europa.eu/sites/portal/files/documents/AER_for_2017-invasive-meningococcal-disease.pdf (accessed January 5, 2021).

[117] J. Finne, M. Leinonen, P.H. Mäkelä, Antigenic similarities between brain components and bacteria causing meningitis. Implications for Vaccine Development and Pathogenesis, Lancet. 2 (1983) 355–357. doi:10.1016/S0140-6736(83)90340-9.

[118] D.M. Granoff, Review of Meningococcal Group B Vaccines, Clin. Infect. Dis. 50 (2010) S54–S65. doi:10.1086/648966.

[119] C.T. Sacchi, A.M. Whitney, T. Popovic, D.S. Beall, M.W. Reeves, B.D. Plikaytis, N.E. Rosenstein, B.A. Perkins, M.L. Tondella, L.W. Mayer, Diversity and prevalence of PorA types in Neisseria meningitidis serogroup B in the United States, 1992-1998., J. Infect. Dis. 182 (2000) 1169–1176. doi:10.1086/315833.

[120] J. Holst, D. Martin, R. Arnold, C.C. Huergo, P. Oster, J. O'Hallahan, E. Rosenqvist, Properties and clinical performance of vaccines containing outer membrane vesicles from Neisseria meningitidis, Vaccine. 27 (2009) B3–B12. doi:10.1016/j.vaccine.2009.04.071.

[121] J. Sevestre, E. Hong, V. Delbos, A. Terrade, E. Mallet, A.E. Deghmane, L. Lemée, M.K. Taha, F. Caron, Durability of immunogenicity and strain coverage of MenBvac, a meningococcal vaccine based on outer membrane vesicles: Lessons of the Normandy campaign, Vaccine. 35 (2017) 4029–4033. doi:10.1016/j.vaccine.2017.05.065.

[122] G. Trzewikoswki de Lima, A.I. Portilho, E. De Gaspari, Cross-reactivity with Brazilian strains of Neisseria meningitidis B after immunization with outer membrane vesicles, Ther. Adv. Vaccines Immunother. 7 (2019) 251513551989482. doi:10.1177/2515135519894825.

[123] B. van de Waterbeemd, M. Streefland, L. van Keulen, J. van den IJssel, A. de Haan, M.H. Eppink, L.A. van der Pol, Identification and optimization of critical process parameters for the production of NOMV vaccine against Neisseria meningitidis, Vaccine. 30 (2012) 3683–3690. doi:10.1016/j.vaccine.2012.03.028.

[124] P. Van Der Ley, L. Steeghs, H.J. Hamstra, J. Ten Hove, B. Zomer, L. Van Alphen, Modification of lipid a biosynthesis in Neisseria meningitidis lpxL mutants: Influence on lipopolysaccharide structure, toxicity, and adjuvant activity, Infect. Immun. 69 (2001) 5981–5990. doi:10.1128/IAI.69.10.5981-5990.2001.

[125] F. Fransen, H.J. Hamstra, C.J. Boog, J.P. Van Putten, G.P.J.M. Van Den Dobbelsteen, P. Van Der Ley, The structure of Neisseria meningitidis lipid A determines outcome in experimental meningococcal disease, Infect. Immun. 78 (2010) 3177–3186. doi:10.1128/IAI.01311-09.

[126] P.B. Keiser, S. Biggs-Cicatelli, E.E. Moran, D.H. Schmiel, V.B. Pinto, R.E. Burden, L.B. Miller, J.E. Moon, R.A. Bowden, J.F. Cummings, W.D. Zollinger, A phase 1 study of a meningococcal native outer membrane vesicle vaccine made from a group B strain with deleted lpxL1 and synX, over-expressed factor H binding protein, two PorAs and stabilized OpcA expression, Vaccine. 29 (2011) 1413–1420. doi:10.1016/j.vaccine.2010.12.039.

[127] M.J.H. Gerritzen, L. Stangowez, B. Van De Waterbeemd, D.E. Martens, R.H. Wijffels, M. Stork, Continuous production of Neisseria meningitidis outer membrane vesicles, Appl. Microbiol. Biotechnol. 103 (2019) 9401–9410. doi:10.1007/s00253-019-10163-z.

[128] J.D. Cherry, Pertussis in young infants throughout the world, Clin. Infect. Dis. 63 (2016) S119–S122. doi:10.1093/cid/ciw550.

[129] P.E. Kilgore, A.M. Salim, M.J. Zervos, H.J. Schmitt, Pertussis: Microbiology, disease, treatment, and prevention, Clin. Microbiol. Rev. 29 (2016) 449–486. doi:10.1128/CMR.00083-15.

[130] J.A. Melvin, E. V. Scheller, J.F. Miller, P.A. Cotter, Bordetella pertussis pathogenesis: Current and future challenges, Nat. Rev. Microbiol. 12 (2014) 274–288. doi:10.1038/nrmicro3235.

[131] B.L. Ligon, Pertussis: An historical review of the research and of the development of whole-cell and acellular vaccines, Semin. Pediatr. Infect. Dis. 9 (1998) 168–178. doi:10.1016/S1045-1870(98)80068-X.

[132] R.H.M. Raeven, J. Brummelman, J.L.A. Pennings, L. Van Der Maas, W. Tilstra, K. Helm, E. Van Riet, W. Jiskoot, C.A.C.M. Van Els, W.G.H. Han, G.F.A. Kersten, B. Metz, Bordetella pertussis outer membrane vesicle vaccine confers equal efficacy in mice with milder inflammatory responses compared to a whole-cell vaccine,

Sci. Rep. 6 (2016) 1–15. doi:10.1038/srep38240.

[133] D. Jayasundara, E. Lee, S. Octavia, R. Lan, M.M. Tanaka, J.G. Wood, Emergence of pertactin-deficient pertussis strains in Australia can be explained by models of vaccine escape, Epidemics. 31 (2020) 100388. doi:10.1016/j.epidem.2020.100388.

[134] A.M. Barkoff, J. Mertsola, D. Pierard, T. Dalby, S.V. Hoegh, S. Guillot, P. Stefanelli, M. van Gent, G. Berbers, D. Vestrheim, M. Greve-Isdahl, L. Wehlin, M. Ljungman, N.K. Fry, K. Markey, Q. He, Pertactin-deficient Bordetella pertussis isolates: Evidence of increased circulation in Europe, 1998 to 2015, Eurosurveillance. 24 (2019). doi:10.2807/1560-7917.ES.2019.24.7.1700832.

[135] N. Burdin, L.K. Handy, S.A. Plotkin, What Is Wrong with Pertussis Vaccine Immunity? The Problem of Waning Effectiveness of Pertussis Vaccines., Cold Spring Harb. Perspect. Biol. 9 (2017) 1–18. doi:10.1101/cshperspect.a029454.

[136] M. V. Pinto, T.J. Merkel, Pertussis disease and transmission and host responses: insights from the baboon model of pertussis, J. Infect. 74 (2017) S114–S119. doi:10.1016/S0163-4453(17)30201-3.

[137] WHO, WHO World Health Organization: Immunization, Vaccines And Biologicals. Vaccine preventable diseases Vaccines monitoring system 2016 Global Summary Reference Time Series: BCG, World Heal. Organ. (2016). http://apps.who.int/immunization_monitoring/globalsummary/timeseries/tsincidencepertussis.html (accessed March 20, 2020).

[138] CDC, Pertussis | Whooping Cough | Surveillance | Cases by Year | CDC, Pertussis (Whooping Cough). (2017). https://www.cdc.gov/pertussis/surv-reporting/cases-by-year.html (accessed March 20, 2020).

[139] M. Rumbo, D. Hozbor, Development of improved pertussis vaccine., Hum. Vaccin. Immunother. 10 (2014) 2450–3. doi:10.4161/hv.29253.

[140] M. Brennan, A New Whooping Cough Vaccine That May Prevent Colonization and Transmission, Vaccines. 5 (2017) 1–2. doi:10.3390/vaccines5040043.

[141] A.-S. Debrie, N. Mielcarek, S. Lecher, X. Roux, J.-C. Sirard, C. Locht, Early Protection against Pertussis Induced by Live Attenuated Bordetella pertussis BPZE1 Depends on TLR4 , J. Immunol. 203 (2019) 3293–3300. doi:10.4049/jimmunol.1901102.

[142] R.H. Raeven, J. Brummelman, J. LA Pennings, L. van der Maas, K. Helm, W. Tilstra, A. van der Ark, A. Sloots, P. van der Ley, W. van Eden, W. Jiskoot, E. van Riet, C.A. van Els, G.F. Kersten, W.G. Han, B. Metz, Molecular and cellular signatures underlying superior immunity against Bordetella pertussis upon pulmonary vaccination, Mucosal Immunol. 11 (2018) 979–993. doi:10.1038/mi.2017.81.

[143] D.F. Hozbor, Outer membrane vesicles: an attractive candidate for pertussis vaccines, Expert Rev. Vaccines. 16 (2017) 193–196. doi:10.1080/14760584.2017.1276832.

[144] D. Hozbor, New Pertussis Vaccines: A Need and a Challenge, in: Adv. Exp. Med. Biol., Springer, 2019: pp. 115–126. doi:10.1007/5584_2019_407.

[145] J. Geurtsen, L. Steeghs, H.J. Hamstra, J. Ten Hove, A. De Haan, B. Kuipers, J. Tommassen, P. Van Der Ley, Expression of the lipopolysaccharide-modifying enzymes PagP and PagL modulates the endotoxic activity of bordetella pertussis, Infect. Immun. 74 (2006) 5574–5585. doi:10.1128/IAI.00834-06.

[146] M.L. Elizagaray, M.T.R. Gomes, E.S. Guimaraes, M. Rumbo, D.F. Hozbor, S.C. Oliveira, G. Moreno, Canonical and Non-canonical Inflammasome Activation by Outer Membrane Vesicles Derived From Bordetella pertussis, Front. Immunol. 11 (2020). doi:10.3389/fimmu.2020.01879.

[147] M. Ormazábal, E. Bartel, M.E. Gaillard, D. Bottero, A. Errea, M.E. Zurita, G. Moreno, M. Rumbo, C. Castuma, D. Flores, M.J. Martín, D. Hozbor, Characterization of the key antigenic components of pertussis vaccine based on outer membrane vesicles, Vaccine. 32 (2014) 6084–6090. doi:10.1016/j.vaccine.2014.08.084.

[148] M.E. Gaillard, D. Bottero, A. Errea, M. Ormazábal, M.E. Zurita, G. Moreno, M. Rumbo, C. Castuma, E. Bartel, D. Flores, P. van der Ley, A. van der Ark, D. F. Hozbor, Acellular pertussis vaccine based on outer membrane vesicles capable of conferring both long-lasting immunity and protection against different strain genotypes, Vaccine. 32 (2014) 931–937. doi:10.1016/j.vaccine.2013.12.048.

[149] R.H.M. Raeven, L. Van Der Maas, W. Tilstra, J.P. Uittenbogaard, T.H.E. Bindels, B. Kuipers, A. Van Der Ark, J.L.A. Pennings, E. Van Riet, W. Jiskoot, G.F.A. Kersten, B. Metz, Immunoproteomic Profiling of Bordetella pertussis Outer Membrane Vesicle Vaccine Reveals Broad and Balanced Humoral Immunogenicity, J. Proteome Res. 14 (2015) 2929–2942. doi:10.1021/acs.jproteome.5b00258.

[150] B. van de Waterbeemd, G. Zomer, J. van den IJssel, L. van Keulen, M.H. Eppink, P. van der Ley, L.A. van der Pol, Cysteine Depletion Causes Oxidative Stress and Triggers Outer Membrane Vesicle Release by Neisseria meningitidis; Implications for Vaccine Development, PLoS One. 8 (2013). doi:10.1371/journal.pone.0054314.

[151] M. von Bergen, F. Steiniger, T. Baumgarten, L.Y. Wick, H.J. Heipieper, J. Seifert, S. Sperling, Membrane Vesicle Formation as a Multiple-Stress Response Mechanism Enhances Pseudomonas putida DOT-T1E Cell Surface Hydrophobicity and Biofilm Formation, Appl. Environ. Microbiol. 78 (2012) 6217–6224. doi:10.1128/aem.01525-12.

[152] C. Eberlein, T. Baumgarten, S. Starke, H.J. Heipieper, Immediate response mechanisms of Gram-negative solvent-tolerant bacteria to cope with environmental stress: cis-trans isomerization of unsaturated fatty acids and outer membrane vesicle secretion, Appl. Microbiol. Biotechnol. 102 (2018) 2583–2593. doi:10.1007/s00253-018-8832-9.

[153] E.F. de Jonge, M.D. Balhuizen, R. van Boxtel, J. Wu, H.P. Haagsman, J. Tommassen, Heat shock enhances outer-membrane vesicle release in Bordetella spp., Curr. Res. Microb. Sci. 2 (2021) 100009. doi:10.1016/j.crmicr.2020.100009.

[154] A.L. Hilchie, K. Wuerth, R.E.W. Hancock, Immune modulation by multifaceted cationic host defense (antimicrobial) peptides, Nat. Chem. Biol. 9 (2013) 761–768. doi:10.1038/nchembio.1393.

[155] R.E.W. Hancock, E.F. Haney, E.E. Gill, The immunology of host defence peptides: Beyond antimicrobial activity, Nat. Rev. Immunol. 16 (2016) 321–334. doi:10.1038/nri.2016.29.

[156] D.J. Davidson, A.J. Currie, G.S.D. Reid, D.M.E. Bowdish, K.L. MacDonald, R.C. Ma, R.E.W. Hancock, D.P. Speert, The Cationic Antimicrobial Peptide LL-37 Modulates Dendritic Cell Differentiation and Dendritic Cell-Induced T Cell Polarization, J. Immunol. 172 (2004) 1146–1156. doi:10.4049/jimmunol.172.2.1146.

[157] A. Van Dijk, M. Van Eldik, E.J.A. Veldhuizen, H.L.M. Tjeerdsma-Van Bokhoven, M.R. De Zoete, F.J. Bikker, H.P. Haagsman, Immunomodulatory and anti-inflammatory activities of chicken cathelicidin-2 derived peptides, PLoS One. 11 (2016) 1–16. doi:10.1371/journal.pone.0147919.

[158] K. Kandler, R. Shaykhiev, P. Kleemann, F. Klescz, M. Lohoff, C. Vogelmeier, R. Bals, The anti-microbial peptide LL-37 inhibits the activation of dendritic cells by TLR ligands, Int. Immunol. 18 (2006) 1729–1736. doi:10.1093/intimm/dxl107.

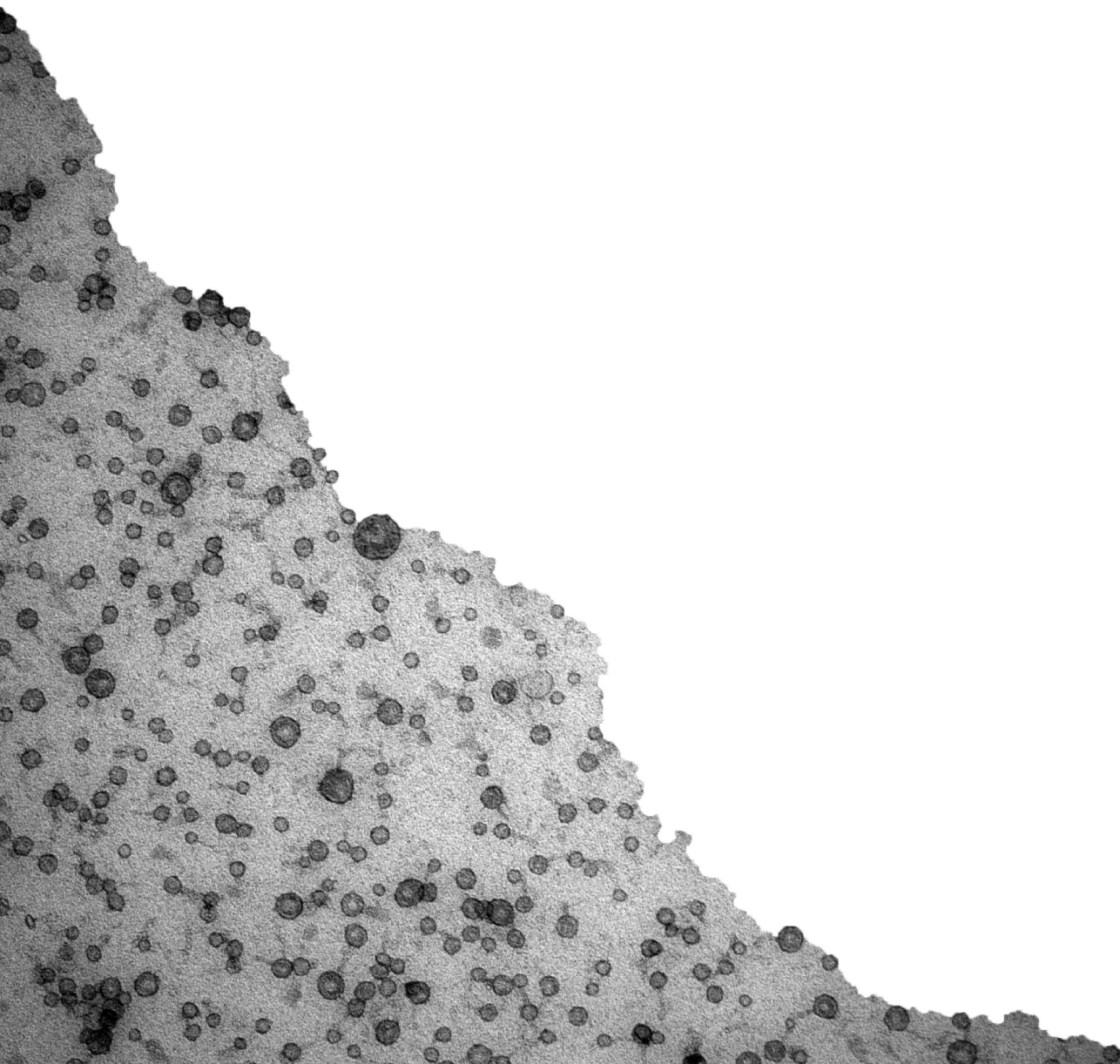
[159] M. Coorens, V.A.F. Schneider, A.M. de Groot, A. van Dijk, M. Meijerink, J.M. Wells, M.R. Scheenstra, E.J.A. Veldhuizen, H.P. Haagsman, Cathelicidins Inhibit Escherichia coli –Induced TLR2 and TLR4 Activation in a Viability-Dependent Manner, J. Immunol. 199 (2017) 1418–1428. doi:10.4049/jimmunol.1602164.

[160] A. Pfalzgraff, W. Correa, L. Heinbockel, A.B. Schromm, C. Lübow, N. Gisch, G. Martinez-de-Tejada, K. Brandenburg, G. Weindl, LPS-neutralizing peptides reduce outer membrane vesicle-induced inflammatory responses, Biochim. Biophys. Acta - Mol. Cell Biol. Lipids. 1864 (2019) 1503–1513. doi:10.1016/j.bbalip.2019.05.018.

[161] K.C. Wuerth, R. Falsafi, R.E.W. Hancock, Synthetic host defense peptide IDR-1002 reduces inflammation in Pseudomonas aeruginosa lung infection, PLoS One. 12 (2017) 1–18. doi:10.1371/journal.pone.0187565.

[162] E.F. Haney, B. Wu, K. Lee, A.L. Hilchie, R.E.W. Hancock, Aggregation and Its Influence on the Immunomodulatory Activity of Synthetic Innate Defense Regulator Peptides, Cell Chem. Biol. 24 (2017) 969-980.e4. doi:10.1016/j.chembiol.2017.07.010.

[163] H. Etayash, D. Pletzer, P. Kumar, S.K. Straus, R.E.W. Hancock, Cyclic Derivative of Host-Defense Peptide IDR-1018 Improves Proteolytic Stability, Suppresses Inflammation, and Enhances In Vivo Activity, J. Med. Chem. 63 (2020) 9228–9236. doi:10.1021/acs.jmedchem.0c00303.



Chapter 2

General introduction on Host Defense Peptides

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Abstract

Host defense peptides (HDPs) are part of the first line of defense and have a dual functionality. HDPs possess both immunomodulatory capabilities and direct antimicrobial activity. Immune modulation by HDPs acts on the mammalian host cell and can be either pro- or anti-inflammatory. Furthermore, HDPs were shown to act on many different immune cell types. The antimicrobial activity of HDPs most often comprises membrane active mechanisms and three models have been described: barrel stave, toroidal pore and carpet model. Several HDPs have shown to interact with lipopolysaccharide (LPS), an abundant molecule in the outer membrane of Gram-negative bacteria, but it is unclear yet whether LPS-binding aids or actually inhibits the membrane active antimicrobial mechanisms of HDPs. Despite HDPs targeting essential and often multiple processes in bacteria, some bacterial evasion or adaptation mechanisms have evolved, such as efflux pumps and bacterial membrane adaptation. This chapter summarizes the current knowledge on these topics and introduces the HDPs used throughout this thesis.

Host defense peptides

The first line of defense is formed by the innate immune system, which comprises of cellular components, including macrophages and NK-cells, and molecular components, including complement, collectins and host defense peptides (HDPs) [1]. These peptides are small, usually less than 50 amino acids, cationic and amphipathic in nature. They are found among all organisms, even in bacteria and fungi to combat competing species [2]. In multicellular organisms, HDPs are expressed by several types of cells, such as epithelial cells and neutrophils [3]. A large variety in sequence and structure exists, as well as number of HDPs per species [4]. Amphibians have the largest repertoire of HDPs, with over 500 described thus far, found mainly in their skin secretions [2].

Classes

HDPs can be divided into several classes, with defensins and cathelicidins being the major classes. Humans possess over 30 defensins but only one cathelicidin while pigs have 13 defensins and 11 cathelicidins and chicken possess over 25 defensins and 4 cathelicidins [5–7].

Defensins are characterized by three internal disulfide bonds between cysteines, and mostly comprise of β-sheet structures. Based on the specific disulfide linkages, they can be divided into α- and β-defensins, of which the β-defensins are most wide spread among organisms. A third class, θ-defensins, also contain internal disulfide bonds but are cyclized peptides, but this class was only found in a limited number of primate species [8]. Defensins are expressed as prepropeptides, with the pre-sequence being a signal peptide that is cleaved in the Golgi apparatus. The pro-sequence can vary and so can the cleavage site. Mature peptides are processed in different manners. In human, mature α-defensins can be stored in granules before secretion while mature β-defensins are directly secreted [9,10].

Most HDPs used in this thesis fall in the cathelicidin class, characterized by a conserved cathelin precursor domain. Cathelicidins are, just like defensins, expressed as prepropeptides, with the pre-sequence being a signal peptide. However, the pro-domain, also called the cathelin domain, is highly conserved among cathelicidins [11]. Cathelicidins are stored in granules as intact propeptides and only get cleaved upon secretion. Mature peptides vary widely in length and structure, with β-hairpins, linear peptides or α-helical structures [12].

LL-37

The only human cathelicidin is LL-37. The gene for LL-37, *CAMP*, was discovered in 1995 by three independent research groups, and it is currently the most studied HDP. The mature

peptide has been found to vary in length, with FALL-39 being a minor variant of 39 amino acids [13]. The major variant, LL-37, is 37 amino acids in length and has a net positive charge of 6 **(Table 1)**. The peptide is expressed, stored and secreted by many different cell types, such as epithelial cells, where it is constitutively secreted, and immune cells, such as neutrophils, natural killer cells and lymphocytes, where secretion is induced by microbial compounds or endogenous signals [14]. Vitamin D3 has been shown to be a potent inducer of LL-37 in several cell types, since the *CAMP* gene contains vitamin D response elements [14]. The propeptide is not processed internally, but externally upon release from granules [15]. Mature LL-37 was shown to adopt an extended structure in aqueous solutions, but readily adopts an α-helical structure when interacting with model membranes. With circular dichroism (CD) spectroscopy it was shown that 70-80% of the structure is α-helical, suggesting that the more hydrophobic N-terminus adopts an extended conformation. Furthermore, LL-37 was found to form dimer, trimers and even higher order oligomers in aqueous solution [16,17]. LL-37 is the only HDP discussed here of which an NMR spectrum has been assigned, in lipopolysaccharide (LPS) micelles, shown in **Figure 1a** [18].

CATH-2

In chicken, 4 cathelicidins are present; CATH-1, -2, -3 and CATH-B1, where CATH-2 is most studied in our group. The mature peptide is a result of cleavage by elastase [19] and is 26 amino acids long with a net positive charge of 8 **(Table 1)**. Similar to LL-37, immune organs were found to have high expression of CATH-2 [19]. However, expression of CATH-2 was not found in monocytes or lymphocytes, but was exclusively found in heterophils, the avian counterpart of mammalian neutrophils [20]. High expression of CATH-2 was also found in epithelial tissue, such as the lungs, probably due to presence of heterophils [6]. NMR studies on fowlicidin-2, containing five additional amino acids at the N-terminus due to a different predicted cleavage site, has shown an α-helical fold like LL-37 but with a proline-induced kink parting the helix into two [21]. A predicted structure for CATH-2 showed a similar structure **(Fig. 1b)**. The proline-induced kink has been shown to be important for penetration of bacterial membranes, as well as immunomodulatory activities [22].

PMAP-23, PMAP-36, PR-39

Of the eleven porcine cathelicidins, only three will be discussed here, PMAP-23, PMAP-36 and PR-39 **(Table 1)**. Porcine cathelicidins are among the first mammalian cathelicidins discovered. PMAP-23 and PMAP-36 are highly expressed in bone marrow [23]. PR-39 on the other hand was preferentially found in granules of intestinal leukocytes [23]. PMAP-23 was found not to interact with *Salmonella* Minnesota LPS [24], but did show conformational changes upon addition of dodecyl phosphocholine (DPC) or sodium dodecyl sulphate (SDS) micelles. Further NMR studies of PMAP-23 in DPC micelles showed

a double helix conformation, with a flexible region in between [25]. The predicted structure of PMAP-23 in **Figure 1c** shows a similar structure, but lacking the second helix, which suggests this might be an interaction induced helix. Structural studies on PMAP-36, using CD spectroscopy, showed an extended conformation in phosphate buffer, but an α-helical structure in trifluoroethanol (TFE) solution. Furthermore, unlike any other cathelicidin discussed here, it has the ability to dimerize through the C-terminal cysteine [26]. The C-terminus of PMAP-36 was indeed shown to be flexible in structure predictions, confirming the availability of the cysteine for dimerization **(Fig. 1d)**. Since PR-39 is a proline-rich peptide, it does not adopt the α-helical conformation found in many other cathelicidins, but rather a polyproline type helix, which could not be structured in the prediction **(Fig. 1e)** [27]. The high proline content of PR-39 has been implicated in resistance to proteolytic cleavage [28].

K9CATH

Similar to humans, dogs only possess a single cathelicidin, K9CATH **(Table 1)**. This cathelicidin is highly expressed in bone marrow with lower levels found in the skin, gastro-intestinal tract, liver, spleen and testes, probably due to the presence of immune cells in these tissues [29]. Similar as cathelicidins discussed above, K9CATH was found to be present in neutrophil granules. In one study K9CATH structure was investigated using CD spectroscopy and showed an extended conformation in aqueous solution, but an α-helical structure in TFE or SDS solution [30]. The predicted structure confirmed this and showed a straight α-helix for K9CATH **(Fig. 1f)**.

Table 1: Selection of peptides used in this thesis and their sequence, organism of origin, number of amino acids (No. aa) and charge.

Peptide	*Sequence*	*Origin*	*No. aa*	*Charge*
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLR NLVPRTES	Human	37	6+
CATH-2	RFGRFLRKIRRFRPKVTITIQGSARF-NH_2	Chicken	26	8+
PMAP-23	RIIDLLWRVRRPQKPKFVTVWVR	Porcine	23	6+
PMAP-36	Ac-GRFRRLRKKTRKRLKKIGKVLKWIPPIVG SIPLGCG	Porcine	36	13+
PR-39	RRRPRPPYLPRPRPPPFFPPRLPPRIPPG FPPRFPPRFP	Porcine	39	10+
K9CATH	RLKELITTGGQKIGEKIRRIGQRIKDFFKN LQPREEKS	Canine	38	5+
IDR-1018	VRLIVAVRIWRR-NH_2	Synthetic	12	5+
IDR-2005	VRLIVRVRIWRR-NH2	Synthetic	12	6+

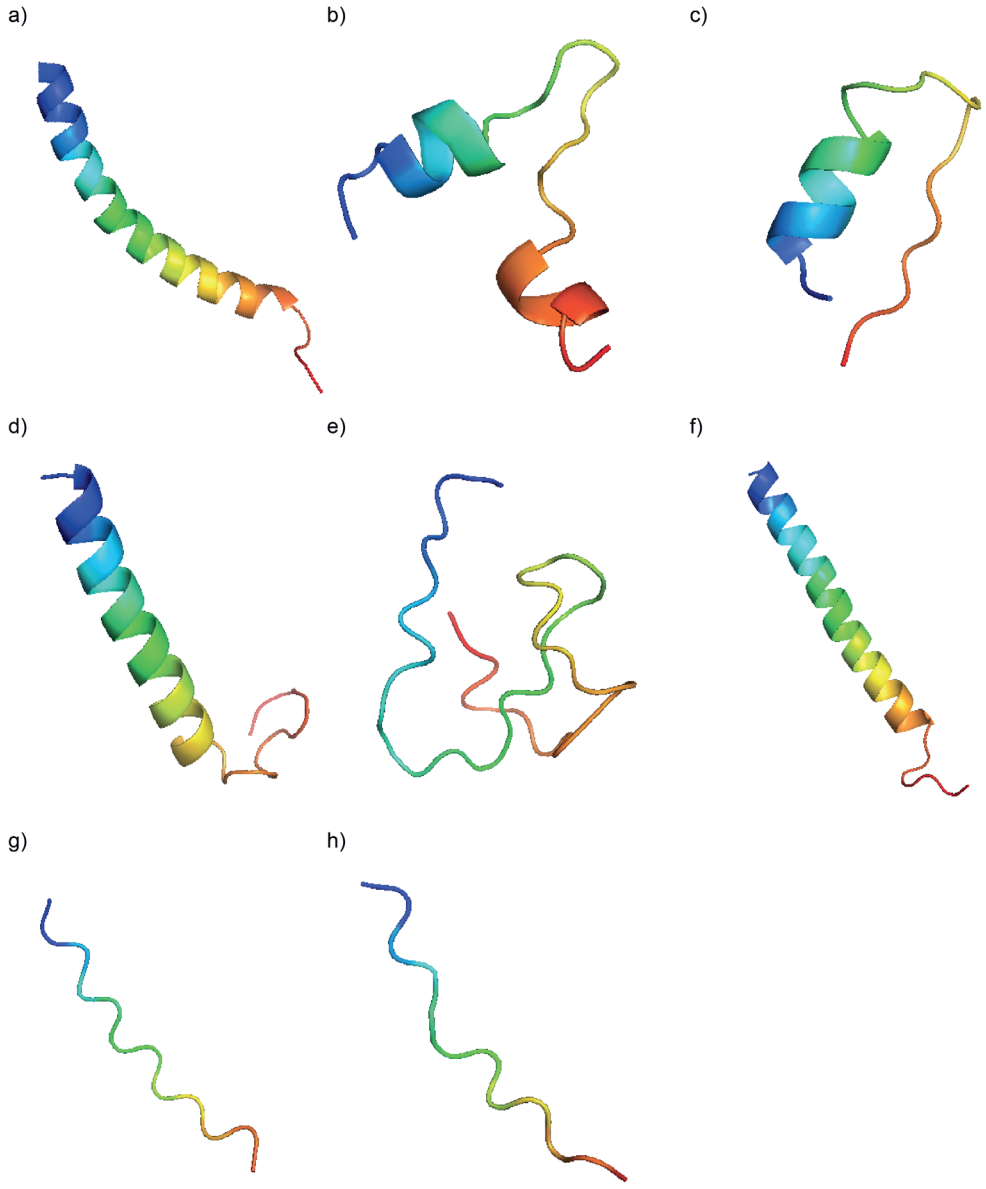


Figure 1: NMR and predicted structures of cathelicidins used in this thesis. *(a) NMR structure of LL-37, determined in LPS micelles. (b-h) Predicted structures of (b) CATH-2, (c) PMAP-23, (d) PMAP-36, (e) PR-39, (f) K9CATH, (g) IDR-1018 and (h) IDR-2005 using the I-TASSER software* [31]*. N-termini are colored in blue, C-termini in red.*

IDR-1018, IDR-2005

Finally, two innate defense regulator (IDR) peptides were used in this thesis which are derived from the bovine cathelicidin bactenecin. The first peptide is IDR-1018, originally designed to exhibit increased immunomodulatory activities [32]. Structural studies revealed IDR-1018 to adopt an extended structure in phosphate buffer, but an α-helical structure in SDS solution and DPC micelles [33]. The second peptide, IDR-2005, was derived from IDR-1018 by one point mutation, which selectively decreased aggregation propensity of the peptide, but retained immunomodulatory properties [34]. Predicted structures for both IDRs show extended structures, that might have an α-helix induced structure upon interaction with bacterial membranes **(Fig. 1g-h)**.

Functions

HDPs were originally discovered for their antimicrobial function, which they exert at the pathogenic surface by lysis or intracellularly by halting essential processes. Hence, these peptides were originally termed antimicrobial peptides (AMPs). However, most HDPs show loss of antimicrobial function in physiological buffers. HDPs also are immune modulating molecules and it is thought that the immunomodulatory properties of most vertebrate HDPs may be more important than their antimicrobial properties. Immunomodulatory functions and membrane-active properties will be discussed below, with examples from several cathelicidins.

Immunomodulatory mechanisms

HDP concentrations *in vivo* can be difficult to detect. Local concentrations can be very high, sufficient to establish antimicrobial functions. However, at lower concentrations HDPs can exhibit immunomodulatory functions. HDPs have shown to modulate differentiation of immune cells like monocytes and macrophages, expression of cytokines and chemokines and function as chemokine themselves [3,35–38]. However, both pro-inflammatory and anti-inflammatory functions have been described for HDPs **(Fig. 2)**, suggesting that they are modulators that can also dampen the immune response and thereby maintain homeostasis.

Pro-inflammatory activity

Several HDPs have been shown to independently induce pro-inflammatory cytokine and chemokine expression by immune cells. The bovine HDP BMAP-28 induced production of TNFα and IL-1β mRNA in a murine macrophage cell line [39]. Similarly, the porcine peptides PR-39 and PMAP-23 were shown to induce release of IL-8 in a porcine macrophage cell line [24,40]. Furthermore, HDPs have been shown to enhance cytokine-

induced immune responses, to aid in faster clearance of pathogens. LL-37 has been shown to synergistically enhance IL-1β induced cytokine and chemokine expression in human peripheral blood mononuclear cells [41].

Not only do HDPs indirectly increase influx of immune cells by enhancement of chemokine release, they also have direct chemotactic effects. LL-37 was shown to attract both neutrophils and eosinophils [42]. For murine CRAMP it was demonstrated to attract not only mouse peripheral blood leukocytes, but also human monocytes, macrophages and neutrophils, demonstrating the chemotactic effect of HDPs across species [43].

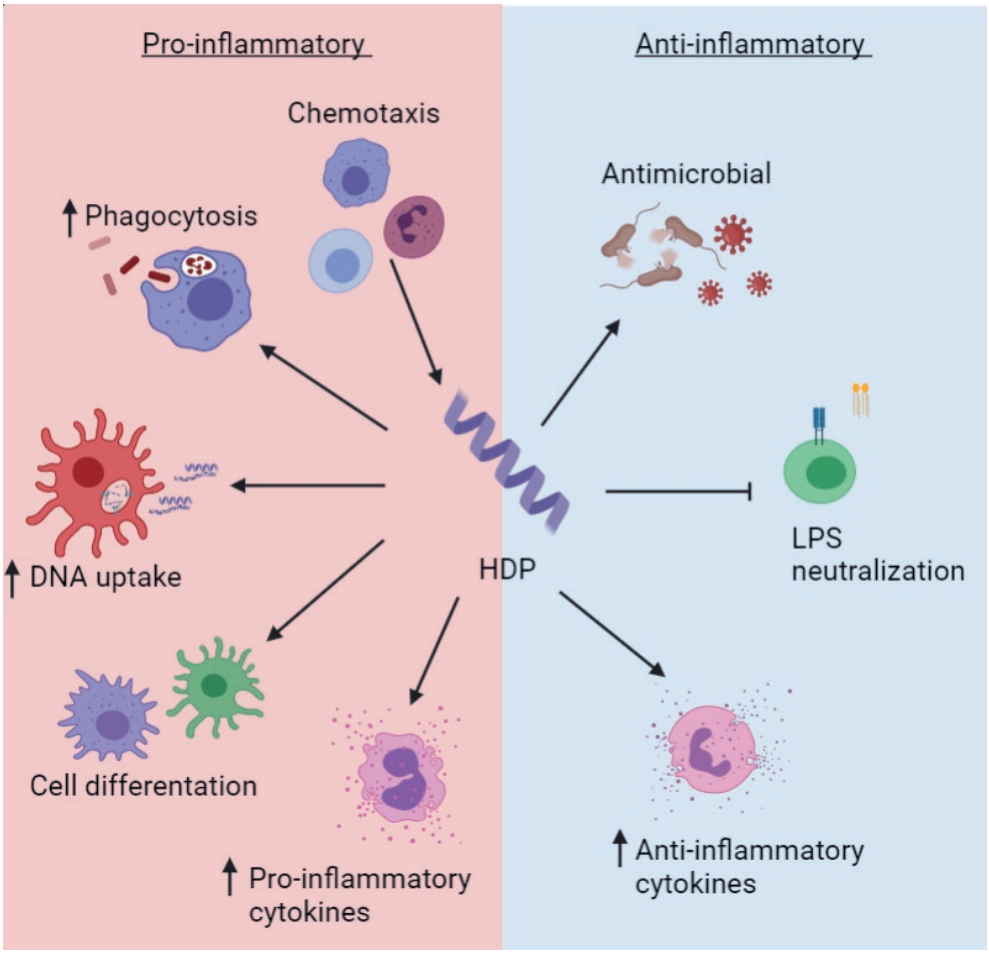


Figure 2: Overview of a selection of HDP functions. *HDPs have both anti-inflammatory effects, by enhancing release of anti-inflammatory cytokine productions, neutralizing LPS activation or directly killing pathogens, as well as pro-inflammatory effects, by enhancing phagocytosis, DNA uptake, pro-inflammatory cell differentiation, release of pro-inflammatory cytokines or direct chemotaxis. Shown here is a non-exhaustive selection of HDP functions. Created with BioRender.com*

Even before an active immune response is taking place, HDPs are capable of influencing immune cells. For instance, LL-37 has been shown to affect dendritic cell (DC) differentiation from monocytes, resulting in enhanced endocytic capacity and secretion of Th-1 inducing cytokines, suggesting a more pro-inflammatory type of DC [44]. Furthermore, LL-37 was demonstrated to enhance development of a proinflammatory macrophage signature during differentiation of human monocytes into macrophages and even was shown to have this effect on differentiated macrophages [45]. Inducing a pro-inflammatory state in immune cells can have protective functions, as shown with several animal models. Prophylactic treatment in zebrafish embryos with CATH-2 showed a protective effect upon *Salmonella enteritidis* infection [46]. Similarly, *in ovo* treatment with the D-amino acid analog of CATH-2 demonstrated a protective effect during challenge with avian pathogenic *Escherichia coli*, perhaps partly due to the increased percentage of peripheral blood lymphocytes and heterophiles [47]. The observed protective effects indicate that HDPs might be capable of inducing innate immune training [48].

Anti-inflammatory activity

Not only have HDPs been implicated in stimulating immune responses, they also have been shown to have a neutralizing effect on pro-inflammatory processes or even enhance anti-inflammatory processes. Peripheral blood mononuclear cells (PBMCs) treated with LL-37 demonstrated enhanced IL-10 production in resulting DCs, CD14-positive monocytes, T cells and B cells, while upon LPS stimulation together with LL-37, it was found that the production of TNFα, IL-6 and IL-8 decreased in all cell types [49]. Likewise, in chicken PBMCs IL-10 mRNA production was increased upon incubation with CATH-2, while upon LPS stimulation, IL-1β mRNA production was decreased by CATH-2 administration [50]. The functional relevance of HDP anti-inflammatory effects is clearly demonstrated in several animal models. In a rat sepsis model, administration of LL-37 showed lower plasma levels of TNFα compared to conventional antibiotic treatment and LL-37 thereby protected against sepsis lethality [51]. In a *Pseudomonas aeruginosa* lung infection model, the synthetic IDR-1002 reduced IL-6 release and overall inflammation in the mouse lungs and thereby reduced lung lesions [52].

Furthermore, HDPs have proven to be very effective in neutralization of LPS-evoked toll like receptor 4 (TLR4) activation, thereby abolishing pro-inflammatory effects. A species-wide comparison of cathelicidins showed LL-37, CRAMP, K9CATH, PMAP-36 and CATH-1, -2 and -3 were able to fully neutralize LPS-induced TLR4 activation in a macrophage system [53]. However, this is a very clean system, with only pure LPS and in a more representative system with live *E. coli*, only CATH-2 and PMAP-36 were able to neutralize TNFα release of macrophages. When macrophages were stimulated with gentamicin- or heat-killed *E. coli*

also LL-37, CRAMP and K9CATH were able to neutralize TNFα release [54]. This process is thought to protect the host for unnecessary and harmful activation of the immune system.

Membrane active mechanisms

Most cathelicidins adopt amphipathic helix structures upon interaction with bacterial membranes and exhibit their antibacterial activity through membrane active mechanisms. At high concentrations, several HDPs show lytic activities towards both Gram-negative and Gram-positive bacteria. This is initiated by an interaction between cationic residues in the HDPs and the negatively charged LPS or lipoteichoic acid (LTA). In this stage, HDPs orient parallel to the bacterial membrane. In the second stage, hydrophobic residues in the HDPs are able to interact with the fatty acid chains of the lipid molecules in the bacterial membrane and thereby insertion is facilitated [3,55]. Several models have been proposed for this membrane active mechanism **(Fig. 3)**, which will be discussed below.

Barrel stave model

First the barrel stave model will be discussed, which describes the formation of HDP pores with a proper channel. In this model it is suggested that HDPs first orient parallel to the bacterial membrane and sometimes form dimers or small oligomers. Upon insertion, hydrophobic regions align with the fatty acid chains of the lipids, while the hydrophilic regions of the HDPs line the inside of the channel. The circular pore shape was confirmed for alamethicin, a peptide with very few charges, by x-ray scattering, with six peptides forming one channel [56]. It appeared to be one of the few peptides adopting this pore model [57].

Toroidal pore model

A second model is the toroidal pore model, where HDPs intercalate into the membrane, causing distortion of the membrane packing. Again, HDPs first orient parallel to the bacterial membrane. Upon insertion, the hydrophilic parts of the HDPs maintain in contact with the polar lipid headgroups, causing the lipids to tilt and eventually connect the outer and inner leaflet of the membrane. Here the lining of the pore is formed by both the hydrophilic part of the HDP and the polar lipid headgroups, indicating this model might be used by heavily charged peptides. Phosphorus NMR confirmed major distortion of lipid headgroup orientation by protegrin-1 [58].

Carpet model

A third model is the carpet model, which describes a full coating of the membrane with HDPs, thereby causing membrane lysis. HDPs again orient parallel to the bacterial

membrane; however, in this model they extensively coat the outer leaflet of the membrane. Transient pores allow HDPs to access the inner leaflet of the membrane, further coating the membrane. This mechanism is thought to be employed by shorter HDPs, which cannot span the bacterial membrane to form proper pores [59]. Only upon higher concentrations of HDPs the membrane will be disrupted, which is thought to happen in a detergent-like manner, eventually leading to the formation of micelles. NMR spectroscopy has been a very powerful tool to distinguish between HDP orientations and has confirmed the carpet model for several HDPs [59].

These different models are not static and are probably all intermittently at play. Not only does the mechanism of action depend on the HDP and concentration, also composition of the target membrane and temperature are factors influencing HDP insertion. For melittin a carpet model was described in 1-palmitoyl, 2-oleoyl phosphatidylglycerol liposomes, while size-restricted pore formation was demonstrated in 1-palmitoyl, 2-oleoyl phosphatidylcholine liposomes [60]. Also temperature was shown to affect melittin orientation: at lower temperatures a parallel orientation was found and at higher temperatures a perpendicular orientation [57]. This orientation depends on the target membrane composition. This example shows that one peptide can exhibit different membrane active mechanisms, depending on several environmental factors.

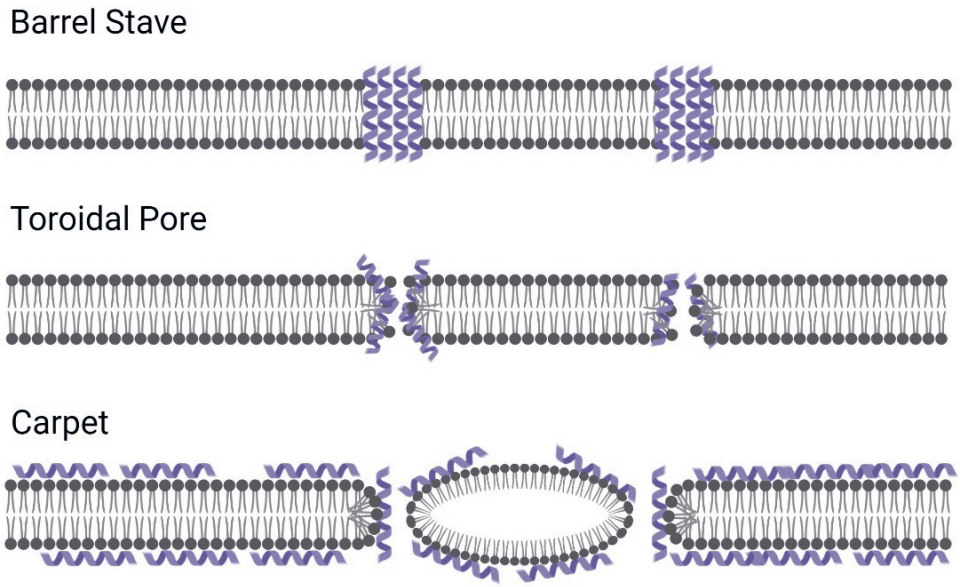


Figure 3: Described models for membrane lysis by HDPs. *The barrel stave, toroidal pore and carpet model have been described as potential mechanisms for membrane lysis by HDPs. The in vivo mechanism of action is most likely a combination of the membranolytic effects described by these three models. Created with BioRender.com*

LPS binding

Currently, it is still up for debate whether LPS binding by HDPs is necessary for killing of Gram-negative bacteria or whether LPS serves as 'sink' and initially prevents killing. Both theories are plausible and supported by data. Affinity between HDPs and LPS is greatly influenced by LPS structure. The number of fatty acid chains in the lipid A portion determines hydrophobicity of the LPS and thereby influences interactions. Furthermore, the phosphate groups can be decorated with sugars and other compounds and thereby charges are neutralized, affecting electrostatic interactions. Lastly, the presence (smooth LPS) or absence (rough LPS) of O-antigen in LPS has been shown to affect HDP binding.

On the one hand, it seems intuitive that HDPs harbor cationic charges in order to interact with anionic lipid molecules and thereby enhance their affinity and activity. This was shown for CAP18, the rabbit analogue of LL-37. Insertion into LPS monolayers and liposomes was studied and revealed a deeper intercalation of CAP18 into LPS membranes of susceptible *Salmonella enterica* compared to LPS membranes of resistant *Proteus mirabilis* [61]. In another study, peptide derivatives were compared for binding to *E. coli* LPS and *E. coli* killing, which showed that peptide derivatives with lower LPS affinity resulted in higher MIC values [62]. This supports the theory that LPS binding promotes bacterial penetration and killing.

In contrast, when HDPs interact with anionic lipid molecules in the bacterial membrane, intracellular targets will never be reached [63]. No binding was observed of CATH-2 with rough LPS of *E. coli* and this correlated with lower MIC values, while binding was observed with smooth LPS of *E. coli*, resulting in higher MIC values [64]. The importance of an O-antigen was also demonstrated in *Bordetella bronchiseptica*, since loss of the O-antigen resulted in increased susceptibility for several cationic peptides, to a similar extent as *Bordetella pertussis* who intrinsically lacks the O-antigen [65]. Lack of an O-antigen might decrease entrapment of HDPs in this complex sugar network. Similarly, in *Salmonella typhimurium* mutants deficient in *pagP*, lacking an acyl chain in their LPS, showed increased outer membrane permeability for C18G, protegrin-1 and polymyxin B and increased susceptibility for killing [66]. This might be due to less dense packing of LPS molecules, creating a more permeable barrier.

Several studies have investigated the correlation between LPS binding and membrane penetration or bacterial killing. In synthetic liposomes, an increase in LPS content did increase binding of melittin, however, it decreased vesicle leakage, suggesting melittin is trapped in the membrane and unable to penetrate [67]. A synthetically designed amphipathic peptide and its diastereomeric counterpart both showed ability to interact with LPS, but only the diastereomeric peptide was able to eliminate bacteria. In SPR

spectra, the diastereomeric peptide was shown to have a much higher dissociation, possibly explaining the enhanced killing [68]. This illustrates the fine balance between a necessary interaction of the peptide with LPS to target the bacterial outer membrane, but also having the ability to dissociate from LPS and traverse through the membrane.

Bacterial evasion of HDPs

Since HDPs target multiple essential molecules or processes in bacteria, it was believed that development of resistance would be uncommon. However, bacteria have co-evolved with our immune system and adapted several mechanisms to evade killing by HDPs [3,69]. Here it is important to note that against HDPs mostly adaptation mechanisms are observed, mechanisms which are developed in the presence of HDPs but are lost again when HDPs are removed from the environment, and not so much resistance mechanisms, which are permanent adaptations [70]. Additionally, transfer of AMP resistance genes was found to be very limited *in vivo*, unlike transfer of antibiotic resistance genes [71].

Peptide modification

The first bacterial defense mechanism is to defuse HDPs before they are able to interact with bacterial membranes, for instance by proteolytic degradation. The outer membrane protease OmpT in *E. coli* was able to rapidly degrade the antimicrobial peptide protamine, as shown with HPLC [72]. Deletion of a protein family member, PgtE, in *S. typhimurium* increased sensitivity towards the α-helical peptide C18G, LL-37 and CRAMP. HPLC analysis of the supernatant after incubation with a strain expressing PgtE confirmed C18G was degraded [73]. Caseinolytic protease X (ClpX) was identified in *Bacillus anthracis* to degrade LL-37 and CRAMP and confer resistance [74], showing multiple protease families have developed in bacteria to aid in protection against HDPs.

Cleavage of peptides is not the only way in which bacteria are able to defuse HDPs, since some bacteria modify HDPs. *Porphyromonas gingivalis* peptidylarginine deiminase citrullinates the arginine residues of LP9, a lysozyme derived peptide. This process neutralizes the cationic charge of LP9 and thereby abrogates its function [75]. Another mechanism is simply the sequestration of HDPs, rendering them unable to reach their target. M1 protein, decorating the surface of *Staphylococcus aureus,* increases resistance to LL-37 or CRAMP by sequestration [76]. All together this shows that several processes have evolved, aiming to defuse HDPs before reaching bacterial targets.

Membrane adaptation

More long-term defense can be achieved by remodeling of the bacterial membrane in order to minimize affinity of HDPs [77]. The multiple peptide resistance factor (MprF) is a

widespread membrane protein that substitutes the two hydroxyl groups on an anionic phosphatidylglycerol with lysine groups, resulting in a net cationic charge. This diminishes the affinity of HDPs for the bacterial membrane, conferring resistance [78]. Not only phospholipids, but also the major component of Gram-negative outer membrane, LPS, was found to be modified. In *B. pertussis*, the phosphate groups of LPS are decorated with glucosamines, shielding negative charges. This was shown to enhance resistance to Polymyxin B, E and LL-37 [79]. *P. aeruginosa* LPS was shown to contain an aminoarabinose group shielding the phosphate group, again conferring resistance to polymyxin B [80].

Efflux pumps

If HDPs have succeeded in entering bacterial cells, several mechanisms are in place to export HDPs. Deletion of the vraFG transporter in *Staphylococcus aureus* increased susceptibility to nisin, indolicidin and LL-37, suggesting its involvement as defense mechanism [81]. Deletion of the *multiple transferable resistance (mtr) CDE*-encoded efflux pump in *Neisseria gonorrhoeae* enhanced susceptibility to protegrin-1 and LL-37 [82]. An entire operon, *ClnRAB*, is upregulated in *Clostridium difficile* upon exposure to LL-37, which among others encodes for an ATP binding cassette (ABC) transporter (*ClnAB*) [83]. However, expression of this ABC transporter alone was unable to confer resistance. This is presumably due to substrate specificity, as has been shown for multiple efflux pumps [84].

OMV release

A more coarse mechanism of defense is secreting larger parts of membrane, affected with HDPs or antibiotics, as an outer membrane vesicle (OMV) [85]. OMV release was induced in *E. coli* upon treatment with polymyxin B and colistin [86]. Addition of isolated OMVs to *E. coli* even showed increased resistance to polymyxin B. Even cross-protection between species was observed by OMVs. OMVs of *Moraxella catarrhalis* were isolated and conferred protection to polymyxin B when added to cultures of *Hemophilus influenzae*, *P. aeruginosa* and *Acinetobacter baumannii* [87]. Addition of OMVs could also protect *Helicobacter pylori* against killing by LL-37 [88]. To date, that was the only study known to show this protective effect of OMVs against HDPs, but this mechanism is further described in **Chapter 3** of this thesis. There, three different Gram-negative bacteria were tested and this gives a strong indication that this might be a conserved mechanism.

HDPs' modulation of OMV reactogenicity

All Gram-negative bacteria produce spherical particles from their outer membrane, so called OMVs. These vesicles resemble the outer membrane of the originating bacteria and have many different functions. Since most HDPs exhibit a membrane active mechanism, OMVs could be an efficient decoy for HDPs. Their biogenesis, function, induction and

isolation have been reviewed in **Chapter 1**, so here only their potential, especially in combination with HDPs, will be highlighted.

Potential

OMVs closely resemble the surface of the originating bacterium, but do not have any replicative function and therefore have great potential to be used as next-generation vaccines. Immunization studies with OMVs in mice have already shown protective effects for *B. pertussis, Borrelia burgdorferi, Brucella melitensis, Francisella, Helicobacter and Vibrio cholerae* [89]. However, only for *Neisseria meningitidis* currently an OMV-based vaccine is used [90,91].

One major drawback for OMV-based vaccines is the presence of LPS, which can increase reactogenicity and provoke unwanted side-effects. Complete removal of LPS, by the use of detergents, is a safe method for OMV-based vaccine preparation [90,91]. However, the loss of LPS also results in loss of loosely attached membrane antigens and loss of the intrinsic adjuvant activity, based on LPS. Therefore, a milder method to neutralize LPS in an OMV-based vaccine is needed. Here HDPs might be promising, since they are known to interact with LPS and thereby neutralize TLR4 responses. A large study of HDPs from several species revealed that LL-37, CRAMP, K9CATH, CATH-1, CATH-2, CATH-3 and PMAP-36 capable of fully neutralizing LPS-induced TLR4 activation [53]. However, this is shown in a setting were pure LPS is studied. In the context of OMVs, LPS is not freely available, so therefore neutralization might be difficult. Additionally, multiple TLRs might be activated by OMVs, so not only LPS neutralization is relevant.

Few studies have shown the effect of HDPs on LPS neutralization in an OMV-based setting. Pep19-2.5 is the only one studied in this context and is a peptide derived from the LPS-binding domain of the *Limulus* anti-LPS factor [92]. This peptide was shown to interact with *E. coli* OMVs using isothermal calorimetry (ITC) and blocked LPS detection in the limulus amebocyte lysate assay [93]. Furthermore, both Pep19-2.5 and polymyxin B reduced TNF and IL-1β release, as well as pyroptosis in THP-1 macrophages after OMV stimulation. A specific TLR4 inhibitor only reduced TNF and IL-1β release of OMV-stimulated THP-1 macrophages, suggesting HDPs are capable of neutralizing also other inflammatory responses. Since this is the only study known to date, a major knowledge gap exists here and provides an opportunity to gain interesting insights into the OMV neutralizing capability of HDPs, useful for development of next-generation vaccines.

Thesis scope and outline

In this thesis, I aim to gain insight into the interplay between host HDPs and bacterial OMVs. Not only can OMVs be a potential defense mechanism against HDPs, but they can

also be used as bacterial vaccine, in conjunction with HDPs as adjuvant to balance the LPS-evoked responses. Several questions regarding OMVs and HDPs were investigated in this thesis.

First it was investigated whether OMVs could act as defense mechanism against HDPs **(Chapter 3)**. To investigate OMV release upon sub-lethal HDP treatment, quantifications and characterizations of released OMVs were performed. Three different HDPs and three different Gram-negative bacterial species were used to investigate how conserved the mechanism is. Furthermore, it was investigated whether addition of external OMVs or overproduction of genetically modified OMVs could protect bacteria against killing by HDPs.

In **Chapter 4** it was investigated whether binding to LPS, an abundant molecule in OMVs, could aid HDPs in their antibacterial function or if it could retain HDPs in the membrane and prevent them from exhibiting their antibacterial function. Binding affinity was studied using several different LPS structures to obtain information on the effect of the O-antigen or acyl chain composition on the HDP-LPS interaction. Furthermore, the binding affinity was correlated to HDP activity. The mechanism of action of the peptides was also investigated in detail by using an assay that is able to distinguish between inner and outer membrane damage of bacteria.

Next the focus was shifted to the immunomodulatory capacity of HDPs and in **Chapter 5** it was investigated whether PMAP-36 used to induce OMVs could also decrease subsequent OMV-evoked immune responses. The peptide-induced OMVs (pOMVs) were extensively characterized using lipidomics studies to investigate the lipidome of pOMVs and electron microscopy studies to investigate the morphology. Furthermore, it was investigated whether PMAP-36 present in the pOMVs could affect pOMV-evoked immune responses in porcine bone marrow-derived M1 macrophages.

In **Chapter 6** the aim was to investigate whether other HDPs could modulate OMV-based immune responses and which TLRs would be involved in OMV-based immune responses. Eight different HDPs were tested for their immune modulation capacity of OMV-evoked responses in macrophages. Cell lines expressing one specific human TLR (hTLR) were used to investigate hTLR activation by OMVs and possible neutralization by HDPs. Endocytosis of OMVs was also investigated with the use of inhibitor molecules.

The results of the thesis are summarized and discussed in **Chapter 7** with respect to future perspectives.

References

[1] B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, P. And Walter, Molecular Biology of the Cell, 4th ed., Garland Science, New York, 2002.

[2] J.M. Ageitos, A. Sánchez-Pérez, P. Calo-Mata, T.G. Villa, Antimicrobial peptides (AMPs): Ancient compounds that represent novel weapons in the fight against bacteria, Biochem. Pharmacol. 133 (2017) 117–138. doi:10.1016/j.bcp.2016.09.018.

[3] N. Mookherjee, M.A. Anderson, H.P. Haagsman, D.J. Davidson, Antimicrobial host defence peptides: functions and clinical potential, Nat. Rev. Drug Discov. 19 (2020) 311–332. doi:10.1038/s41573-019-0058-8.

[4] M. Zasloff, Antimicrobial peptides of multicellular organisms, Nature. 415 (2002) 389–395. doi:10.1038/415389a.

[5] L. Zhao, W. Lu, Defensins in innate immunity, Curr. Opin. Hematol. 21 (2014) 37–42. doi:10.1097/MOH.0000000000000005.

[6] T. Cuperus, M. Coorens, A. van Dijk, H.P. Haagsman, Avian host defense peptides, Dev. Comp. Immunol. 41 (2013) 352–369. doi:10.1016/j.dci.2013.04.019.

[7] Y. Sang, F. Blecha, Porcine host defense peptides: Expanding repertoire and functions, Dev. Comp. Immunol. 33 (2009) 334–343. doi:10.1016/j.dci.2008.05.006.

[8] L. Hazlett, M. Wu, Defensins in innate immunity, Cell Tissue Res. 343 (2011) 175–188. doi:10.1007/s00441-010-1022-4.

[9] M.E. Selsted, A.J. Ouellette, Mammalian defensins in the antimicrobial immune response, Nat. Immunol. 6 (2005) 551–557. doi:10.1038/ni1206.

[10] J. Jarczak, E.M. Kościuczuk, P. Lisowski, N. Strzałkowska, A. Jóźwik, J. Horbańczuk, J. Krzyzewski, L. Zwierzchowski, E. Bagnicka, Defensins: Natural component of human innate immunity, Hum. Immunol. 74 (2013) 1069–1079. doi:10.1016/j.humimm.2013.05.008.

[11] M. Zanetti, Cathelicidins, multifunctional peptides of the innate immunity, J. Leukoc. Biol. 75 (2004) 39–48. doi:10.1189/jlb.0403147.

[12] E.M. Kościuczuk, P. Lisowski, J. Jarczak, N. Strzałkowska, A. Jóźwik, J. Horbańczuk, J. Krzyżewski, L. Zwierzchowski, E. Bagnicka, Cathelicidins: family of antimicrobial peptides. A review., Mol. Biol. Rep. 39 (2012) 10957–10970. doi:10.1007/s11033-012-1997-x.

[13] B. Agerberth, H. Gunne, J. Odeberg, P. Kogner, H.G. Boman, G.H. Gudmundsson, FALL-39, a putative human peptide antibiotic, is cysteine-free and expressed in

bone marrow and testis, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 195–199. doi:10.1073/pnas.92.1.195.

[14] D. Vandamme, B. Landuyt, W. Luyten, L. Schoofs, A comprehensive summary of LL-37, the factoctum human cathelicidin peptide, Cell. Immunol. 280 (2012) 22–35. doi:10.1016/j.cellimm.2012.11.009.

[15] D. Xhindoli, S. Pacor, M. Benincasa, M. Scocchi, R. Gennaro, A. Tossi, The human cathelicidin LL-37 - A pore-forming antibacterial peptide and host-cell modulator, Biochim. Biophys. Acta - Biomembr. 1858 (2016) 546–566. doi:10.1016/j.bbamem.2015.11.003.

[16] U.H.N. Dürr, U.S. Sudheendra, A. Ramamoorthy, LL-37, the only human member of the cathelicidin family of antimicrobial peptides, Biochim. Biophys. Acta - Biomembr. 1758 (2006) 1408–1425. doi:10.1016/j.bbamem.2006.03.030.

[17] J. Johansson, G.H. Gudmundsson, M.E. Rottenberg, K.D. Berndt, B. Agerberth, Conformation-dependent Antibacterial Activity of the Naturally Occurring Human Peptide LL-37, J. Biol. Chem. 273 (1998) 3718–3724. doi:10.1074/JBC.273.6.3718.

[18] G. Wang, Structures of human host defense cathelicidin LL-37 and its smallest antimicrobial peptide KR-12 in lipid micelles, J. Biol. Chem. 283 (2008) 32637–32643. doi:10.1074/jbc.M805533200.

[19] A. Van Dijk, E.J.A. Veldhuizen, A.J.A.M. Van Asten, H.P. Haagsman, CMAP27, a novel chicken cathelicidin-like antimicrobial protein, Vet. Immunol. Immunopathol. 106 (2005) 321–327. doi:10.1016/j.vetimm.2005.03.003.

[20] A. van Dijk, M.H.G. Tersteeg-Zijderveld, J.L.M. Tjeerdsma-van Bokhoven, A.J.M. Jansman, E.J.A. Veldhuizen, H.P. Haagsman, Chicken heterophils are recruited to the site of Salmonella infection and release antibacterial mature Cathelicidin-2 upon stimulation with LPS, Mol. Immunol. 46 (2009) 1517–1526. doi:10.1016/j.molimm.2008.12.015.

[21] Y. Xiao, A.I. Herrera, Y.R. Bommineni, J.L. Soulages, O. Prakash, G. Zhang, The central kink region of fowlicidin-2, an α-helical host defense peptide, is critically involved in bacterial killing and endotoxin neutralization, J. Innate Immun. 1 (2009) 268–280. doi:10.1159/000174822.

[22] A. van Dijk, E.M. Molhoek, E.J.A. Veldhuizen, J.L.M.T. van Bokhoven, E. Wagendorp, F. Bikker, H.P. Haagsman, Identification of chicken cathelicidin-2 core elements involved in antibacterial and immunomodulatory activities, Mol. Immunol. 46 (2009) 2465–2473. doi:10.1016/j.molimm.2009.05.019.

[23] G. Zhang, C.R. Ross, F. Blecha, Porcine antimicrobial peptides: New prospects for ancient molecules of host defense, Vet. Res. 31 (2000) 277–296.

doi:10.1051/vetres:2000121.

[24] E.J.A. Veldhuizen, M.R. Scheenstra, J.L.M. Tjeerdsma-van Bokhoven, M. Coorens, V.A.F. Schneider, F.J. Bikker, A. van Dijk, H.P. Haagsman, Antimicrobial and Immunomodulatory Activity of PMAP-23 Derived Peptides, Protein Pept. Lett. 24 (2017) 1–36. doi:10.2174/0929866524666170428150925.

[25] K. Park, D. Oh, Y. Kim, S. Yub Shin, K.S. Hahm, Structural studies of porcine myeloid antibacterial peptide PMAP-23 and its analogues in DPC micelles by NMR spectroscopy, Biochem. Biophys. Res. Commun. 290 (2002) 204–212. doi:10.1006/bbrc.2001.6173.

[26] M. Scocchi, I. Zelezetsky, M. Benincasa, R. Gennaro, A. Mazzoli, A. Tossi, Structural aspects and biological properties of the cathelicidin PMAP-36, FEBS J. 272 (2005) 4398–4406. doi:10.1111/j.1742-4658.2005.04852.x.

[27] V. Cabiaux, B. Agerberths, J. Johansson, F. Homble, E. Goormaghtigh, J.-M. Ruysschaert, Secondary Structure and Membrane Interaction of PR-39, a Pro+Arg-rich Antibacterial Peptide, Eur. J. Biochem. 224 (1994) 1019–1027. doi:10.1111/j.1432-1033.1994.01019.x.

[28] R. Holani, C. Shah, Q. Haji, G.D. Inglis, R.R.E. Uwiera, E.R. Cobo, Proline-arginine rich (PR-39) cathelicidin: Structure, expression and functional implication in intestinal health, Comp. Immunol. Microbiol. Infect. Dis. 49 (2016) 95–101. doi:10.1016/j.cimid.2016.10.004.

[29] B.C. Leonard, V.K. Affolter, C.L. Bevins, Antimicrobial peptides: Agents of border protection for companion animals, Vet. Dermatol. 23 (2012) 177-e36. doi:10.1111/j.1365-3164.2012.01037.x.

[30] Y. Sang, M. Teresa Ortega, K. Rune, W. Xiau, G. Zhang, J.L. Soulages, G.H. Lushington, J. Fang, T.D. Williams, F. Blecha, T. Melgarejo, Canine cathelicidin (K9CATH): Gene cloning, expression, and biochemical activity of a novel pro-myeloid antimicrobial peptide, Dev. Comp. Immunol. 31 (2007) 1278–1296. doi:10.1016/J.DCI.2007.03.007.

[31] J. Yang, Y. Zhang, I-TASSER server: new development for protein structure and function predictions, Web Serv. Issue Publ. Online. 43 (2015). doi:10.1093/nar/gkv342.

[32] S.C. Mansour, C. de la Fuente-Núñez, R.E.W. Hancock, Peptide IDR-1018: modulating the immune system and targeting bacterial biofilms to treat antibiotic-resistant bacterial infections, J. Pept. Sci. 21 (2015) 323–329. doi:10.1002/psc.2708.

[33] M. Wieczorek, H. Jenssen, J. Kindrachuk, W.R.P. Scott, M. Elliott, K. Hilpert, J.T.J. Cheng, R.E.W. Hancock, S.K. Straus, Structural studies of a peptide with immune

modulating and direct antimicrobial activity, Chem. Biol. 17 (2010) 970–980. doi:10.1016/j.chembiol.2010.07.007.

[34] E.F. Haney, B. (Catherine) Wu, K. Lee, A.L. Hilchie, R.E.W. Hancock, Aggregation and Its Influence on the Immunomodulatory Activity of Synthetic Innate Defense Regulator Peptides, Cell Chem. Biol. 24 (2017) 969-980.e4. doi:10.1016/j.chembiol.2017.07.010.

[35] R.E.W. Hancock, E.F. Haney, E.E. Gill, The immunology of host defence peptides: Beyond antimicrobial activity, Nat. Rev. Immunol. 16 (2016) 321–334. doi:10.1038/nri.2016.29.

[36] A.L. Hilchie, K. Wuerth, R.E.W. Hancock, Immune modulation by multifaceted cationic host defense (antimicrobial) peptides, Nat. Chem. Biol. 9 (2013) 761–768. doi:10.1038/nchembio.1393.

[37] R. van Harten, E. van Woudenbergh, A. van Dijk, H. Haagsman, Cathelicidins: Immunomodulatory Antimicrobials, Vaccines. 6 (2018) 63. doi:10.3390/vaccines6030063.

[38] Y. Lai, R.L. Gallo, AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense, Trends Immunol. 30 (2009) 131–141. doi:10.1016/j.it.2008.12.003.

[39] F. D'Este, L. Tomasinsig, B. Skerlavaj, M. Zanetti, Modulation of cytokine gene expression by cathelicidin BMAP-28 in LPS-stimulated and -unstimulated macrophages, Immunobiology. 217 (2012) 962–971. doi:10.1016/j.imbio.2012.01.010.

[40] E.J.A. Veldhuizen, V.A.F. Schneider, H. Agustiandari, A. Van Dijk, J.L.M. Tjeerdsma-van Bokhoven, F.J. Bikker, H.P. Haagsman, Antimicrobial and immunomodulatory activities of PR-39 derived peptides, PLoS One. 9 (2014) e95939. doi:10.1371/journal.pone.0095939.

[41] J. Yu, N. Mookherjee, K. Wee, D.M.E. Bowdish, J. Pistolic, Y. Li, L. Rehaume, R.E.W. Hancock, Host Defense Peptide LL-37, in Synergy with Inflammatory Mediator IL-1β, Augments Immune Responses by Multiple Pathways, J. Immunol. 179 (2007) 7684–7691. doi:10.4049/jimmunol.179.11.7684.

[42] G.S. Tjabringa, D.K. Ninaber, J.W. Drijfhout, K.F. Rabe, P.S. Hiemstra, Human Cathelicidin LL-37 Is a Chemoattractant for Eosinophils and Neutrophils That Acts via Formyl-Peptide Receptors, Int. Arch. Allergy Immunol. 140 (2006) 103–112. doi:10.1159/000092305.

[43] K. Kurosaka, Q. Chen, F. Yarovinsky, J.J. Oppenheim, D. Yang, Mouse Cathelin-Related Antimicrobial Peptide Chemoattracts Leukocytes Using Formyl Peptide Receptor-Like 1/Mouse Formyl Peptide Receptor-Like 2 as the Receptor and Acts

as an Immune Adjuvant, J. Immunol. 174 (2005) 6257–6265. doi:10.4049/jimmunol.174.10.6257.

[44] D.J. Davidson, A.J. Currie, G.S.D. Reid, D.M.E. Bowdish, K.L. MacDonald, R.C. Ma, R.E.W. Hancock, D.P. Speert, The Cationic Antimicrobial Peptide LL-37 Modulates Dendritic Cell Differentiation and Dendritic Cell-Induced T Cell Polarization, J. Immunol. 172 (2004) 2704.2-2704. doi:10.4049/jimmunol.172.4.2704-a.

[45] A.M. van der Does, H. Beekhuizen, B. Ravensbergen, T. Vos, T.H.M. Ottenhoff, J.T. van Dissel, J.W. Drijfhout, P.S. Hiemstra, P.H. Nibbering, LL-37 Directs Macrophage Differentiation toward Macrophages with a Proinflammatory Signature, J. Immunol. 185 (2010) 1442–1449. doi:10.4049/jimmunol.1000376.

[46] V.A.F. Schneider, A. van Dijk, A.M. van der Sar, M.D. Kraaij, E.J.A. Veldhuizen, H.P. Haagsman, Prophylactic administration of chicken cathelicidin-2 boosts zebrafish embryonic innate immunity, Dev. Comp. Immunol. 60 (2016) 108–114. doi:10.1016/j.dci.2016.02.023.

[47] T. Cuperus, A. Van Dijk, M.G.R. Matthijs, E.J.A. Veldhuizen, H.P. Haagsman, Protective effect of in ovo treatment with the chicken cathelicidin analog D-CATH-2 against avian pathogenic E. coli, Sci. Rep. 6 (2016) 26622. doi:10.1038/srep26622.

[48] M.G. Netea, L.A.B. Joosten, E. Latz, K.H.G. Mills, G. Natoli, H.G. Stunnenberg, L.A.J. ONeill, R.J. Xavier, Trained immunity: A program of innate immune memory in health and disease, Science. 352 (2016) aaf1098. doi:10.1126/science.aaf1098.

[49] N. Mookherjee, P. Hamill, J. Gardy, D. Blimkie, R. Falsafi, A. Chikatamarla, D.J. Arenillas, S. Doria, T.R. Kollmann, R.E.W. Hancock, Systems biology evaluation of immune responses induced by human host defence peptide LL-37 in mononuclear cells, Mol. Biosyst. 5 (2009) 483–496. doi:10.1039/b813787k.

[50] M.D. Kraaij, A. van Dijk, H.P. Haagsman, CATH-2 and LL-37 increase mannose receptor expression, antigen presentation and the endocytic capacity of chicken mononuclear phagocytes, Mol. Immunol. 90 (2017) 118–125. doi:10.1016/j.molimm.2017.07.005.

[51] O. Cirioni, A. Giacometti, R. Ghiselli, C. Bergnach, F. Orlando, C. Silvestri, F. Mocchegiani, A. Licci, B. Skerlavaj, M. Rocchi, V. Saba, M. Zanetti, G. Scalise, LL-37 protects rats against lethal sepsis caused by gram-negative bacteria, Antimicrob. Agents Chemother. 50 (2006) 1672–1679. doi:10.1128/AAC.50.5.1672-1679.2006.

[52] K.C. Wuerth, R. Falsafi, R.E.W. Hancock, Synthetic host defense peptide IDR-1002

reduces inflammation in Pseudomonas aeruginosa lung infection, PLoS One. 12 (2017) 1–18. doi:10.1371/journal.pone.0187565.

[53] M. Coorens, M.R. Scheenstra, E.J.A. Veldhuizen, H.P. Haagsman, Interspecies cathelicidin comparison reveals divergence in antimicrobial activity, TLR modulation, chemokine induction and regulation of phagocytosis, Sci. Rep. 7 (2017) 1–11. doi:10.1038/srep40874.

[54] M. Coorens, V.A.F. Schneider, A.M. de Groot, A. van Dijk, M. Meijerink, J.M. Wells, M.R. Scheenstra, E.J.A. Veldhuizen, H.P. Haagsman, Cathelicidins Inhibit Escherichia coli –Induced TLR2 and TLR4 Activation in a Viability-Dependent Manner, J. Immunol. 199 (2017) 1418–1428. doi:10.4049/jimmunol.1602164.

[55] K.A. Brogden, Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria?, Nat. Rev. Microbiol. 3 (2005) 238–250. doi:10.1038/nrmicro1098.

[56] A. Spaar, C. Münster, T. Salditt, Conformation of peptides in lipid membranes, studied by x-ray grazing incidence scattering, Biophys. J. 87 (2004) 396–407. doi:10.1529/biophysj.104.040667.

[57] L. Yang, T.A. Harroun, T.M. Weiss, L. Ding, H.W. Huang, Barrel-Stave Model or Toroidal Model? A Case Study on Melittin Pores, Biophys. J. 81 (2001) 1475–1485. doi:10.1016/S0006-3495(01)75802-X.

[58] S. Yamaguchi, T. Hong, A. Waring, R.I. Lehrer, M. Hong, Solid-state NMR investigations of peptide-lipid interaction and orientation of a β-sheet antimicrobial peptide, protegrin, Biochemistry. 41 (2002) 9852–9862. doi:10.1021/bi0257991.

[59] B. Bechinger, The structure, dynamics and orientation of antimicrobial peptides in membranes by multidimensional solid-state NMR spectroscopy, Biochim. Biophys. Acta - Biomembr. 1462 (1999) 157–183. doi:10.1016/S0005-2736(99)00205-9.

[60] A.S. Ladokhin, S.H. White, "Detergent-like" permeabilization of anionic lipid vesicles by melittin, Biochim. Biophys. Acta. 1514 (2001) 253–260. doi:10.1016/S0005-2736(01)00382-0.

[61] T. Gutsmann, M. Fix, J.W. Larrick, A. Wiese, Mechanisms of action of rabbit CAP18 on monolayers and liposomes made from endotoxins or phospholipids, J. Membr. Biol. 176 (2000) 223–236. doi:10.1007/s002320001092.

[62] M.G. Scott, H. Yan, R.E.W. Hancock, Biological properties of structurally related α-helical cationic antimicrobial peptides, Infect. Immun. 67 (1999) 2005–2009. doi:10.1128/iai.67.4.2005-2009.1999.

[63] Y. Rosenfeld, Y. Shai, Lipopolysaccharide (Endotoxin)-host defense antibacterial

peptides interactions: Role in bacterial resistance and prevention of sepsis, Biochim. Biophys. Acta - Biomembr. 1758 (2006) 1513–1522. doi:10.1016/j.bbamem.2006.05.017.

[64] V.A.F. Schneider, M. Coorens, S.R. Ordonez, J.L.M. Tjeerdsma-Van Bokhoven, G. Posthuma, A. Van Dijk, H.P. Haagsman, E.J.A. Veldhuizen, Imaging the antimicrobial mechanism(s) of cathelicidin-2, Sci. Rep. 6 (2016) 1–11. doi:10.1038/srep32948.

[65] A. Banemann, H. Deppisch, R. Gross, The lipopolysaccharide of Bordetella bronchiseptica acts as a protective shield against antimicrobial peptides, Infect. Immun. 66 (1998) 5607–5612. doi:10.1128/iai.66.12.5607-5612.1998.

[66] L. Guo, K.B. Lim, C.M. Poduje, M. Daniel, J.S. Gunn, M. Hackett, S.I. Miller, Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides, Cell. 95 (1998) 189–198. doi:10.1016/S0092-8674(00)81750-X.

[67] D. Allende, T.J. McIntosh, Lipopolysaccharides in bacterial membranes act like cholesterol in eukaryotic plasma membranes in providing protection against melittin-induced bilayer lysis, Biochemistry. 42 (2003) 1101–1108. doi:10.1021/bi026932s.

[68] N. Papo, Y. Shai, A molecular mechanism for lipopolysaccharide protection of gram-negative bacteria from antimicrobial peptides, J. Biol. Chem. 280 (2005) 10378–10387. doi:10.1074/jbc.M412865200.

[69] D.I. Andersson, D. Hughes, J.Z. Kubicek-Sutherland, Mechanisms and consequences of bacterial resistance to antimicrobial peptides, Drug Resist. Updat. 26 (2016) 43–57. doi:10.1016/j.drup.2016.04.002.

[70] B. El Shazely, A. Urbański, P.R. Johnston, J. Rolff, In vivo exposure of insect AMP resistant Staphylococcus aureus to an insect immune system, Insect Biochem. Mol. Biol. 110 (2019) 60–68. doi:10.1016/j.ibmb.2019.04.017.

[71] B. Kintses, O. Méhi, E. Ari, M. Számel, Á. Györkei, P.K. Jangir, I. Nagy, F. Pál, G. Fekete, R. Tengölics, Á. Nyerges, I. Likó, A. Bálint, T. Molnár, B. Bálint, B.M. Vásárhelyi, M. Bustamante, B. Papp, C. Pál, Phylogenetic barriers to horizontal transfer of antimicrobial peptide resistance genes in the human gut microbiota, Nat. Microbiol. 2018 43. 4 (2018) 447–458. doi:10.1038/s41564-018-0313-5.

[72] S. Stumpe, R. Schmid, D.L. Stephens, G. Georgiou, E.P. Bakker, Identification of OmpT as the Protease That Hydrolyzes the Antimicrobial Peptide Protamine before It Enters Growing Cells of Escherichia coli, J. Bacteriol. 180 (1998) 4002–4006. doi:10.1128/JB.180.15.4002-4006.1998.

[73] T. Guina, E.C. Yi, H. Wang, M. Hackett, S.I. Miller, A PhoP-regulated outer membrane protease of Salmonella enterica serovar typhimurium promotes

resistance to alpha-helical antimicrobial peptides., J. Bacteriol. 182 (2000) 4077–86. doi:10.1128/jb.182.14.4077-4086.2000.

[74] S.M. McGillivray, C.M. Ebrahimi, N. Fisher, M. Sabet, D.X. Zhang, Y. Chen, N.M. Haste, R. V. Aroian, R.L. Gallo, D.G. Guiney, A.M. Friedlander, T.M. Koehler, V. Nizet, ClpX Contributes to Innate Defense Peptide Resistance and Virulence Phenotypes of *Bacillus anthracis*, J. Innate Immun. 1 (2009) 494–506. doi:10.1159/000225955.

[75] T. Stobernack, M. du T. Espina, L.M. Mulder, L.M.P. Medina, D.R. Piebenga, G. Gabarrini, X. Zhao, K.M.J. Janssen, J. Hulzebos, E. Brouwer, T. Sura, D. Becher, A.J. van Winkelhoff, F. Götz, A. Otto, J. Westra, J.M. van Dijl, A secreted bacterial peptidylarginine deiminase can neutralize human innate immune defenses, MBio. 9 (2018) 1–15. doi:10.1128/mbio.01704-18.

[76] X. Lauth, M. von Köckritz-Blickwede, C.W. McNamara, S. Myskowski, A.S. Zinkernagel, B. Beall, P. Ghosh, R.L. Gallo, V. Nizet, M1 Protein Allows Group A Streptococcal Survival in Phagocyte Extracellular Traps through Cathelicidin Inhibition, J. Innate Immun. 1 (2009) 202–214. doi:10.1159/000203645.

[77] A. Peschel, How do bacteria resist human antimicrobial peptides?, Trends Microbiol. 10 (2002) 179–186. doi:10.1016/S0966-842X(02)02333-8.

[78] C.M. Ernst, P. Staubitz, N.N. Mishra, S.-J. Yang, G. Hornig, H. Kalbacher, A.S. Bayer, D. Kraus, A. Peschel, The Bacterial Defensin Resistance Protein MprF Consists of Separable Domains for Lipid Lysinylation and Antimicrobial Peptide Repulsion, PLoS Pathog. 5 (2009) e1000660. doi:10.1371/journal.ppat.1000660.

[79] N.R. Shah, R.E.W. Hancock, R.C. Fernandez, Bordetella pertussis lipid a glucosamine modification confers resistance to cationic antimicrobial peptides and increases resistance to outer membrane perturbation, Antimicrob. Agents Chemother. 58 (2014) 4931–4934. doi:10.1128/AAC.02590-14.

[80] S.M. Moskowitz, R.K. Ernst, S.I. Miller, PmrAB, a Two-Component Regulatory System of Pseudomonas aeruginosa That Modulates Resistance to Cationic Antimicrobial Peptides and Addition of Aminoarabinose to Lipid A, J. Bacteriol. 186 (2004) 575–579. doi:10.1128/JB.186.2.575-579.2004.

[81] M. Li, D.J. Cha, Y. Lai, A.E. Villaruz, D.E. Sturdevant, M. Otto, The antimicrobial peptide-sensing system aps of Staphylococcus aureus, Mol. Microbiol. 66 (2007) 1136–1147. doi:10.1111/j.1365-2958.2007.05986.x.

[82] W.M. Shafer, X.D. Qu, A.J. Waring, R.I. Lehrer, Modulation of Neisseria gonorrhoeae susceptibility to vertebrate antibacterial peptides due to a member of the resistance/nodulation/division efflux pump family, Proc. Natl. Acad. Sci. 95 (1998) 1829–1833. doi:10.1073/pnas.95.4.1829.

[83] E.C. Woods, A.N. Edwards, K.O. Childress, J.B. Jones, S.M. McBride, The C. difficile clnRAB operon initiates adaptations to the host environment in response to LL-37, PLoS Pathog. 14 (2018) e1007153. doi:10.1371/journal.ppat.1007153.

[84] H.-S. Joo, C.I. Fu, M. Otto, Bacterial strategies of resistance to antimicrobial peptides, Philos. Trans. R. Soc. B Biol. Sci. 371 (2016) 20150292. doi:10.1098/rstb.2015.0292.

[85] A.J. Manning, M.J. Kuehn, Functional advantages conferred by extracellular prokaryotic membrane vesicles, J. Mol. Microbiol. Biotechnol. 23 (2013) 131–141. doi:10.1159/000346548.

[86] A.J. Manning, M.J. Kuehn, Contribution of bacterial outer membrane vesicles to innate bacterial defense, BMC Microbiol. 11 (2011) 1–14. doi:10.1186/1471-2180-11-258.

[87] J. Roszkowiak, P. Jajor, G. Guła, J. Gubernator, A. Żak, Z. Drulis-Kawa, D. Augustyniak, Interspecies outer membrane vesicles (OMVs) modulate the sensitivity of pathogenic bacteria and pathogenic yeasts to cationic peptides and serum complement, Int. J. Mol. Sci. 20 (2019) 1–21. doi:10.3390/ijms20225577.

[88] B.O. Murray, R.A. Dawson, L.M. Alsharaf, J. Anne Winter, Protective effects of Helicobacter pylori membrane vesicles against stress and antimicrobial agents, Microbiology. 166 (2020) 751–758. doi:10.1099/mic.0.000934.

[89] L. van der Pol, M. Stork, P. van der Ley, Outer membrane vesicles as platform vaccine technology, Biotechnol. J. 10 (2015) 1689–1706. doi:10.1002/biot.201400395.

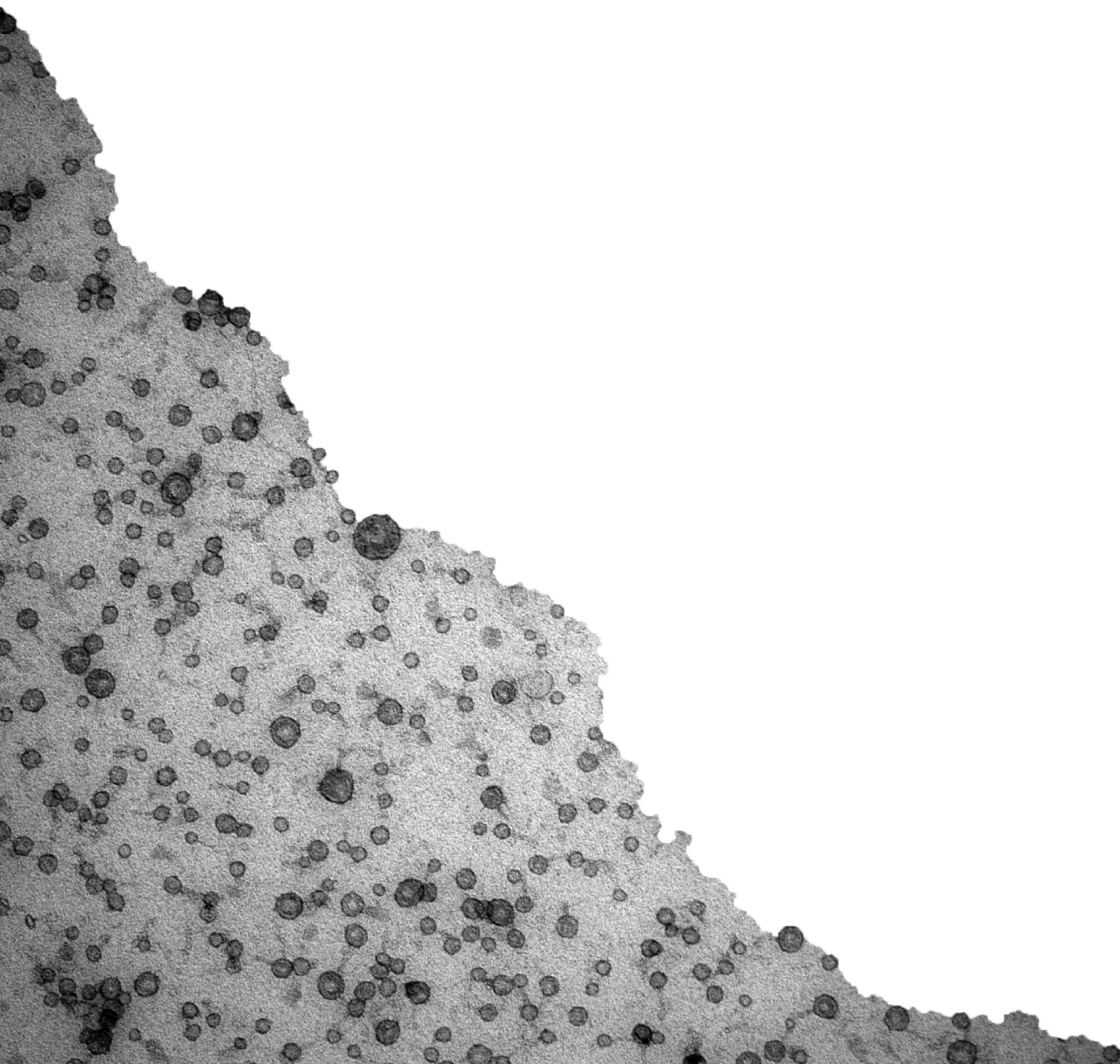
[90] D.M. Granoff, Review of Meningococcal Group B Vaccines, Clin. Infect. Dis. 50 (2010) S54–S65. doi:10.1086/648966.

[91] J. Holst, P. Oster, R. Arnold, M. Tatley, L. Næss, I. Aaberge, Y. Galloway, A. McNicholas, J. O'Hallahan, E. Rosenqvist, S. Black, Vaccines against meningococcal serogroup B disease containing outer membrane vesicles (OMV): Lessons from past programs and implications for the future, Hum. Vaccin. Immunother. 9 (2013) 1241–1253. doi:10.4161/hv.24129.

[92] L. Heinbockel, S. Sánchez-Gómez, G.M. De Tejada, S. Dömming, J. Brandenburg, Y. Kaconis, M. Hornef, A. Dupont, S. Marwitz, T. Goldmann, M. Ernst, T. Gutsmann, T. Schürholz, K. Brandenburg, Preclinical investigations reveal the broad-spectrum neutralizing activity of peptide pep19-2.5 on bacterial pathogenicity factors, Antimicrob. Agents Chemother. 57 (2013) 1480–1487. doi:10.1128/AAC.02066-12.

[93] A. Pfalzgraff, W. Correa, L. Heinbockel, A.B. Schromm, C. Lübow, N. Gisch, G. Martinez-de-Tejada, K. Brandenburg, G. Weindl, LPS-neutralizing peptides

reduce outer membrane vesicle-induced inflammatory responses, Biochim. Biophys. Acta - Mol. Cell Biol. Lipids. 1864 (2019) 1503–1513. doi:10.1016/j.bbalip.2019.05.018.



Chapter 3

Outer Membrane Vesicles protect Gram-negative Bacteria against Host Defense Peptides

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Abstract

Host defense peptides (HDPs) are part of the innate immune system and constitute a first line of defense against invading pathogens. They possess antimicrobial activity against a broad spectrum of pathogens. However, pathogens have been known to adapt to hostile environments. Therefore, the bacterial response to treatment with HDPs was investigated. Previous observations suggested that sub-lethal concentrations of HDPs increase the release of outer membrane vesicles (OMVs) in *Escherichia coli.* First, the effects of sub-lethal treatment with HDPs CATH-2, PMAP-36 and LL-37 on OMV release of several Gram-negative bacteria was analyzed. Treatment with PMAP-36 and CATH-2 induced release of OMVs, but treatment with LL-37 did not. The OMVs were further characterized with respect to morphological properties. The HDP-induced OMVs often had disc-like shapes. The beneficial effect of bacterial OMV release was studied by determining the susceptibility of *E. coli* towards HDPs in the presence of OMVs. The minimal bactericidal concentration was increased in the presence of OMVs. It is concluded that OMV release is a means of bacteria to dispose of HDP-affected membrane. Furthermore, OMVs act as a decoy for HDPs and thereby protect the bacterium.

Introduction

When pathogens enter a host, they enter a hostile environment. Host species have developed many measures to eliminate and remove pathogens, however, pathogens have been evolving simultaneously. Well known is the development of antibiotic resistance, but pathogens also have found ingenious mechanisms to evade the host's intrinsic immune system [1]. One of the first innate defense molecules pathogens will encounter are host defense peptides (HDPs). HDPs are small, cationic molecules and have antibacterial activities against a broad range of pathogens. They are amphipathic and this enables them to interact with bacterial membranes. Therefore, many HDPs are membrane active and exert their antibacterial function through membrane lysis [2].

One extensively studied HDP is LL-37. It is the only human cathelicidin and much is known about its mechanism of action. LL-37 is an α-helical amphipathic peptide which was shown to interact with the bacterial membrane with its helical axis in a parallel fashion to the bacterial surface. It can form small, toroidal pores that cause cytoplasmic leakage but also provide opportunity for translocation of the peptide [3]. LL-37 can bind to components of the peptidoglycan layer and interfere with its synthesis. Furthermore, LL-37 can interact with DNA and ribosomes and cause clustering of these components [4,5]. A second well studied cathelicidin is the chicken cathelicidin CATH-2. It was shown to interact with LPS and very rapidly localizes to the bacterial membrane where it internalizes and, at higher concentrations, causes membrane permeabilization [4,6]. Another very active cathelicidin is porcine PMAP-36. It has a helical fold, similar to LL-37 and CATH-2, but differs from them by its ability to covalently dimerize which enhances its pore forming ability. Its mechanism of action is not fully understood although it was shown to permeabilize bacterial membranes, as well as cause clustering of intracellular targets, suggesting a multitarget mode of action [4,7].

HDPs target multiple and vital parts of the bacterium, which makes it difficult to develop resistance. However, there are some bacteria that have developed mechanisms to counteract the antibacterial activity of HDPs [8–13]. The most common mechanism is the secretion of molecules that render HDPs inactive. For example, the M1 protein of group A *Streptococcus* is able to confer protection against HDPs, even when expressed in other bacteria, by sequestering HDPs [14]. PgtE from *Salmonella* does not only interact with α-helical antimicrobial peptides, but was also shown to cleave these peptides [15]. Furthermore, the secreted peptidylarginine deiminase (PAD) from *Porphyromonas gingivalis* is able to citrullinate peptides and thereby decrease the cationic charge which is essential for the peptide's function [16]. An entire operon is upregulated in *Clostridioides difficile* to confer resistance against HDPs, of which the mechanism is not yet fully understood [17].

Since HDPs are membrane active molecules, bacteria also have been shown to alter their membranes to render HDPs inactive. Modification of phospholipids happens in multiple species through a conserved protein, MprF, which adds a lysine to phosphatidylglycerol and thereby neutralizes the negative charge [18]. Furthermore, it has been shown that addition of external membrane, in the form of outer membrane vesicles (OMVs) [19], protects *E. coli* against polymyxin B and colistin, two peptide antibiotics [20]. Similarly, addition of OMVs protected *Helicobacter pylori* against LL-37 [21]. In this work we investigated bacterial defense against HDPs by exposing Gram-negative bacteria, *E. coli*, *Bordetella bronchiseptica* and *Pseudomonas aeruginosa*, to sub-lethal concentrations of three HDPs: PMAP-36, CATH-2 and LL-37. OMV release was quantified and the resulting OMVs were characterized. Furthermore, external OMVs were added to bacterial cultures to investigate whether this could increase resistance to HDPs. The results showed that CATH-2 and PMAP-36, but not LL-37, were able to induce OMV release. However, addition of OMVs to bacterial cultures showed protection against all three peptides.

Material and Methods

Peptide synthesis

PMAP-36 and CATH-2 were synthesized by Fmoc-chemistry at China Peptides (CPC scientific, Sunnyvale, CA, USA). LL-37 was synthesized by Fmoc-chemistry at the Academic Centre for Dentistry Amsterdam (Amsterdam, the Netherlands). All peptides were purified to a purity of >95% by reverse phase high-performance liquid chromatography. Sequences and characteristics of the peptides are shown in **Table 1**.

Bacterial species and growth conditions

E. coli ATCC 25922, a clinical isolate of *B. bronchiseptica* from pig (BB-P19, Veterinary Microbiological Diagnostic Center (VMDC), Utrecht University) and the laboratory strain *P. aeruginosa* PAO1 were used throughout this study. Both *E. coli* and *P. aeruginosa* were grown on tryptone soy agar (TSA) plates (Oxoid Ltd, Basingstoke, Hampshire, UK). Liquid cultures were grown in lysogeny broth (LB) containing 1% yeast extract (Becton, Dickinson and Company, Sparks, USA), 1% NaCl (Merck, Darmstadt, Germany) and 0.5% tryptone (Becton, Dickinson and Company). *B. bronchiseptica* was grown on Difco™ Bordet-Gengou

Table 1: Sequence, number of amino acids (No. aa) and charge of studied peptides [4].

Peptide	***Sequence***	***No. aa***	***Charge***
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	37	6+
CATH-2	RFGRFLRKIRRFRPKVTITIQGSARF-NH_2	26	8+
PMAP-36	Ac-GRFRRLRKKTRKRLKKIGKVLKWIPPIVGSIPLGCG	36	13+

(BG) agar plates (Becton, Dickinson and Company), containing 1% glycerol (Merck) supplemented with 15% (v/v) defibrinated sheep blood (Oxoid Ltd). Liquid cultures were grown in Verwey medium (pH 7.4) [22] containing 0.1% (w/v) starch from potato (S2004, Sigma-Aldrich, St. Louis, MO, USA), 0.02% (w/v) KCl, 0.05% (w/v) KH_2PO_4, 0.01% (w/v) $MgCl_2 \bullet 6\ H_2O$ (all from Merck), 0.002% (w/v) nicotinic acid (Sigma-Aldrich), 1.4% (w/v) Bacto™ casamino acids (Becton, Dickinson and Company), and 0.001% (w/v) L-glutathione reduced (Sigma-Aldrich).

OMV isolation

OMVs were isolated as described before [23]. In short, bacteria were grown overnight to an optical density of approximately 1.5. Before OMV isolation was initiated, bacteria were treated for 1 h. Subsequently, bacterial cells were removed by centrifugation for 30 min at 4700 x g. The supernatant was passed through a 0.45 µm Whatman filter (GE healthcare, Chicago, Illinois, USA) and centrifuged at 40,000 rpm for 2 h at 4°C (Ti-70 rotor, Beckman coulter, Brea, California, USA). The supernatant was decanted, and the transparent pellet was dissolved in 2 mM Tris (pH 7.5, Sigma-Aldrich) in a volume corresponding to 2% of the bacterial culture.

Generation of PMAP-36 antibody

Rabbit polyclonal antibody against synthetic PMAP-36 peptide (GRFRRLRKKTRKRLKKIGKVLKWIPPIVGSIPLGCG-amide) was generated at Biogenes (Berlin, Germany). 25 mg of PMAP-36 peptide was synthesized by Fmoc chemistry at a purity >80% with quality control by HPLC and mass spectrometry. Five mg of PMAP-36 was conjugated to limulus polyphemus hemocyanin (LPH) with 4-(N-maleimidomethyl)-cyclohexane-1-carboxylic acid N-hydroxysuccinimide ester and used to immunize two rabbits. Immunization followed a schedule with several boosts during several months and ELISA titer testing of antisera after which antisera from both rabbits were collected after final bleeding. Hundred milliliters of pooled antiserum was purified by affinity chromatography on a PMAP-36 coated CNBr-Sepharose column. Monospecific IgG was then eluted from the column with 0.2 M Glycine–HCl buffer containing 250 mM NaCl (pH 2.2), neutralized with 2 M Tris–HCl (pH 7.5) and filtered (pore width: 0.45 µm) to remove any remaining debris. To conserve the antibody, 0.1 % ProClin 300 was added.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

Acrylamide gels (14%) were prepared as previously described [24]. For Coomassie staining OMVs were diluted in 2x sample buffer containing 5% v/v β-mercaptoethanol (Sigma-Aldrich), boiled for 10 min at 95°C, and 20 µL were loaded on gel. Gels were run for 30 min at 50 V and then another 60 min at 150 V. Gels were stained with 0.1% (w/v) Coomassie

Brilliant Blue R-250 (Serva, Heidelberg, Germany) in 50:40:10 MilliQ (MQ): Methanol: Acetic acid (Sigma-Aldrich, Honeywell, Charlotte, North Carolina, USA) and destained overnight in 80:10:10 MQ: Methanol: Acetic Acid. For western blots, gels were transferred to activated nitrocellulose membranes (Biorad, Hercules, California, USA) using Transblot Turbo (Biorad) according to manufacturer's protocol. Membranes were blocked for 1 h with 5% Bovine Serum Albumin (BSA, Sigma-Aldrich) in phosphate-buffered saline (PBS, Thermo Fisher Scientific) at RT and washed three times with TBS-T (0.9 M Tris, 25 M NaCl, both Merck and 0.1 v/v% Tween-20, Serva). Primary antibodies were diluted 1:2500 for CATH-2 [25] and PMAP-36 and 1:1000 for LL-37 (Phoenix Pharmaceuticals, CA, USA) in 1% BSA in PBS and blots were incubated overnight at 4°C. After three washes with TBS-t, blots were incubated with goat anti-rabbit peroxidase antibodies, diluted 1:5000, for 1 h at RT. Then, blots were washed again three times with TBS-t and once with PBS and developed using the clarity western ECL substrate kit (Biorad), according to manufacturer's protocol. Gels and blots were imaged with a Universal Hood III (Biorad).

Bicinchoninic acid (BCA) assay

Total protein concentration of isolated OMVs was determined using the Pierce BCA assay (Thermo Fisher Scientific). In short, 25 µL of sample, supplemented with 2% SDS (Invitrogen, Carlsbad, California, USA), was incubated with 200 µL of working reagent at 37°C for 2 h. Absorbance was measured at 562 nm with the Fluostar omega (BMG Labtech, Ortenberg, Germany). BSA was used as reference.

FM4-64 assay

Total lipid concentration of isolated OMVs was determined using the membrane-inserting fluorescent dye FM4-64 (Invitrogen). Samples (25 µL) were incubated with 200 µL FM4-64 (2.25 µg/mL) at 37°C for 10 min. Samples were excited at 485 nm and fluorescence was measured at 670 nm with the Fluostar omega.

Dynamic light scattering (DLS)

Samples for DLS were diluted 10-fold in 2 mM Tris. Samples were measured in micro-volume cuvettes (Sarstedt, Nümbrecht, Germany) on a Zetasizer nano (Malvern Panalytical, Malvern, UK) with a scatter angle of 173°. The standard polystyrene latex was used with a refractive index of 1.590 and absorbance of 0.010. Water was used as solvent (viscosity of 0.8872, refractive index of 1.330). Three measurements of 10-20 samplings were performed at 25°C.

Lipidomics

OMV pellets were obtained as described above. Lipids from OMVs were extracted using the method described by Bligh and Dyer [26]. Lipid extracts were dried under N_2, dissolved in 100 µL of chloroform and methanol (1:1), and injected (10 µL) into a hydrophilic interaction liquid chromatography column (2.6 µm HILIC 100 Å, 50x4.6 mm, Phenomenex, CA). Lipid classes were separated by gradient elution on an Infinity II 1290 UPLC (Agilent, CA) at a flow rate of 1 mL/min. A mixture of acetonitrile and acetone (9:1, v/v) was used as solvent A, while solvent B consisted of a mixture of acetonitrile, MQ (7:3, v/v) with 50 mM ammonium formate. Both A and B contained 0.1% formic acid (v/v). Gradient elution was done as follows (time in min, % B): (0, 0), (1, 50), (3, 50), (3.01, 100), (4, 100). No re-equilibration of the column was necessary between successive samples. The column effluent was connected to a heated electrospray ionization source of an Orbitrap Fusion mass spectrometer (Thermo Scientific, MA) operated at -3600 V in the negative ionization mode. The vaporizer and ion transfer tube were set at a temperature of 450°C and 350°C, respectively. Full scan measurements (MS1) in the mass range from 450 to 1100 amu were collected at a resolution of 120.000. Data processing was based on the package 'XCMS' version 3.12 running under R version 4.0.3 for peak recognition and integration [27]. Lipid classes were identified based on retention time and molecular species were then matched against an *in silico* generated lipid database. Mass accuracy of annotated lipids was typically below 2 ppm.

Electron microscopy (EM)

For negative staining of OMVs, a protocol was provided by the Cell Microscopy Center (CMC, University Medical Center, Utrecht) [23]. In short, copper grids were carbon activated, incubated with 10 µL vesicle solution for 10-30 min and washed three times with PBS. The solution was fixed on the grids using 1% glutaraldehyde (Sigma-Aldrich) in PBS for 10 min and washed two times with PBS and subsequently four times with MQ. The grids were then briefly rinsed with methylcellulose/uranyl acetate (pH 4, provided by the CMC) and incubated for 5 min with methylcellulose/uranyl acetate (pH 4) on ice. Grids were looped out of the solution and air dried. Samples were imaged on a Tecnai-12 electron microscope (FEI, Hillsboro, Oregon, USA).

Track dilution assay

Bacterial killing by HDPs was assessed using track dilution assays, as described before [4]. In short, $2x10^6$ colony forming units (CFU)/mL bacteria were incubated with different concentrations of peptide for three hours at 37°C in a U-bottom microtiter plate (Corning, New York, USA). For assays with hypervesiculating mutants, supernatant of

hypervesiculating and wild-type *E. coli* was collected by 10 min centrifugation at 4700 x g, filtered over 0.45 µm filters and used to dilute wild-type bacteria. For OMV protection studies isolated OMVs of *E. coli* were added in a final concentration of 500 A.U. as defined by the FM4-64 lipid dye. After incubation, the mixture was diluted 2- or 5-fold of which 10-fold serial dilutions were prepared using medium and 10 µL of each dilution was plated on appropriate agar plates. Plates were incubated at 37°C for 24 h. Minimal bactericidal concentration (MBC) was defined as <200 CFU/mL, the detection limit of this assay.

Results

CATH-2 and PMAP-36, but not LL-37, stimulate the release of OMVs in Gram-negative bacteria

To investigate the effect of HDPs on OMV release by Gram-negative bacteria we selected three bacterial species for our experiments, *E. coli*, *B. bronchiseptica* and *P. aeruginosa*. Bacterial cultures were stimulated with two sub-lethal concentrations of different peptides, PMAP-36, CATH-2 and LL-37, and peptide-induced OMVs (pOMVs) were isolated. Heat treatment was applied as control stressor, since it has been shown to induce OMV release, resulting in heat-induced OMVs (hOMVs) [23,28]. Isolated pOMVs and hOMVs were analyzed using Coomassie stained SDS-PAGE and compared to spontaneous OMVs (sOMVs, **Fig. 1)**. This confirmed that heat treatment indeed induced OMVs, as shown by an increase of protein band intensity. For the higher concentrations of PMAP-36 and CATH-2 a slight increase in protein band intensity was observed. As shown previously, PMAP-36 is present in the isolated pOMVs **(Fig. 1a**, black arrow**)** [23]. CATH-2 is also present in the pOMV fraction **(Fig. 1a**, red arrow**)** but LL-37 is not. This indicates a difference in mechanism of action between the three peptides.

To quantify differences in OMV release, first a BCA assay was used **(Fig. S1)**. The high signal of medium alone interfered with accurate assessment of differences between treatments. However, heat treatment resulted in a large significant increase of OMV release by *B. bronchiseptica* and *P. aeruginosa*, as measured by the BCA assay. To quantify differences in OMV release based on lipids, the fluorescent FM4-64 membrane dye was used **(Fig. 2**, top**)**. A significant increase in OMV release of all bacteria upon heat treatment was observed, as well as an increase in OMV release upon treatment with 2.5 µM of PMAP-36 and CATH-2 for *E. coli* and *B. bronchiseptica*. However, for *P. aeruginosa*, only treatment with 2.5 µM of PMAP-36 resulted in an increase of OMV release, indicating that the effect of CATH-2 might be bacteria-specific.

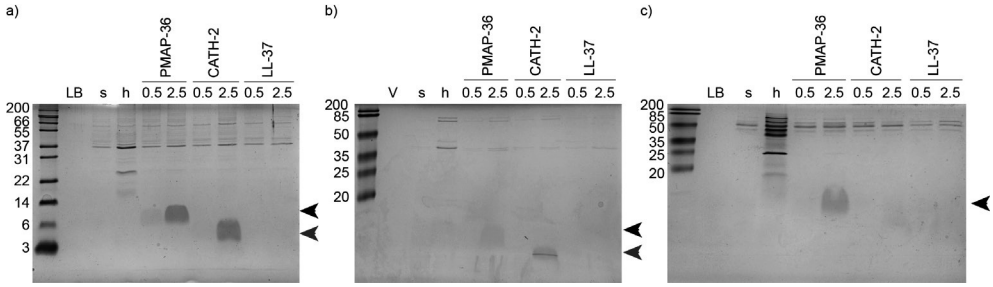


Figure 1: Coomassie stained SDS-PAGE of isolated OMVs. *OMVs were induced by HDPs and isolated from (a) E. coli, (b) B. bronchiseptica and (c) P. aeruginosa. Heat was applied as stress control. LB/V = Lysogeny Broth/Verwey medium, s = sOMVs, h = hOMVs, 0.5 = 0.5 μM and 2.5 = 2.5 μM of the corresponding peptide. Black and red arrow point to PMAP-36 and CATH-2 respectively. Shown is a representative image of three experiments.*

Since HDPs are membrane active and the intercalation of FM4-64 into the membrane might be influenced by the presence of peptides, an orthogonal technique was used to support the FM4-64 quantifications. Therefore, DLS was also used to estimate the number of OMV particles **(Fig. 2,** bottom**)**. Since individual particles can be counted several times by this technique, it will not result in an absolute number, but relative outcomes can still be compared. The particle counts of the DLS overall corresponded to the results of the FM4-64 quantification, although in this assay not all differences reached statistical significance.

When comparing minimal bactericidal concentrations of the peptides used in this study, it was found that for *B. bronchiseptica* MBCs of PMAP-36 and CATH-2 were similar, 0.25 μM and 0.5 μM respectively. For *E. coli* MBCs of PMAP-36 and CATH-2 were 1.25 μM and 5 μM respectively, only 4-fold different. However, for *P. aeruginosa*, the MBC of CATH-2 (20 μM), was 16-fold higher than the MBC of PMAP-36 (1.25 μM), possibly explaining the lack of OMV induction by CATH-2 for this bacterial specie **(Table 2)**. MBCs for LL-37 were consistently higher, possibly related to the lack of OMV induction by this peptide. Therefore higher, but still sublethal, concentrations of LL-37 were tested for OMV induction of *E. coli* and *P. aeruginosa* **(Fig. S2)**. Neither 5 nor 10 μM of LL-37 was able to induce any OMVs for both species tested, suggesting that even at higher concentrations OMVs are not used as defense against LL-37.

Table 2: MBC values of PMAP-36, CATH-2 and LL-37 for E. coli, B. bronchiseptica and P. aeruginosa. *Concentrations were determined with track dilution assays and depicted in μM. Values for E. coli and P. aeruginosa were determined in LB, values for B. bronchiseptica in Verwey medium.*

	E. coli ATCC 25922	*B. bronchiseptica*	*P. aeruginosa PAO1*
PMAP-36	*1.25*	*0.25*	*1.25*
CATH-2	*5*	*0.5*	*20*
LL-37	*10*	*1.25*	*>40*

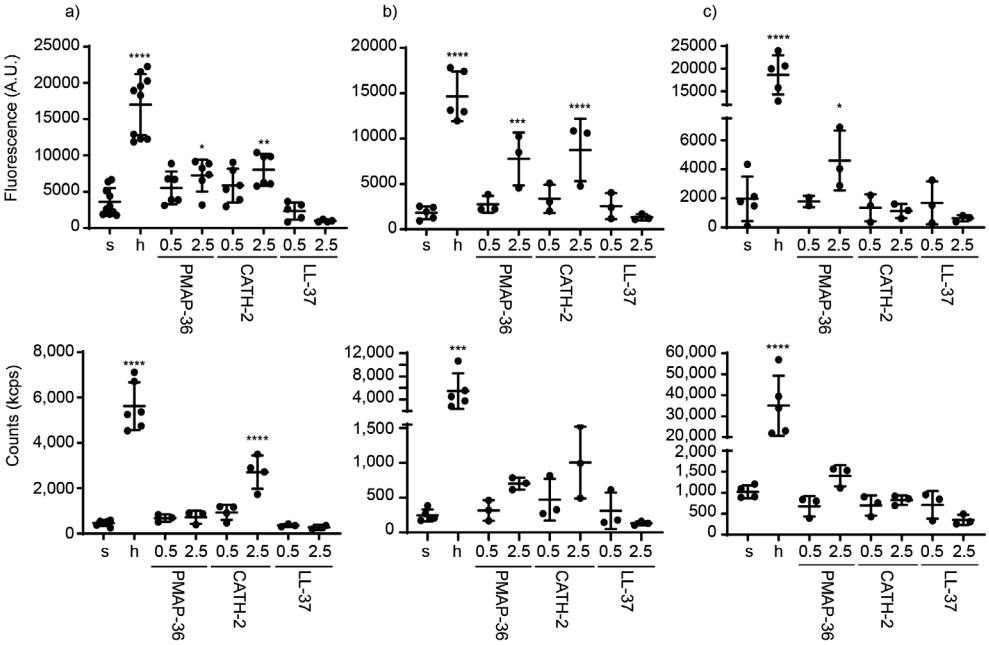


Figure 2: Quantification of isolated OMVs. *OMVs were induced by HDPs and isolated from (a) E. coli, (b) B. bronchiseptica and (c) P. aeruginosa. TOP: FM4-64 lipid quantification of isolated OMVs. BOTTOM: particle count of isolated OMVs using DLS. Heat was applied as stress control. Results were corrected for medium signal. s = sOMVs, h = hOMVs, 0.5 = 0.5 μM and 2.5 = 2.5 μM of the corresponding peptide. Statistical analysis was performed using a linear mixed-model with post-hoc Dunnett (n=3-9). *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.*

CATH-2 and PMAP-36, but not LL-37, are present in the isolated OMVs

To confirm the presence of HDPs in the OMVs, these were investigated on western blot and stained with the corresponding antibody **(Fig. 3)**. This indeed confirmed that the low molecular weight patches observed before on Coomassie-stained SDS-PAGE corresponded to PMAP-36 and CATH-2. No LL-37 was detected in the OMV fraction, which is in line with the absence of peptide on Coomassie stained SDS-PAGE. If bacteria utilize OMV release as means to dispose of HDP-affected membrane, one would expect that OMVs would be enriched in HDPs compared to bacterial membranes. Therefore, the presence of HDPs was also investigated in the bacterial pellet, separated from the OMVs with centrifugation, after HDP treatment. To analyze this, equal ratios of the bacterial pellet and isolated OMV fraction was loaded. This allows for comparison between bacterial cell pellet and OMV fraction of corresponding samples. Analysis between corresponding samples showed that PMAP-36 is preferentially found in the OMV fraction for *E. coli*, roughly equally distributed between OMVs and bacterial pellet for *B. bronchiseptica* and preferentially found in the bacterial pellet for *P. aeruginosa* **(Fig. 3a)**. CATH-2 was found equally in the bacterial pellet and OMV fraction for *E. coli*, but preferentially in the bacterial

pellet for *B. bronchiseptica* and *P. aeruginosa* **(Fig. 3b)**. This already shows differences between the two peptides. Remarkably, LL-37 was found in the OMV fraction of *E. coli*, but not in the bacterial pellet. It was not detected in the bacterial pellet or in the OMV fraction of *B. bronchiseptica* and *P. aeruginosa* **(Fig. 3c)**. This could indicate that the peptide mainly resided in the OMV supernatant after ultracentrifugation, but this showed no peptide either (data not shown). These results clearly suggest that bacterial species have different defense mechanisms towards HDPs.

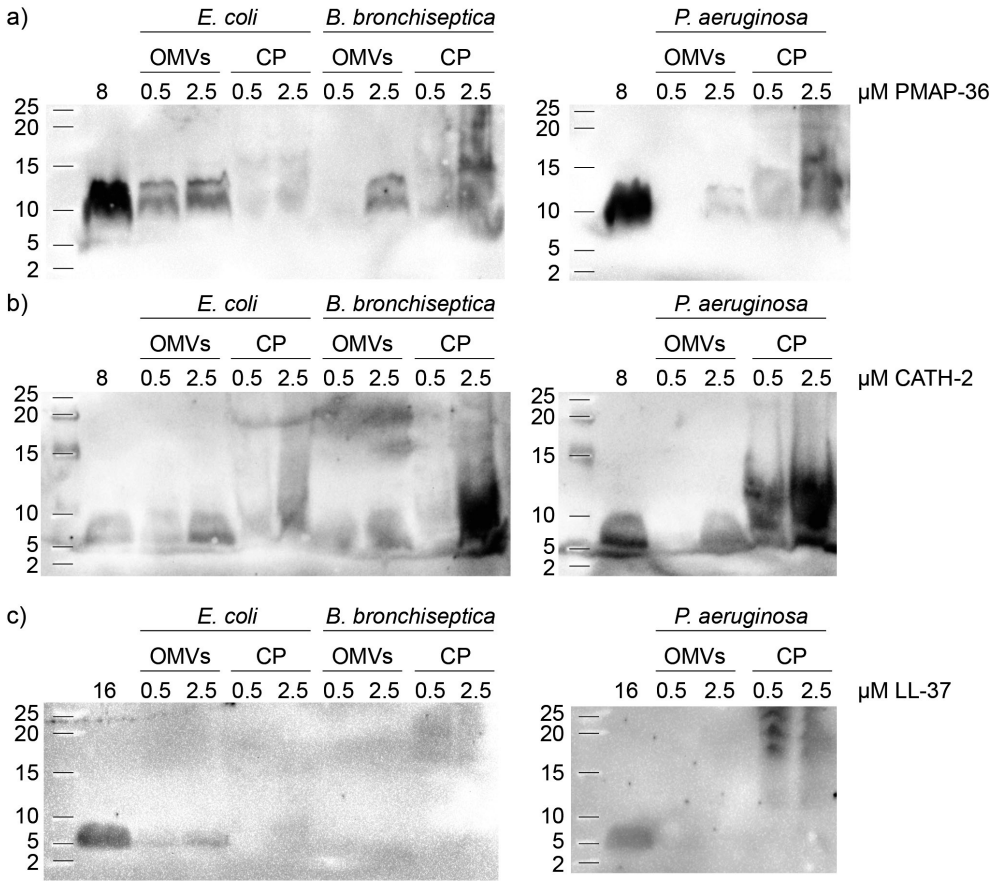


Figure 3: Western blot analysis of isolated OMVs and bacterial cell pellet (CP), stained for (a) PMAP-36, (b) CATH-2 and (c) LL-37. *OMVs were induced by two concentrations of HDPs (0.5 and 2.5 µM) and isolated from E. coli, B. bronchiseptica and P. aeruginosa and bacterial cell pellets (CP) were collected during isolation. Equal volumes of OMV and CP fraction were loaded to compare HDP presence. Synthetic peptide (8 µM for PMAP-36 and CATH-2, 16 µM for LL-37) was loaded as positive control.*

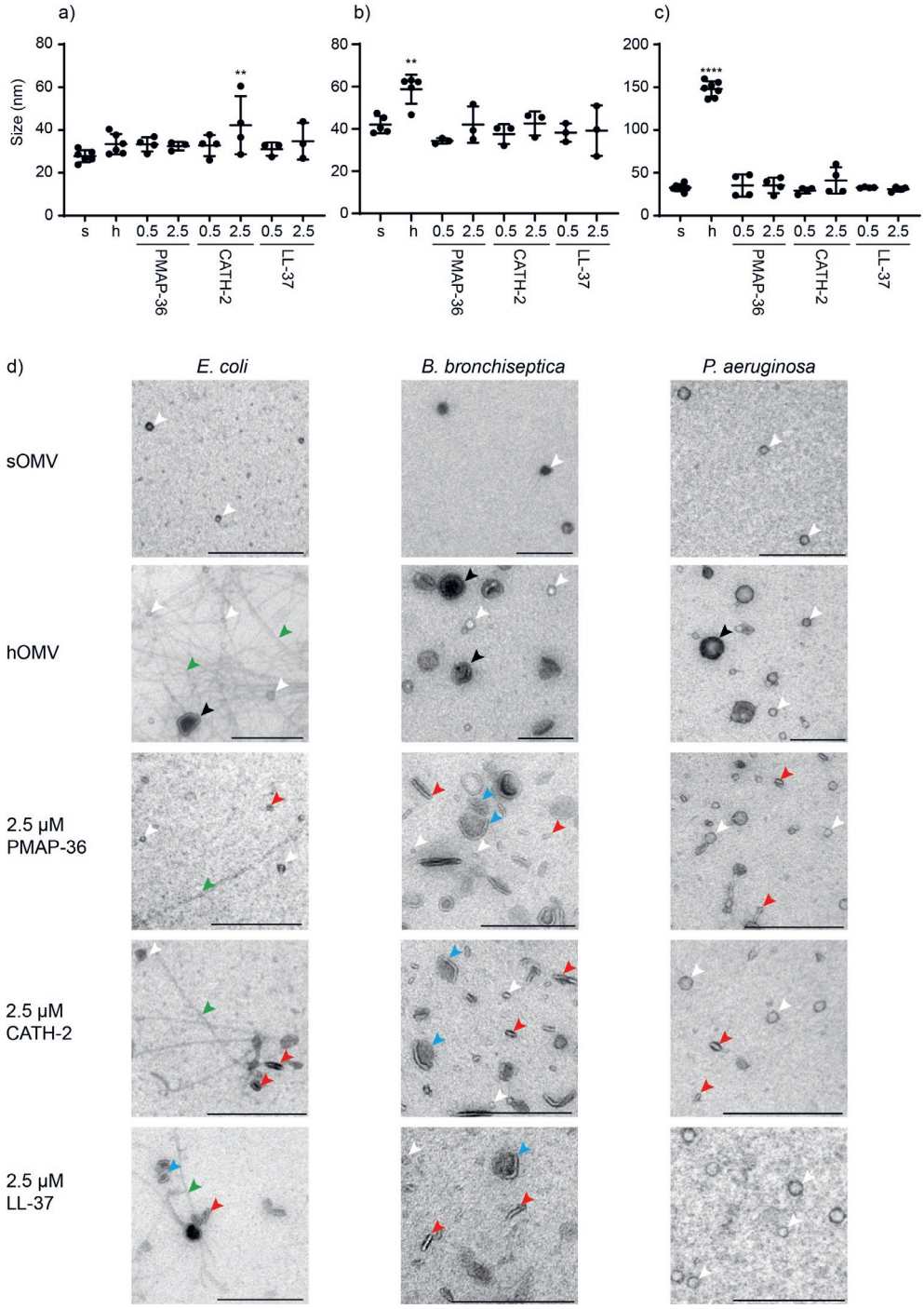
HDP induced OMVs differ in morphology

The effect of HDP-treatment of bacteria on the resulting pOMVs size was determined using DLS. This revealed that spontaneous, as well as HDP-induced OMVs had a diameter of approximately 30-40 nm, for all three Gram-negative bacteria **(Fig. 4a-c)**. However, heat treatment affected the size of resulting hOMVs, but not to the same extent for all bacteria. For *B. bronchiseptica*, hOMVs of 60 nm were measured, while hOMVs of *E. coli* were similar in size to sOMVs. However, a very large effect was observed for *P. aeruginosa*, where hOMVs were found to have an average diameter of 150 nm.

In order to assess morphology and integrity OMVs were visualized using EM **(Fig. 4d, Fig. S3)**. This revealed that OMVs were indeed quite small, in concordance with the DLS results **(Fig. 4d**, white arrows**)**. It also showed that heat treatment affects OMV appearance, with larger and darker OMVs present **(Fig. 4d**, black arrows**)**. Peptide treatment did not affect OMV size, but did affect morphology as observed by EM. OMVs induced by all three HDPs revealed disc-like shapes **(Fig. 4d**, red arrows**)**. The association of cargo, represented by darker patches along these disc-like OMVs, suggests that the OMVs have split open after release from the bacterium **(Fig. 4d**, blue arrows**)**. Remarkably, not only differences in OMV morphology were observed for *E. coli* upon treatment, also flagellae were observed **(Fig. 4d**, green arrows**)**. These were mostly observed after heat treatment of *E. coli*, but also after peptide treatment. For *B. bronchiseptica* and *P. aeruginosa* flagellae were occasionally observed after heat treatment, but not to the same extent as for *E. coli* (data not shown). For *P. aeruginosa* smaller fragments of flagellae were also observed, possibly interfering with the DLS size measurement and explaining the large increase in diameter upon heat treatment.

To further investigate OMV characteristics, lipidomic analysis was performed using mass-spectrometry. In this analysis not only phospholipids were measured, but also ornithine lipids for *B. bronchiseptica* as these were described before to be present in OMVs [23]. OMVs of all three different bacterial species consisted mainly of phosphatidyl-ethanolamine (PE) and phosphatidylglycerol (PG). Slight differences between species were observed, such as relatively more acyl-phosphatidylglycerol (aPG) in *P. aeruginosa* compared to *E. coli* and *B. bronchiseptica* **(Table S1)**.

Figure 4: Morphological properties of isolated OMVs. *OMVs were induced by HDPs and isolated from (a) E. coli, (b) B. bronchiseptica and (c) P. aeruginosa. Heat was applied as stress control. (a-c) Size determination by DLS. s = sOMVs, h = hOMVs, 0.5 = 0.5 μM and 2.5 = 2.5 μM of the corresponding peptide. Statistical analysis was performed using a linear mixed-model with post-hoc Dunnett (n=3-6). **=p<0.01, ****=p<0.0001. (d) Electron microscopy. White arrows point to sOMVs, black arrows to larger hOMVs. Red arrows show disc-like pOMVs and blue arrows show pOMVs that have split open and still have cargo associated. Green arrows show flagellae. Scale bars represent 200 nm. Shown is a representative image of three experiments.*


a)
b)
c)
Size (nm)
s
h
0.5
2.5
PMAP-36
CATH-2
LL-37
d)
E. coli
B. bronchiseptica
P. aeruginosa
sOMV
hOMV
2.5 µM PMAP-36
2.5 µM CATH-2
2.5 µM LL-37

When peptide- and heat induced OMVs were compared to spontaneous OMVs several differences in lipid composition were observed. A relative increase in negatively charged PG occurred in OMVs induced with 2.5 µM PMAP-36 and 2.5 µM CATH-2, but not 2.5 µM LL-37, supporting the observation that LL-37 does not induce OMV release **(Fig. 5)**. Furthermore, OMVs from *P. aeruginosa* induced with 2.5 µM CATH-2 do not display an increase in PG lipids, confirming earlier results where CATH-2 was unable to induce *P. aeruginosa* OMVs **(Fig. 5c)**. A relative increase of lysophospholipids was found in OMVs of all three bacterial species upon heat treatment [23].

OMVs protect bacteria from HDPs

To investigate whether the release of OMVs in response to peptide treatment is indeed a means of the bacterium to defend itself, track dilution assays were performed. Two sets of *E. coli* hypervesiculating mutants were investigated, one with a deletion of the outer membrane protein OmpA, the other with a deletion of the lipoprotein Lpp, both important for outer membrane tethering to either the peptidoglycan or inner membrane. These genetically modified bacteria have an increased production of OMVs [29,30], which might protect them against HDPs, but the deletion may also influence membrane stability of the bacterium, which should be taken into consideration. Therefore, the supernatant of the hypervesiculating mutants and wild-type bacteria was used to investigate protective capabilities against HDP killing of the wild-type bacteria, to eliminate bacterial differences. This revealed a protective effect against CATH-2, PMAP-36 and LL-37 **(Fig S4)**.

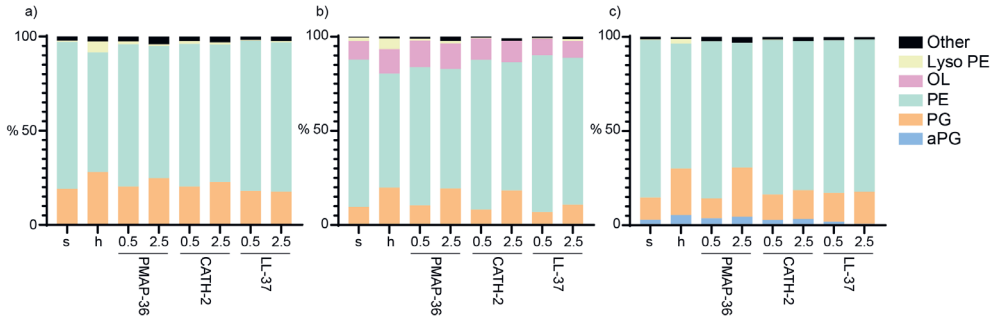


Figure 5: Lipidomic analysis of isolated OMVs from (a) E. coli, (b) B. bronchiseptica and (c) P. aeruginosa induced by no treatment (s), heat treatment (h) or different concentrations (0.5 or 2.5 µM) of indicated HDPs. *The main OMV components are phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). Ornithine lipid (OL) was only found in B. bronchiseptica. aPG = Acyl-Phosphatidylglycerol. Other include phosphatidic acid (PA), phosphatidylserine (PS), dilyso-cardiolipin (DLCL) and lipid groups <2%.*

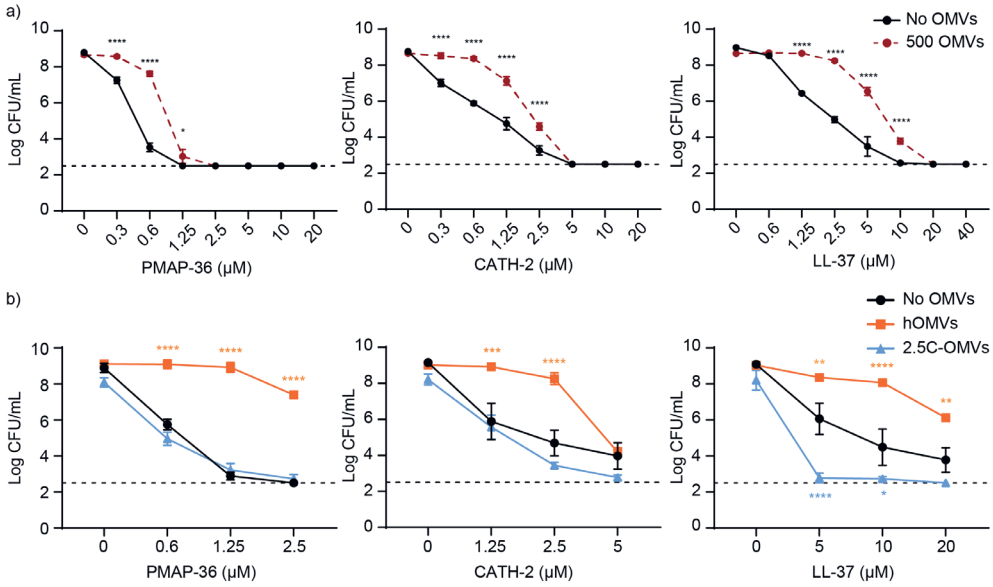


Figure 6: Assessment of killing of E. coli by HDPs with and without the addition of external (a) sOMVs, (b) hOMVs or OMVs induced by 2.5 μM CATH-2 (2.5C-OMVs). *OMVs from E. coli were isolated and added to the bacteria-HDP mixture during incubation. In the control, an equal volume of Tris buffer was added to the bacteria-HDP mixture. (a) Shown is the mean of four independent experiments with SEM. Statistical analysis was performed for each peptide concentration using a two-way ANOVA with post-hoc Sidak. *=p<0.05, ****=p<0.0001. (b) Shown is the mean of three independent experiments with SEM. Statistical analysis was performed compared to the control without OMVs added using a two-way ANOVA with post-hoc Dunnet. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.*

Since the supernatant contains several components, also isolated *E. coli* sOMVs were added during track dilution assays to investigate the potential protective effect of OMVs against the antibacterial activity of HDPs. The concentration of sOMVs added in these track dilution assays was equated with the amount of sOMVs isolated after two hours of logarithmic growth of *E. coli*, being 500 A.U. as defined using the FM4-64 lipid dye. Addition of isolated sOMVs during the incubation of HDPs with bacteria in the track dilution assay resulted in a protective effect of sOMVs against CATH-2, PMAP-36 and LL-37 killing **(Fig. 6a)**. This suggests that the mere presence of outer membrane vesicles can act as a decoy for HDPs. Additionally, the protective effect of hOMVs and OMVs induced by 2.5 μM CATH-2 (2.5C-OMVs) were investigated, since these conditions significantly increased OMV release. This revealed that hOMVs also protected *E. coli* against killing by all three peptides, even more than sOMVs for PMAP-36. However, 2.5C-OMVs did not protect *E. coli* and even enhanced killing by LL-37 **(Fig. 6b)**.

Discussion

In this study, the Gram-negative bacteria *E. coli*, *B. bronchiseptica* and *P. aeruginosa* were exposed to sub-lethal concentrations of the HDPs PMAP-36, CATH-2 and LL-37 and OMV release was studied to determine the role of OMVs as a defense mechanism against these HDPs. Furthermore, it was assessed whether an increase in OMVs in the culture medium could protect *E. coli* from HDP killing. Treatment with either PMAP-36 or CATH-2 induced OMV release but treatment with LL-37 did not have this effect **(Fig. 2)**. Remarkably, CATH-2 was only effective for *E. coli* and a clinical isolate of *B. bronchiseptica*, but not *P. aeruginosa*. It would be interesting to investigate the OMV induction by HDPs further for more clinically relevant bacterial species and strains. Experiments were performed in rich media to exclude other environmental factors affecting OMV release and observed effects were solely caused by heat or HDP treatment.

Peptide-dependent differences in OMV release may be partially explained by their different antimicrobial mechanisms. For PMAP-36 and CATH-2 it was shown that they both interact strongly with LPS of *E. coli*, in a biphasic manner. LL-37 only interacts weakly with LPS, in a monophasic manner [4]. Furthermore, electron micrographs showed clustered DNA and ribosomes for LL-37, demonstrating an intracellular-active mechanism, while CATH-2 localized intracellularly at sub-lethal concentrations but disrupted membranes at lethal concentrations, demonstrating a membrane-active mechanism [4,6]. PMAP-36 was shown to disrupt membranes [7,31], but also showed clustered DNA and ribosomes in electron microscopic analysis of *E. coli* [4], suggesting a combination of membrane and intracellular targets for PMAP-36. This shows that the three HDPs have different antibacterial mechanisms of action and may explain why LL-37 does not induce OMV release, even at higher concentrations. Since LL-37 targets intracellular processes and is not localized to the membrane, the bacterium does not require to dispose of membrane in the form of an OMV. This suggests that OMV release might be a means of the bacterium to dispose of membrane affected by peptide.

Western blot analysis of bacterial pellets showed PMAP-36 and CATH-2 present in both the OMV fraction and the bacterial cell pellet, but LL-37 was not present in most samples, except for the OMV pellet of *E. coli* **(Fig. 3)**. The distribution was different per bacteria, where *P. aeruginosa* and *B. bronchiseptica* both contained mostly peptide in the bacterial cell pellet and *E. coli* contained mostly peptide in the OMVs. This suggests that PMAP-36 and CATH-2 can be neutralized by incorporation into OMVs but that this defense mechanism may differ between bacterial species. Perhaps some species rely more on other defense mechanisms instead of elimination of HDPs by OMVs. LL-37 could barely be detected in the bacterial cell pellet, OMV fraction or supernatant, suggesting either concentrations are too low to be detected on western blot, for example by breakdown of

the peptide, or the antibody is not powerful enough. Only a low signal was detected in the OMVs of *E. coli* induced with 2.5 μM LL-37. The positive control for LL-37 had to be doubled in concentration, being 16 μM instead of 8 μM, to be properly detected by the antibody used, suggesting a higher detection limit. Therefore, western blot detection of LL-37 with this antibody might not be conclusive.

A difference was also observed in the bacterial response to CATH-2 specifically. When MBC values were investigated it was found that the MBC of CATH-2 for *P. aeruginosa* was 16-fold higher than that of PMAP-36, while for *E. coli* and *B. bronchiseptica* MBCs for CATH-2 and PMAP-36 were only 2- or 4-fold different. Perhaps CATH-2 fails to induce OMVs in *P. aeruginosa* simply because the concentration used is too low. A similar mechanism could be at play for LL-37.

Still the question remains why the MBC of CATH-2 is so much higher for *P. aeruginosa* compared to *E. coli* and *B. bronchiseptica*. The first molecule CATH-2 will encounter in all of these bacteria is LPS, which differs per bacterium. *B. bronchiseptica* LPS is 90% penta-acylated, and 10% hexa-acylated, with glucosamine groups attached to the phosphates [32]. Similarly, *P. aeruginosa* PAO1 LPS molecules are 75% penta-acylated, and 25% hexa-acylated [33]. On the other hand, *E. coli* ATCC 25922 LPS is fully hexa-acylated [34,35]. All LPS structures contain an O-antigen [36–39] and are comparable in Lipid A composition, but perhaps the differences in the core and O-antigen will influence binding by CATH-2 and its effectiveness. This could be investigated in the future by affinity studies.

When OMV characteristics were assessed, it was shown that heat treatment significantly influenced OMV size for *B. bronchiseptica* and *P. aeruginosa* **(Fig. 4b-c)**. It was also observed that heat treatment influenced the lipid classes present in the hOMVs, which was already described for *B. bronchiseptica* [23]. An increase in lysophospholipids was observed in OMVs of all species **(Fig. 5)**, although less pronounced in *P. aeruginosa*. This could be due to the lack of outer membrane phospholipase A (pldA) in *P. aeruginosa*, which was implicated in *B. bronchiseptica* to cause the increase of lysophospholipids in hOMVs [23]. Protein BLAST searches using the sequence of *E. coli* pldA as a query did not reveal the presence of a pldA homolog in *P. aeruginosa* PAO1. These lysophospholipids contain only one fatty acid tail and therefore induce a positive curvature in the membrane [40].

The presence of many flagella-like structures was observed in the electron microscopic graphs of isolated OMVs. In literature, *P. aeruginosa* is described as monotrichious, while *E. coli* and *B. bronchiseptica* are described as peritrichous [41,42]. However, *Bordetellae* flagellar synthesis is regulated by the Bvg-regulon and is decreased at growth temperatures of 37°C and above [43]. This was consistent with number of flagellar structures observed, where the most were observed for *E. coli* **(Fig 4d**, green arrows**)**. It was

shown that this elevated temperature alters the OMV composition by an increase of lysophospholipids **(Fig. 5)** and thereby may also affect bacterial membrane fluidity and proper attachment of flagellae. These flagellae are connected to the outer membrane by the L-ring and placement is thought to be regulated by marker proteins that interact with phospholipids [41]. However, the effect of phospholipid composition on flagellar connection is currently unknown. For *E. coli*, flagellae were also observed in samples treated with higher peptide concentrations. HDPs could disrupt membrane integrity, in the case of PMAP-36 or CATH-2, or perhaps interfere with translation of flagellar protein components, in the case of LL-37 [4] and thereby prevent proper flagella attachment or cause flagella to detach.

Furthermore, the lipidome and morphology of peptide-induced OMVs was altered. pOMVs obtained a disc-like morphology, which was especially pronounced for *B. bronchiseptica* **(Fig. 4d**, red arrows**)**. Additionally, OMVs induced with 2.5 μM PMAP-36 or CATH-2 showed a relative increase in negatively charged PG lipids, possibly due to a preferred interaction with the positively charged peptides **(Fig. 5)**. In accordance with the quantifications, this effect was not observed for 2.5 μM CATH-2 in *P. aeruginosa*. However, an increase in PG lipids would not explain the observed disc-like morphology of pOMVs, indicating that other mechanisms are at play.

The association of darker patches along these disc-like pOMVs suggest they may have split open after formation and still have cargo associated **(Fig. 4d**, blue arrows**)**. However, not all disc-like pOMVs have darker patches associated with them, which may indicate that these discs have been poked out of the outer membrane of the bacterium directly. This would resemble the mechanism described for nanodisc-formation. Nanodiscs are small phospholipid bilayer discs encircled by an amphipathic scaffold protein [44]. HDPs are amphipathic peptides and could function as nanodisc scaffold protein. Natural lipoproteins, like apolipoprotein J, involved in lipid and cholesterol transport were previously shown to be able to form nanodiscs [45]. A different explanation for the presence of these disc-like pOMVs could be that pOMVs have been compressed during sample preparation, due to decreased stability because of the HDPs present in the pOMVs. Whether this is a true phenomenon or a sample preparation artefact has to be investigated using orthogonal imaging techniques, but this will be difficult due to the small size of these OMVs. However, when 4 μM PMAP-36 was added to isolated *B. bronchiseptica* sOMVs, this did not lead to altered morphology or decreased stability (data not shown), suggesting the phenomenon occurs during bacterial stimulation.

When assessing the protective effect of OMVs against the bactericidal action of HDPs, two sets of different hypervesiculating mutants were utilized. Initially these were tested in HDP killing assays, but no differences were observed (data not shown). This was presumably

due to decreased membrane stability in the mutant counteracting the protective effect of increased OMV release. Therefore, the assays were performed using wildtype bacteria diluted in supernatant, containing sOMVs, of wildtype or hypervesiculating mutant *E.coli*. This indeed showed a protective effect of the sOMVs in the supernatant of the hypervesiculating mutants **(Fig. S3)**. Albeit not quantified, SDS-PAGE did suggest that deletion of *lpp* had a larger increase in OMV formation than deletion of o*mpA* (data not shown). This can explain the larger protective effect observed in the *Δlpp* mutant compared to the *ΔompA* mutant **(Fig. S3)**.

Addition of isolated sOMVs or hOMVs also protected *E. coli* from killing by PMAP-36, CATH-2 and LL-37. However, 2.5C-OMVs did not protect *E. coli* and even enhanced killing by LL-37. Potentially this is caused by pore formation due to the CATH-2 that is present in the OMVs, and apparently still active, which enables easier access for LL-37. Interestingly, addition of sOMVs protected the bacteria against LL-37 **(Fig. 6a)**, but bacteria did not produce OMVs in response to LL-37 even at higher concentrations **(Fig. 2, Fig. S2)**. The rationale suggests that, since LL-37 targets intracellular processes, the bacterium does not need to dispose of large quantities of membrane since that is not where most of the peptide is localized. However, some LL-37 was detected in OMVs of E. coli induced with 2.5 μM LL-37 **(Fig. 3)**. Perhaps lower amounts of OMVs can be sufficient to dispose of all the LL-37 in the membrane. However, to reach the intracellular target, LL-37 needs to traverse through the membrane, possibly by interacting and diffusing through it, since it was shown to interact with membranes [3]. Therefore, the addition of external membranes, in the form of OMVs, might slow the entry of LL-37 into bacteria, since it will interact with both OMVs and bacterial membranes. A similar principle of protection by OMV was observed for bacteriophages where OMV were shown by EM to interact with these bacteriophages and thereby protect the bacterial culture against killing by these viruses [20]. OMVs can not only act as a physical barrier, but perhaps also contain proteases or other factors that decrease HDP function and enhance bacterial survival. Membrane vesicles from *Streptococcus suis* for instance were shown to contain a serine protease [46]. However, in the OMVs induced with HDPs intact peptide was observed on western blot **(Fig. 3)**, suggesting the peptide induced OMVs of *B. bronchiseptica* do not contain proteases.

Concluding remarks

Altogether, these data show OMVs as a possible defense mechanism against membrane-active antibacterial compounds. We hypothesize that bacteria try to dispose of membranes affected by membrane-active antibacterial compounds in the form of an OMV. This mechanism was even found in a clinically isolated *B. bronchiseptica*, suggesting this process is relevant *in vivo.* This does suggest that an anti-vesiculation drug will increase effectiveness of membrane-active antibacterial compounds. Furthermore, previous

studies have shown that non-vesiculating mutants are often lethal, suggesting that OMV formation is an essential process for bacteria [47] and an interesting drug target.

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References

[1] L.E. Reddick, N.M. Alto, Bacteria fighting back: How pathogens target and subvert the host innate immune system, Mol. Cell. 54 (2014) 321–328. doi:10.1016/j.molcel.2014.03.010.

[2] L. Zhang, R.L. Gallo, Antimicrobial Peptides, Springer Singapore, Singapore, 2019. doi:10.1007/978-981-13-3588-4.

[3] D. Xhindoli, S. Pacor, M. Benincasa, M. Scocchi, R. Gennaro, A. Tossi, The human cathelicidin LL-37 - A pore-forming antibacterial peptide and host-cell modulator, Biochim. Biophys. Acta - Biomembr. 1858 (2016) 546–566. doi:10.1016/j.bbamem.2015.11.003.

[4] M.R. Scheenstra, M. van den Belt, J.L.M. Tjeerdsma-van Bokhoven, V.A.F. Schneider, S.R. Ordonez, A. van Dijk, E.J.A. Veldhuizen, H.P. Haagsman, Cathelicidins PMAP-36, LL-37 and CATH-2 are similar peptides with different modes of action, Sci. Rep. 9 (2019) 4780. doi:10.1038/s41598-019-41246-6.

[5] D. Vandamme, B. Landuyt, W. Luyten, L. Schoofs, A comprehensive summary of LL-37, the factoctum human cathelicidin peptide, Cell. Immunol. 280 (2012) 22–35. doi:10.1016/j.cellimm.2012.11.009.

[6] V.A.F. Schneider, M. Coorens, S.R. Ordonez, J.L.M. Tjeerdsma-Van Bokhoven, G. Posthuma, A. Van Dijk, H.P. Haagsman, E.J.A. Veldhuizen, Imaging the antimicrobial mechanism(s) of cathelicidin-2, Sci. Rep. 6 (2016) 1–11. doi:10.1038/srep32948.

[7] M. Scocchi, I. Zelezetsky, M. Benincasa, R. Gennaro, A. Mazzoli, A. Tossi, Structural aspects and biological properties of the cathelicidin PMAP-36, FEBS J. 272 (2005) 4398–4406. doi:10.1111/j.1742-4658.2005.04852.x.

[8] K.A. Brogden, Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria?, Nat. Rev. Microbiol. 3 (2005) 238–250. doi:10.1038/nrmicro1098.

[9] H.-S. Joo, C.-I. Fu, M. Otto, Bacterial strategies of resistance to antimicrobial peptides, Philos. Trans. R. Soc. B Biol. Sci. 371 (2016) 20150292. doi:10.1098/rstb.2015.0292.

[10] D.I. Andersson, D. Hughes, J.Z. Kubicek-Sutherland, Mechanisms and consequences of bacterial resistance to antimicrobial peptides, Drug Resist. Updat. 26 (2016) 43–57. doi:10.1016/j.drup.2016.04.002.

[11] B. Bechinger, S.U. Gorr, Antimicrobial Peptides: Mechanisms of Action and Resistance, J. Dent. Res. 96 (2017) 254–260. doi:10.1177/0022034516679973.

[12] A. Peschel, H.G. Sahl, The co-evolution of host cationic antimicrobial peptides

and microbial resistance, Nat. Rev. Microbiol. 4 (2006) 529–536. doi:10.1038/nrmicro1441.

[13] N. Mookherjee, M.A. Anderson, H.P. Haagsman, D.J. Davidson, Antimicrobial host defence peptides: functions and clinical potential, Nat. Rev. Drug Discov. 19 (2020) 311–332. doi:10.1038/s41573-019-0058-8.

[14] X. Lauth, M. von Köckritz-Blickwede, C.W. McNamara, S. Myskowski, A.S. Zinkernagel, B. Beall, P. Ghosh, R.L. Gallo, V. Nizet, M1 Protein Allows Group A Streptococcal Survival in Phagocyte Extracellular Traps through Cathelicidin Inhibition, J. Innate Immun. 1 (2009) 202–214. doi:10.1159/000203645.

[15] T. Guina, E.C. Yi, H. Wang, M. Hackett, S.I. Miller, A PhoP-regulated outer membrane protease of Salmonella enterica serovar typhimurium promotes resistance to alpha-helical antimicrobial peptides., J. Bacteriol. 182 (2000) 4077–86. doi:10.1128/jb.182.14.4077-4086.2000.

[16] T. Stobernack, M. du T. Espina, L.M. Mulder, L.M.P. Medina, D.R. Piebenga, G. Gabarrini, X. Zhao, K.M.J. Janssen, J. Hulzebos, E. Brouwer, T. Sura, D. Becher, A.J. van Winkelhoff, F. Götz, A. Otto, J. Westra, J.M. van Dijl, A secreted bacterial peptidylarginine deiminase can neutralize human innate immune defenses, MBio. 9 (2018) 1–15. doi:10.1128/mbio.01704-18.

[17] E.C. Woods, A.N. Edwards, K.O. Childress, J.B. Jones, S.M. McBride, The C. difficile clnRAB operon initiates adaptations to the host environment in response to LL-37, PLoS Pathog. 14 (2018) e1007153. doi:10.1371/journal.ppat.1007153.

[18] C.M. Ernst, P. Staubitz, N.N. Mishra, S.-J. Yang, G. Hornig, H. Kalbacher, A.S. Bayer, D. Kraus, A. Peschel, The Bacterial Defensin Resistance Protein MprF Consists of Separable Domains for Lipid Lysinylation and Antimicrobial Peptide Repulsion, PLoS Pathog. 5 (2009) e1000660. doi:10.1371/journal.ppat.1000660.

[19] M.D. Balhuizen, E.J.A. Veldhuizen, H.P. Haagsman, Outer Membrane Vesicle Induction and Isolation for Vaccine Development, Front. Microbiol. 12 (2021) 629090. doi:10.3389/fmicb.2021.629090.

[20] A.J. Manning, M.J. Kuehn, Contribution of bacterial outer membrane vesicles to innate bacterial defense, BMC Microbiol. 11 (2011) 1–14. doi:10.1186/1471-2180-11-258.

[21] B.O. Murray, R.A. Dawson, L.M. Alsharaf, J. Anne Winter, Protective effects of Helicobacter pylori membrane vesicles against stress and antimicrobial agents, Microbiology. 166 (2020) 751–758. doi:10.1099/mic.0.000934.

[22] W.F. Verwey, E.H. Thiele, D.N. Sage, L.F. Schuchardt, A simplified liquid culture medium for the growth of Hemophilus pertussis, J. Bacteriol. 58 (1949) 127–134. doi:10.1128/jb.58.2.127-134.1949.

[23] M.D. Balhuizen, C.M. Versluis, R.M. van Harten, E.F. de Jonge, J.F. Brouwers, C.H.A. van de Lest, E.J.A. Veldhuizen, J. Tommassen, H.P. Haagsman, PMAP-36 reduces the innate immune response induced by Bordetella bronchiseptica-derived outer membrane vesicles, Curr. Res. Microb. Sci. 2 (2021) 100010. doi:10.1016/j.crmicr.2020.100010.

[24] Biorad, General tips for handcasting, (2014). http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6201.pdf (accessed May 1, 2019).

[25] A. van Dijk, M.H.G. Tersteeg-Zijderveld, J.L.M. Tjeerdsma-van Bokhoven, A.J.M. Jansman, E.J.A. Veldhuizen, H.P. Haagsman, Chicken heterophils are recruited to the site of Salmonella infection and release antibacterial mature Cathelicidin-2 upon stimulation with LPS, Mol. Immunol. 46 (2009) 1517–1526. doi:10.1016/j.molimm.2008.12.015.

[26] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification., Can. J. Biochem. Physiol. 37 (1959) 911–917. doi:10.1139/059-099.

[27] C.A. Smith, E.J. Want, G. O'Maille, R. Abagyan, G. Siuzdak, XCMS: Processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification, Anal. Chem. 78 (2006) 779–787. doi:10.1021/ac051437y.

[28] E.F. de Jonge, M.D. Balhuizen, R. van Boxtel, J. Wu, H.P. Haagsman, J. Tommassen, Heat shock enhances outer-membrane vesicle release in Bordetella spp., Curr. Res. Microb. Sci. 2 (2021) 100009. doi:10.1016/j.crmicr.2020.100009.

[29] I. Sonntag, H. Schwarz, Y. Hirota, U. Henning, Cell envelope and shape of Escherichia coli: multiple mutants missing the outer membrane lipoprotein and other major outer membrane proteins, J. Bacteriol. 136 (1978) 280–285. doi:10.1128/jb.136.1.280-285.1978.

[30] D.W. Yem, H.C. Wu, Physiological characterization of an Escherichia coli mutant altered in the structure of murein lipoprotein, J. Bacteriol. 133 (1978) 1419–1426. doi:10.1128/jb.133.3.1419-1426.1978.

[31] Y. Lv, J. Wang, H. Gao, Z. Wang, N. Dong, Q. Ma, A. Shan, Antimicrobial Properties and Membrane-Active Mechanism of a Potential α-Helical Antimicrobial Derived from Cathelicidin PMAP-36, PLoS One. 9 (2014) e86364. doi:10.1371/journal.pone.0086364.

[32] J. Pérez-Ortega, R.M. Van Harten, R. Van Boxtel, M. Plisnier, M. Louckx, D. Ingels, H.P. Haagsman, J. Tommassen, Reduction of endotoxicity in Bordetella bronchiseptica by lipid A engineering: Characterization of lpxL1 and pagP mutants, Virulence. 12 (2021) 1452–1468. doi:10.1080/21505594.2021.1929037.

[33] G. Bedoux, K. Vallée-Réhel, O. Kooistra, U. Zähringer, D. Haras, Lipid A components fromPseudomonas aeruginosa PAO1(serotype O5) and mutant strains investigated by electrospray ionization ion-trap mass spectrometry, J. Mass Spectrom. 39 (2004) 505–513. doi:10.1002/jms.611.

[34] C. Sohlenkamp, O. Geiger, Bacterial membrane lipids: Diversity in structures and pathways, FEMS Microbiol. Rev. 40 (2015) 133–159. doi:10.1093/femsre/fuv008.

[35] O. Holst, S. Muller-Loennies, B. Lindner, H. Brade, Chemical structure of the lipid A of Escherichia coli J-5, Eur. J. Biochem. 214 (1993) 695–701. doi:10.1111/j.1432-1033.1993.tb17970.x.

[36] A. Preston, B.O. Petersen, J. Duus, J. Kubler-Kielb, G. Ben-Menachem, J. Li, E. Vinogradov, Complete structures of Bordetella bronchiseptica and Bordetella parapertussis lipopolysaccharides, J. Biol. Chem. 281 (2006) 18135–18144. doi:10.1074/jbc.M513904200.

[37] M. Caroff, L. Aussel, H. Zarrouk, A. Martin, J.C. Richards, H. Thérisod, M.B. Perry, D. Karibian, Structural variability and originality of the Bordetella endotoxins, J. Endotoxin Res. 7 (2001) 63–68. doi:10.1179/096805101101532567.

[38] J.S. Lam, V.L. Taylor, S.T. Islam, Y. Hao, D. Kocíncová, Genetic and Functional Diversity of Pseudomonas aeruginosa Lipopolysaccharide, Front. Microbiol. 2 (2011). doi:10.3389/fmicb.2011.00118.

[39] R. Stenutz, A. Weintraub, G. Widmalm, The structures of Escherichia coli O-polysaccharide antigens, FEMS Microbiol. Rev. 30 (2006) 382–403. doi:10.1111/j.1574-6976.2006.00016.x.

[40] N. Fuller, R.P. Rand, The influence of lysolipids on the spontaneous curvature and bending elasticity of phospholipid membranes, Biophys. J. 81 (2001) 243–254. doi:10.1016/S0006-3495(01)75695-0.

[41] J.S. Schuhmacher, K.M. Thormann, G. Bange, How bacteria maintain location and number of flagella?, FEMS Microbiol. Rev. 39 (2015) 812–822. doi:10.1093/femsre/fuv034.

[42] G.W. Richter, Y. Kress, Electron microscopy of a strain of Bordetella bronchiseptica., J. Bacteriol. 94 (1967) 1216–1224. doi:10.1128/jb.94.4.1216-1224.1967.

[43] B.J. Akerley, D.M. Monack, S. Falkow, J.F. Miller, The bvgAS locus negatively controls motility and synthesis of flagella in Bordetella bronchiseptica, J. Bacteriol. 174 (1992) 980–990. doi:10.1128/jb.174.3.980-990.1992.

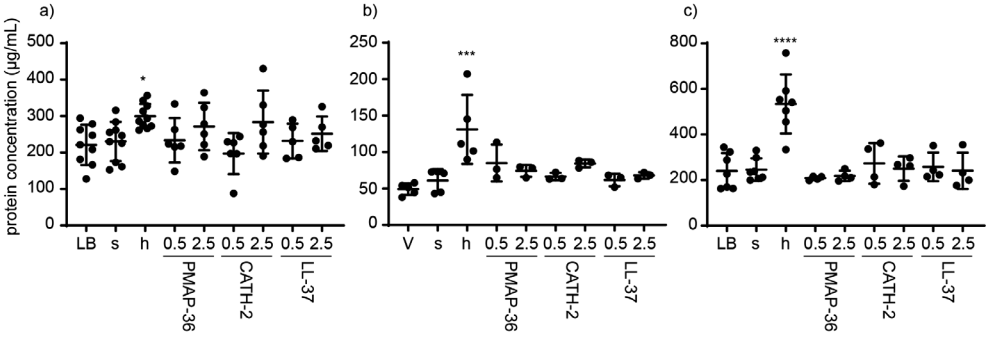
[44] I.G. Denisov, S.G. Sligar, Nanodiscs in Membrane Biochemistry and Biophysics, Chem. Rev. 117 (2017) 4669–4713. doi:10.1021/acs.chemrev.6b00690.

[45] S. Fernández-De-Retana, M. Cano-Sarabia, P. Marazuela, J.L. Sánchez-Quesada, A. Garcia-Leon, A. Montañola, J. Montaner, D. Maspoch, M. Hernández-Guillamon, Characterization of ApoJ-reconstituted high-density lipoprotein (rHDL) nanodisc for the potential treatment of cerebral β-amyloidosis, Sci. Rep. 7 (2017) 1–13. doi:10.1038/s41598-017-15215-w.

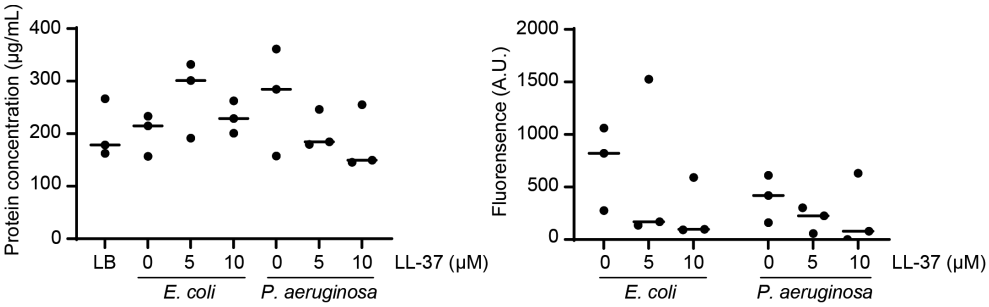
[46] B. Haas, D. Grenier, Isolation, characterization and biological properties of membrane vesicles produced by the swine pathogen Streptococcus suis, PLoS One. 10 (2015). doi:10.1371/journal.pone.0130528.

[47] A.J. McBroom, A.P. Johnson, S. Vemulapalli, M.J. Kuehn, Outer membrane vesicle production by Escherichia coli is independent of membrane instability, J. Bacteriol. 188 (2006) 5385–5392. doi:10.1128/JB.00498-06.

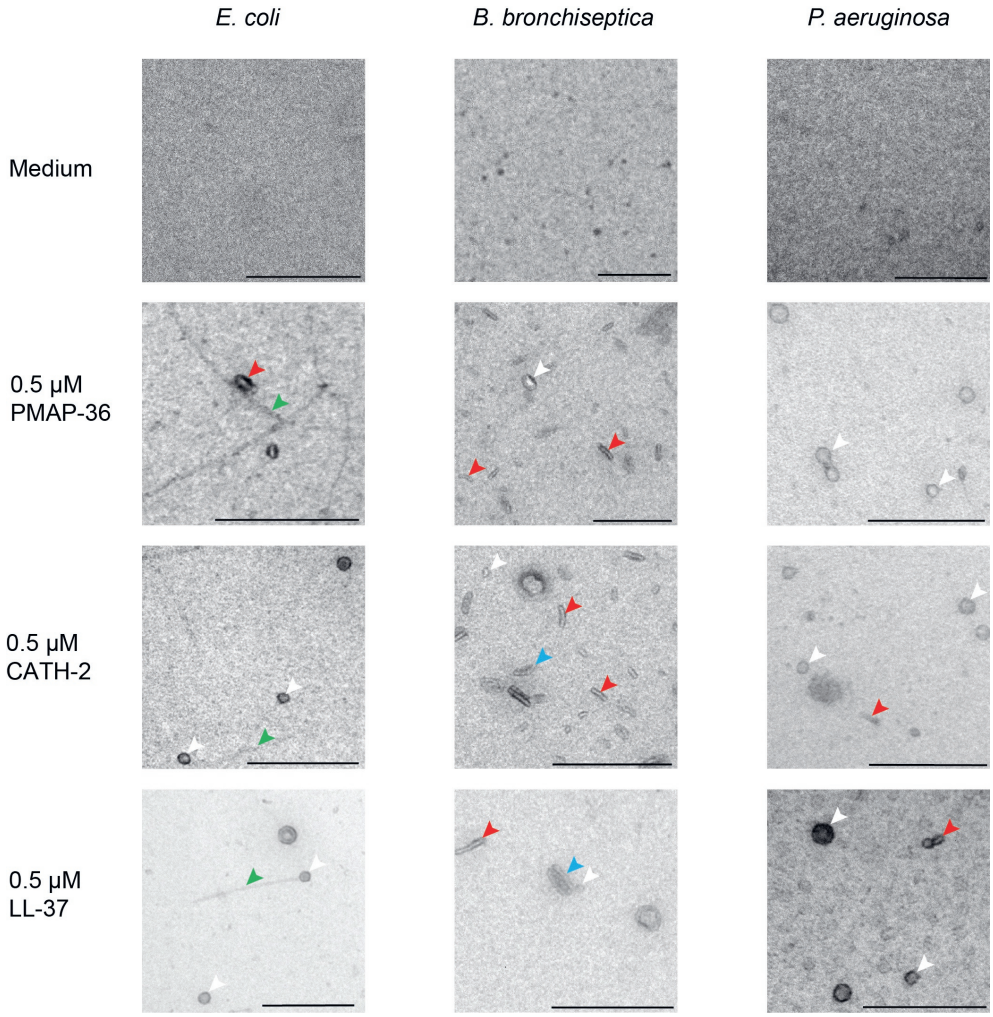
Supplementary Information



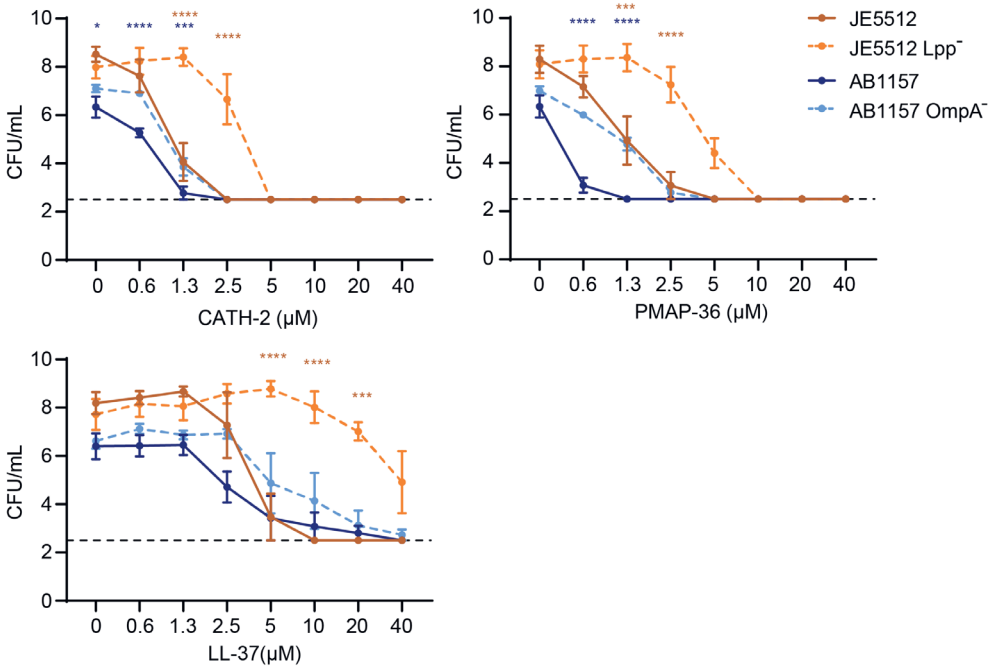
Supplementary Figure 1: BCA quantification of isolated OMVs. *OMVs were induced by HDPs and isolated from (a) E. coli, (b) B. bronchiseptica and (c) P. aeruginosa. Medium was taken along in the isolation protocol as control. Heat was applied as stress control. LB/V = Lysogeny Broth/Verwey medium, s = sOMVs, h = hOMVs, 0.5 = 0.5 µM and 2.5 = 2.5 µM of the corresponding peptide. Statistical analysis was performed using a linear mixed-model with post-hoc Dunnett (n=3-9). *=p<0.05, ***=p<0.001, ****=p<0.0001.*



Supplementary Figure 2: Quantification of E. coli and P. aeruginosa OMVs induced by 5 and 10 µM LL-37. *OMVs were induced by LL-37 and isolated from E. coli and P. aeruginosa. Left: BCA quantification Right: FM4-64 lipid quantification of isolated OMVs. Results were corrected for medium signal.*



Supplementary Figure 3: Morphology of OMVs resulting from bacteria treated with 0.5 µM peptide. *OMVs were induced by 0.5 µM of the respective HDP and isolated from E. coli, B. bronchiseptica and P. aeruginosa. Medium shows the corresponding growth medium of the respective bacterium. White arrows point to sOMVs, black arrows to larger hOMVs. Red arrows show disc-like pOMVs and blue arrows show pOMVs that have split open and still have cargo associated. Green arrows show flagellae. Scale bars represent 200 nm. Shown is a representative image of three experiments.*



Supplementary Figure 4: MBC comparisons of two E. coli strains and corresponding hypervesiculating mutant. *Track dilution assays were performed for two E. coli strains and corresponding hypervesiculating mutant using three different peptides. Shown is the mean with SEM (n=3). Statistical analysis was performed using a two-way ANOVA with post-hoc Sidak between corresponding mutant and wildtype for each concentration. *=p<0.05, ***=p<0.001, ****=p<0.0001.*

Supplementary Table 1: Percentages of lipid species observed in isolated OMVs. *OMVs were isolated from E. coli, B. bronchiseptica and P. aeruginosa, either induced by no treatment (sOMV), heat treatment (hOMVs) or different concentrations of indicated HDPs. aPG = Acyl-phosphatidylglycerol, DLCL = dilyso-cardiolipin, lyso PE = lyso-phosphatidylethanolamine, OL = ornithine lipid, PA = phosphatidic acid, PE = phosphatidylethanolamine, PG = phosphatidylglycerol, PS = phosphatidylserine.*

E. coli

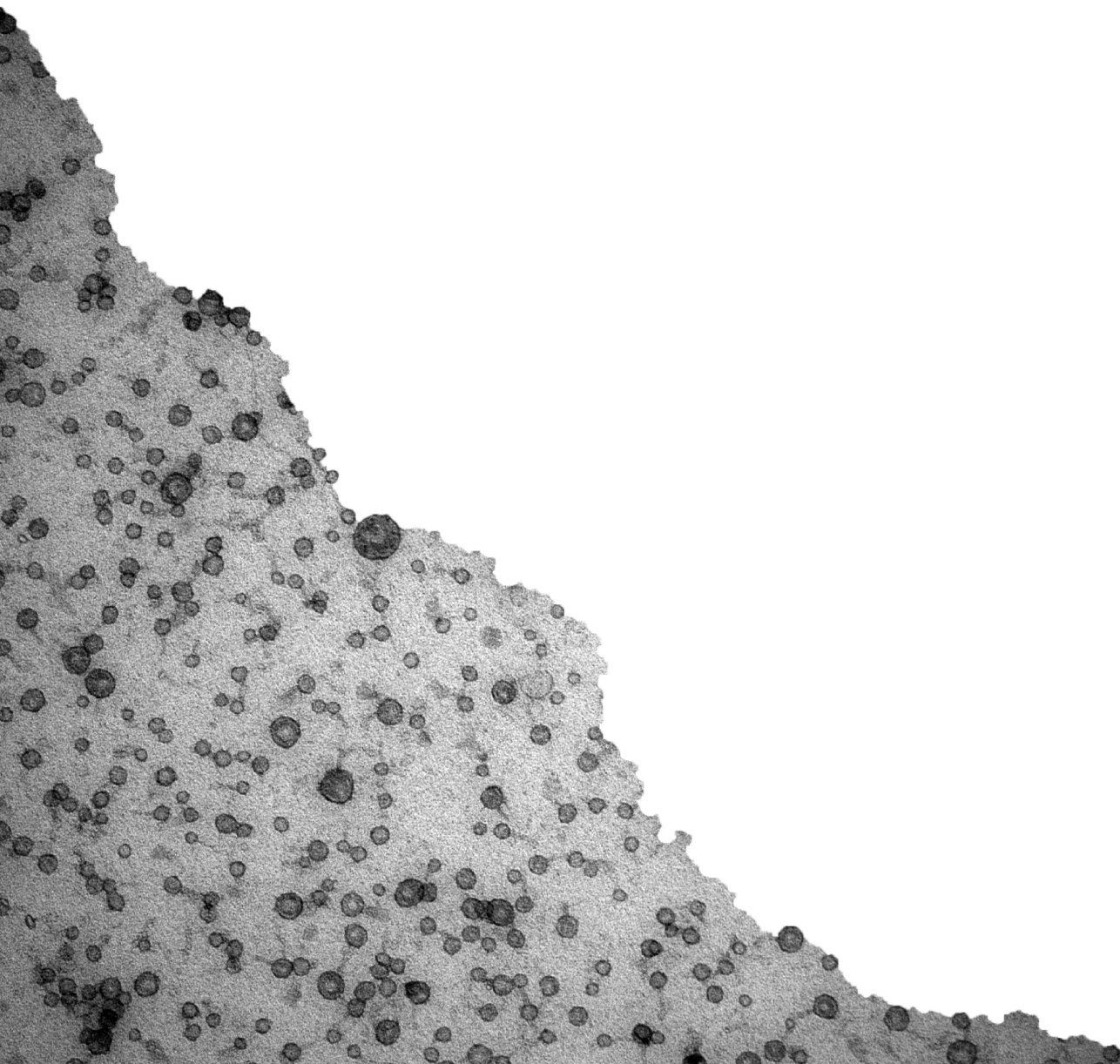
	sOMV	hOMV	0.5 µM PMAP-36	2.5 µM PMAP-36	0.5 µM CATH-2	2.5 µM CATH-2	0.5 µM LL-37	2.5 µM LL-37
aPG	1,2%	2,0%	1,5%	2,8%	1,4%	1,5%	1,1%	1,4%
DLCL	0,3%	0,2%	0,3%	0,3%	0,4%	0,5%	0,2%	0,3%
lyso PE	0,8%	5,8%	1,5%	1,0%	1,5%	1,1%	0,5%	0,6%
OL	0,0%	0,0%	0,0%	0,0%	0,1%	0,0%	0,0%	0,2%
PA	0,6%	0,3%	0,7%	0,8%	0,7%	0,9%	0,5%	0,5%
PE	77,9%	63,5%	75,6%	70,1%	75,7%	73,1%	79,7%	79,4%
PG	19,1%	28,0%	20,3%	24,8%	20,3%	22,7%	18,0%	17,5%
PS	0,1%	0,2%	0,1%	0,1%	0,1%	0,1%	0,0%	0,1%

B. bronchiseptica

	sOMV	hOMV	0.5 µM PMAP-36	2.5 µM PMAP-36	0.5 µM CATH-2	2.5 µM CATH-2	0.5 µM LL-37	2.5 µM LL-37
aPG	0,3%	0,9%	0,7%	1,8%	0,4%	0,8%	0,4%	1,0%
DLCL	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
lyso PE	1,9%	5,7%	1,0%	1,3%	0,4%	0,0%	0,4%	0,9%
OL	9,8%	12,9%	14,1%	13,5%	11,4%	11,4%	8,9%	9,0%
PA	0,1%	0,1%	0,3%	0,4%	0,1%	0,5%	0,3%	0,4%
PE	78,1%	60,6%	73,4%	63,5%	79,5%	68,1%	83,1%	77,9%
PG	9,7%	19,8%	10,4%	19,4%	8,2%	18,3%	6,9%	10,8%
PS	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%

P. aeruginosa

	sOMV	hOMV	0.5 µM PMAP-36	2.5 µM PMAP-36	0.5 µM CATH-2	2.5 µM CATH-2	0.5 µM LL-37	2.5 µM LL-37
aPG	2,9%	5,4%	3,6%	4,5%	2,8%	3,4%	1,9%	0,8%
DLCL	0,1%	0,1%	0,1%	0,1%	0,1%	0,2%	0,1%	0,1%
lyso PE	0,2%	2,4%	0,0%	0,0%	0,1%	0,0%	0,1%	0,0%
OL	0,4%	0,0%	0,1%	0,0%	0,0%	0,0%	0,0%	0,1%
PA	0,7%	0,9%	2,1%	2,4%	1,2%	1,9%	1,4%	1,2%
PE	83,7%	66,4%	83,4%	66,3%	82,2%	79,2%	81,0%	80,8%
PG	11,8%	24,6%	10,6%	26,1%	13,4%	15,1%	15,3%	16,9%
PS	0,1%	0,2%	0,1%	0,4%	0,1%	0,2%	0,1%	0,0%



Chapter 4

Host Defense Peptides' Affinity for Lipopolysaccharide does not Correlate with Killing Efficiency

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MANUSCRIPT IN PREPARATION

Abstract

Host Defense Peptides (HDPs) are part of the first line of defense and can have direct antimicrobial activities. Most HDPs exert their antimicrobial function through membrane-active mechanisms whereby they often interact with lipopolysaccharides (LPS) and phospholipids. LPS has a conserved structure, consisting of sugars, phosphate groups and acyl chains, but the exact composition can differ substantially between species. This has consequences for the properties of the LPS molecule and, most probably, also for the interaction with HDPs. In this study the influence of O-antigen and acyl chain composition on HDP killing and binding was investigated. It revealed that the presence of an O-antigen facilitates killing by HDPs and increases binding affinity for two of the four HDPs tested, PMAP-36 and PR-39. Acyl chain composition did not influence killing or LPS-binding by most HDPs, except for PR-39. Enhanced killing by PR-39 correlates with stronger LPS-binding. Furthermore, assessment of membrane damage revealed that CATH-2 and PMAP-36 are profoundly membrane-active and disrupt the inner and outer membrane of *Escherichia coli* completely, while PMAP-23 and PR-39 showed little to no membrane damage. Altogether the data suggests that LPS binding can serve as anchor for intracellularly-active HDPs, but that LPS binding does not significantly aid membrane-active HDPs.

Introduction

Host Defense Peptides (HDPs) are antimicrobial molecules that are part of the innate immune system. They are short, cationic and amphipathic peptides [1], and they often exert their antimicrobial activity by targeting bacterial membranes. Different models have been proposed to describe this membrane interaction, all eventually resulting in bacterial lysis [2]. The first step in several models is the interaction between the cationic parts of the HDPs and the anionic lipopolysaccharide (LPS) or lipoteichoic acid (LTA) molecules in the membrane [3]. This electrostatic interaction could facilitate the hydrophobic interaction with acyl chains, and thereby facilitate insertion in or translocation through the bacterial membrane.

LPS is the main molecule in the outer leaflet of the outer membrane of Gram-negative bacteria. It generally consists of three domains, the conserved lipid A, the core and the O-antigen that protrudes into the extracellular environment [4]. The lipid A portion consists of acyl chains, attached to a phosphorylated N-acetylglucosamine (NAG) dimer. The number of acyl chains can vary, as well as their length. Acyl chains can be directly attached to the NAG (a primary acyl chain) or attached to the 3-hydroxyl group that is present on the primary acyl chains (a secondary acyl chain) [4]. Despite all these variations, the main lipid A structure is conserved and properties are maintained among bacterial species. Attached to the lipid A is the core moiety. The core is an oligosaccharide (mostly six to twelve sugar moieties), which includes common sugars and sugars that are unique to bacteria, such as 2-keto-3-deoxyoctanoic acid (KDO) and L-glycerol-D-manno-heptose, both of which are often phosphorylated. The core can be divided into an inner core and outer core and where the inner core is well conserved within one bacterial species, the outer core is slightly more variable. The outermost part of the LPS is the O-antigen. This polysaccharide consists of repeating sugar subunits, comprised of one to five different sugars. The length of the O-antigen can vary up to forty repeats of sugar subunits. However, not all bacterial LPS contains this O-antigen. LPS without O-antigen is more hydrophobic than LPS with O-antigen and is often called lipooligosaccharide (LOS) [5,6]. LPS containing an O-antigen is called smooth, while LPS without O-antigen is called rough. All variations present in the LPS structure could affect HDP effectivity [7].

In this study LPS structures of *Bordetella pertussis* and two *Escherichia coli* strains were compared **(Fig. 1)**. It is important to specify the exact strains used, since LPS structures between *E. coli* strains can already differ substantially. In this study *E. coli* O111 LPS was used, which consists of six acyl chains, two KDO moieties and one to two phosphates in the core and an O-antigen of four to forty repeats of a five-sugar moiety, identical to that of *Salmonella enterica* O35 [8–11]. *E. coli* K-12 LPS has an identical lipid A moiety as *E. coli* O111, but lacks the O-antigen and differs slightly in the core sugar [12]. *E. coli* K-12 can

possess a third KDO moiety or a rhamnose attached to the two KDO moieties. Furthermore, where the outer core sugar of *E. coli* K-12 contains three glucose moieties, one galactose and a heptose, the outer core sugar of *E. coli* O111 contains a glucosamine instead of a heptose and a slightly different linkage [13,14]. *B. pertussis* B213 lipid A differs from *E. coli* lipid A, since it only contains five acyl chains, of which one is only 10 carbon atoms long. *B. pertussis* LPS lacks an O-antigen, similar to *E. coli* K-12, but does contain a complex core sugar with modified sugars, such as a fucose decorated with a methyl and an acetic acid moiety [15–17].

Most models and theories suggest that an initial interaction with LPS is advantageous for HDP function. However, recent theories also suggest that LPS can actually inhibit peptides from exhibiting their antimicrobial function. It could do so by binding to HDPs and thereby preventing access to the bacterial inner membrane. Furthermore, LPS could be excreted in outer membrane vesicles (OMVs) from the membrane and even function as decoy target for HDPs. It was shown for *E. coli* that addition of isolated OMVs protects the bacteria from killing by CATH-2, PMAP-36 and LL-37 [18]. Likewise, OMVs of *Helicobacter pylori* protected the bacterium against LL-37 [19].

In this study, the binding affinity of several HDPs to LPS was correlated with HDP killing activity to determine whether LPS binding aids in HDP killing or acts as a sink to inhibit HDPs from reaching their bacterial target. Four HDPs were studied in detail, CATH-2 and PMAP-36, both membrane-active peptides [20,21], PR-39, an intracellularly active peptide that also was shown to affect the bacterial membrane potential [22–24], and PMAP-23, a dual active peptide which showed antibacterial activity through membrane perturbations and intracellular activity but was shown not to interact with *Salmonella* Minnesota LPS [25–27]. Binding affinities of these HDPs to LPS from *B. pertussis* and *E. coli* (differing in O-antigen and lipid A acyl chains) were determined using isothermal calorimetry and an LPS competitive binding assay, while antimicrobial activity was determined using track dilution assays. Furthermore, a genetically modified *E. coli* strain expressing mCherry in the periplasm and GFP in the cytoplasm was used to investigate the antibacterial mechanism of the HDPs tested.

Materials & Methods

Peptide synthesis

Peptides CATH-2, PMAP-36, PR-39, PMAP-23 and K9CATH were synthesized by Fmoc solid-phase synthesis at China Peptides (CPC scientific, Sunnyvale, CA, USA). LL-37 was synthesized by Fmoc solid-phase synthesis at the Academic Centre for Dentistry Amsterdam (Amsterdam, the Netherlands). pBD-2 was synthesized by Genosphere

Biotechnologies (Paris, France) using Fmoc solid-phase synthesis on a Symphony synthesizer (Protein Technology Inc., Tucson, AZ). All peptides were purified to a purity of >95% by reverse phase high-performance liquid chromatography. Sequences and characteristics of the peptides are shown in **Table 1**.

Table 1: Sequence, number of amino acids (No. aa) and charge of studied peptides [20,24,25,28,29]**.**

Peptide	***Sequence***	***No. aa***	***Charge***
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	37	6+
CATH-2	RFGRFLRKIRRFRPKVTITIQGSARF-NH_2	26	8+
PMAP-36	Ac-GRFRRLRKKTRKRLKKIGKVLKWIPPIVGSIPLGCG	36	13+
PR-39	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFP	39	10+
pBD-2	DHYICAKKGGTCNFSPCPLFNRIEGTCYSGKAKCCIR	37	5+
PMAP-23	RIIDLLWRVRRPQKPKFVTVWVR	23	6+
K9CATH	RLKELITTGGQKIGEKIRRIGQRIKDFFKNLQPREEKS	38	5+

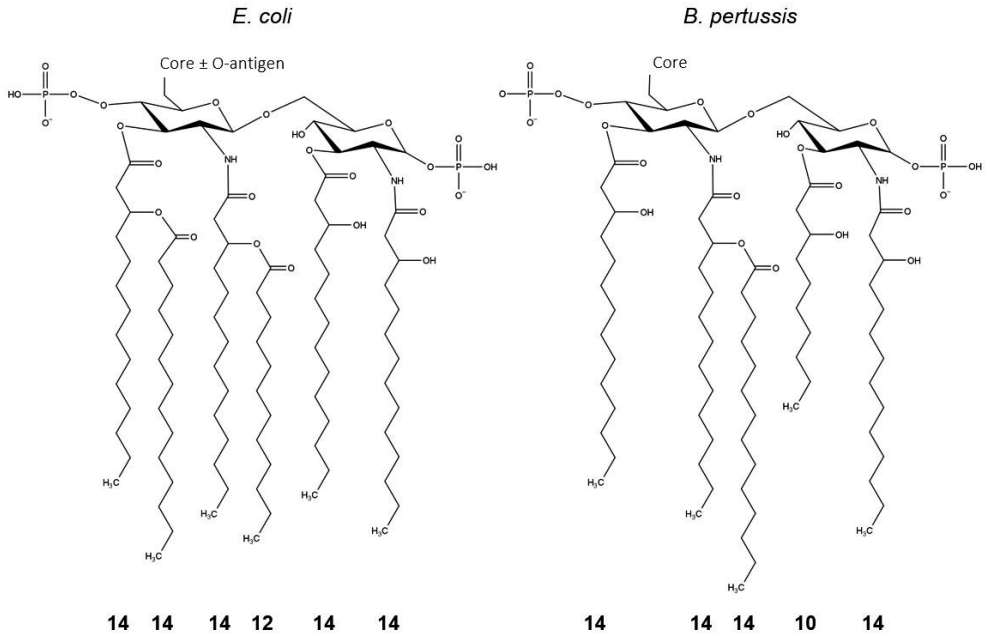

Figure 1: Chemical structures of LPS species used. *B. pertussis LPS is penta-acylated, while E. coli K-12 and E. coli O111 LPS are hexa-acylated. Acyl chain lengths are indicated with numbers below. The core and O-antigen differ for each strain and are not depicted here.*

Bacterial strains

E. coli K-12, a clinical *E. coli* O111 isolate (University Medical Center Groningen) and *B. pertussis* B213, a streptomycin-resistant derivative of *B. pertussis* strain Tohama [30] were used throughout this study. Both *E. coli* strains were grown on tryptic soy agar (TSA) plates (Oxoid Ltd, Basingstoke, Hampshire, UK). Liquid cultures were grown in lysogeny broth (LB) containing 1% yeast extract (Becton, Dickinson and Company, Sparks, USA), 1% NaCl (Merck, Darmstadt, Germany) and 0.5% tryptone (Becton, Dickinson and Company) or Mueller-Hinton Broth (MHB, Becton, Dickinson and Company). *Bordetella* strains were grown on Difco™ Bordet-Gengou (BG) agar plates (Becton, Dickinson and Company), containing 1% glycerol (Merck) supplemented with 15% (v/v) defibrinated sheep blood (Oxoid Ltd). Liquid cultures were grown in Verwey medium (pH 7.4) [31] containing 0.1% (w/v) starch from potato (S2004, Sigma-Aldrich, St. Louis, MO, USA), 0.02% (w/v) KCl, 0.05% (w/v) KH_2PO_4, 0.01% (w/v) $MgCl_2 \bullet 6\ H_2O$ (all from Merck), 0.002% (w/v) nicotinic acid (Sigma-Aldrich), 1.4% (w/v) Bacto™ casamino acids (Becton, Dickinson and Company), and 0.001% (w/v) L-glutathione reduced (Sigma-Aldrich).

LPS isolation

E. coli K-12 LPS and *E. coli* O111 LPS were commercially obtained (**Fig. 1**, Invivogen, San Diego, California, USA). *B. pertussis* LPS was isolated from bacteria according to the Tri-reagent method. In short, lyophilized cells were resuspended in TRIzol Reagent (Invitrogen) by intensive vortexing. After 10 min incubation at room temperature to allow for complete cell homogenization, 20 µl of chloroform (HPLC grade) per mg of cells were added. After vigorous vortexing, the mixture was incubated at room temperature for another 10 min and, subsequently, centrifuged for 10 min to separate phases. The aqueous phase was collected and three additional extractions were performed by adding Milli-Q water to the organic phase, vortexing, incubation at room temperature and centrifugation. All the aqueous phases collected were combined, and the water was evaporated using a speed vacuum concentrator. The pellet was washed with 0.375 M $MgCl_2$ (Merck) in 95% ethanol, pelleted again by centrifugation, and resuspended in Milli-Q water. The extracted LPS was lyophilized, weighed for quantification, and resuspended in endotoxin-free HyPure cell culture grade water (HyClone) for further use. The purity and integrity of purified samples were evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) combined with silver staining of LPS or Coomassie staining of proteins.

Track dilution assay

Bacterial killing by HDPs was assessed using track dilution assays, as described before [18]. In short, 10^6 colony forming units (CFU)/mL bacteria were incubated with different concentrations of peptide for 3 h at 37°C in a U-bottom microtiter plate (Corning, New York, USA). After incubation, 10-fold dilutions were prepared using the corresponding medium and 10 µL of each dilution was plated on appropriate agar plates. Plates were incubated at 37°C and colonies were counted after 24 or 48 h. Minimal bactericidal concentration (MBC) was defined as <250 CFU/mL.

Isothermal Titration Calorimetry (ITC)

ITC was performed with a Low Volume NanoITC (TA Instruments-Waters LLC, New Castle, DE, USA). The 50 µl syringe was filled with 200 µM peptide in 1:3 H_2O:phosphate buffered saline (PBS) for titration into 164 µl 62.5 µM LPS in 1:3 H_2O:PBS, unless stated otherwise. Titrations were incremental with 2 µl injections at 300 s intervals. Experiments were performed at 37°C. Data were analyzed with the NanoAnalyze software (TA Instruments-Waters LLC).

Dansyl-polymyxin B competition assay

Different concentrations of peptide (25 µL) were incubated with 15 µg/mL of LPS (25 µL) in a flat-bottom 96-wells plate at 37°C for 30 min. Afterwards, 50 µL of 8 µM dansyl-labelled polymyxin B was added (end concentration was 4 µM), mixed and fluorescence was determined immediately using the Fluostar Omega. Samples were excited at 340 nm and the signal was measured at 490±10 nm. Signals were corrected for dansyl-polymxyin B background. Bound dansyl-labelled polymyxin B gives a high fluorescent signal at 485 nm, which decreased with increasing peptide concentrations, indicating less dansyl-labelled polymyxin B was able to bind. This was converted in percentages of bound dansyl-polymyxin B.

Bacterial membrane leakage

Recombinant *E. coli* expressing mCherry in the periplasm and Green Fluorescent Protein (GFP) in the cytoplasm ($_{Peri}$mCherry$_{/cyto}$GFP) was prepared as previously described [32]. Bacteria were grown overnight in LB medium containing 100 µg/ml ampicillin. The next day, subcultures were grown to mid-log phase (optical density, OD, of approx. 0.5), washed and resuspended to an OD of approx. 1 in RPMI supplemented with 0.05% human serum albumin (RPMI-HSA). All further incubations were done in RPMI-HSA. Bacterial cultures with an OD of approx. 0.05 were mixed with 1 µM Sytox Blue Dead Cell Stain (Thermofisher) and exposed to a concentration range of HDPs for 30 minutes at 37°C. For

the kinetic experiments, every 5 min bacteria were mixed with the HDPs (concentrations indicated in figure legends) up until 45 min and incubated at 37°C. After the incubations, bacteria were diluted ten times after which the Sytox blue, mCherry and GFP intensity was analyzed by flow cytometry (MACSQuant). Data was analyzed in FlowJo, where the percentage of mCherry and GFP negative or Sytox positive bacteria was determined by gating on the buffer control.

Results

Influence of O-antigen in resistance to host defense peptides

To determine the effect of the presence of an O-antigen on HDP effectivity, two *E. coli* strains were used, *E. coli* O111 and *E. coli* K-12, with LPS structures that only differ in the core and O-antigen. To determine the susceptibility of the two strains to a range of HDPs, MBCs were determined using track dilution assays and this revealed that *E. coli* O111 is more susceptible to HDP killing than *E. coli* K-12 **(Fig. 2, Fig. S1)**, with the exception of pBD-2 and PMAP-23, both described as non-LPS binding peptides [25,29]. This is despite the fact that *E. coli* O111 multiplies faster than *E. coli* K-12 and therefore shows a higher bacterial density after 3 h incubation without peptide. This suggests that the presence of O-antigen might enhance HDP antibacterial activity, since *E. coli* O111 LPS contains an O-antigen where *E. coli* K-12 LPS does not.

Influence of O-antigen on affinity of host defense peptides for LPS

To investigate whether the observed difference in MBCs for the two *E. coli* strains is linked to differences in affinity between HDPs and LPS, binding was assessed using ITC. Since the lipid A portion of the LPS is equal, any differences observed would be due to differences in the core and O-antigen. Two membrane-active peptides were selected, CATH-2 and PMAP-36, as well as a HDP with an intracellular antimicrobial mechanism, PR-39, and a peptide shown not to interact with *Salmonella* Minnesota LPS, PMAP-23 [20,24,25]. Different binding mechanisms were observed for the different HDPs. CATH-2 binding to both LPS species was exothermic and similar exothermic binding was, surprisingly, observed for PMAP-23. This would imply that the O-antigen of LPS plays a relatively small role in binding to these HDPs. On the other hand, PMAP-36 binding to LPS was biphasic with an initial exothermic, but subsequent mainly endothermic binding to *E. coli* O111 LPS **(Fig. 3)**. No, or almost no binding was observed for PMAP-36 to *E. coli* K12 LPS, indicating that the O-antigen is involved in binding of PMAP-36, and that this binding is more hydrophobic in nature compared to CATH-2 binding. PR-39 binding to LPS was initially endothermic for *E. coli* O111 LPS with very low, if any, exothermic binding in later phases of the titration scheme, while no binding was observed for *E. coli* K-12 LPS. Overall, these

data indicate that the binding of HDPs to LPS is not only dependent on the structure of LPS but also differs per HDP.

When dissociation constants (Kd) were compared, it revealed a similar Kd for CATH-2 for binding with both *E. coli* O111 and *E. coli* K-12 LPS, but no binding was observed for PMAP-36 and PR-39 for *E. coli* K-12 **(Table 2)**. A stronger binding between PMAP-23 and *E. coli* K-12 LPS was observed, compared to *E. coli* O111 LPS. Differences in stoichiometry also indicated different binding mechanisms for the different HDPs, but numbers were somewhat comparable between the two LPS structures **(Table 2)**. Altogether, the stoichiometry and Kds show that LPS-binding is dependent on the HDP studied and is quite different per HDP. A stronger binding between PMAP-36 or PR-39 and LPS correlated with more efficient killing, but this was not observed for CATH-2 and PMAP-23.

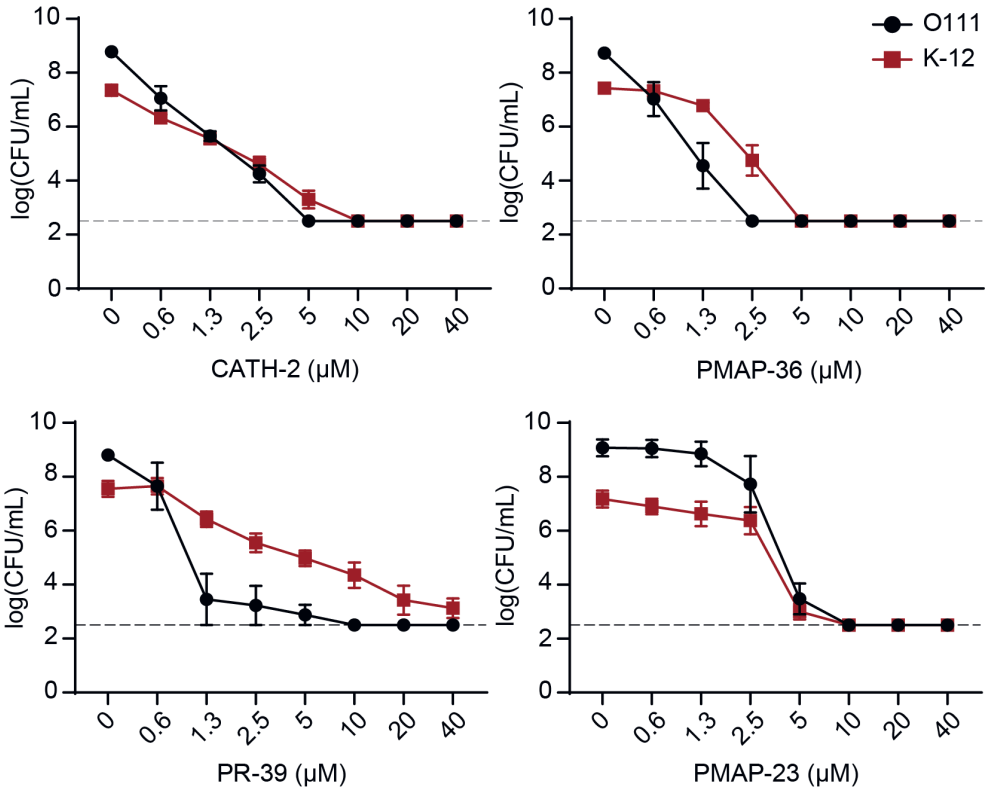

Figure 2: Determinations of MBC values of CATH-2, PMAP-36, PR-39 and PMAP-23 for two E. coli strains. *Surviving bacterial colonies were detected after incubation with HDPs for 3 h in MHB (n=3). Shown is the mean with SEM, dashed line shows the detection limit.*

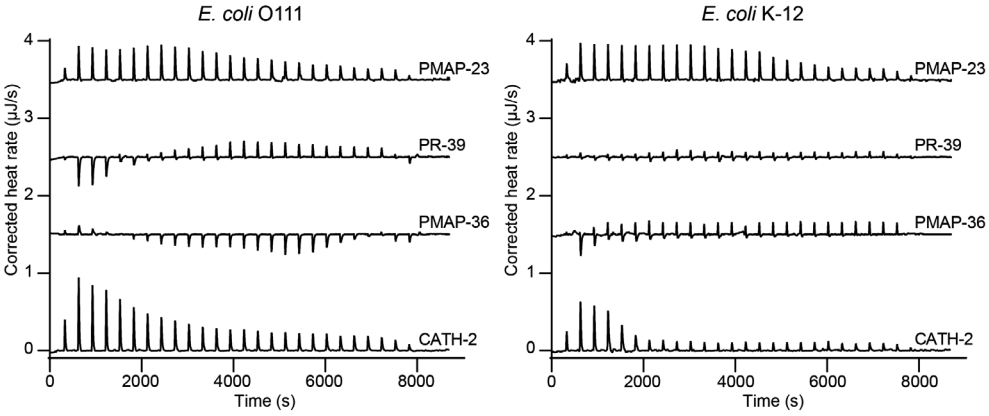

Figure 3: ITC spectra of the interaction between LPS species and peptides. *CATH-2, PMAP-36, PR-39 or PMAP-23 (200 µM) was titrated into E. coli O111 or E. coli K-12 LPS solution (0.5 mg/mL, 0.25 mg/mL for E. coli O111 and CATH-2) and heat rates were recorded. Shown is a representative of two measurements.*

4

Influence of acyl chain composition on resistance to HDPs

To assess the influence of acyl chain composition on HDP effectivity, killing of *E. coli* K-12 was compared to killing of *B. pertussis*. Both lack an O-antigen and are therefore rough LPS structures, but *E. coli* K-12 is hexa-acylated while *B. pertussis* is penta-acylated **(Fig. 1)**. Some differences in the core exist as well, which needs to be taken into account upon analysis of the data. To properly compare killing, *E. coli* K-12 killing by HDPs was also assessed in Verwey medium, since medium composition can have strong effects on HDP activity. This revealed that CATH-2 and PMAP-36 very efficiently eradicated both *E. coli* K-12 and *B. pertussis* in Verwey medium **(Fig. 4)** with comparable MBCs of 0.6 to 1.3 µM. PMAP-23 required a higher concentration of 2.5 µM to eradicate both species. However, PR-39 efficiently killed *B. pertussis* at 0.6 µM, while even 40 µM was not sufficient to eradicate *E. coli* K-12, indicating that the number of lipid chains or specific core elements had an effect on the activity of PR-39.

Table 2: Dissociation constants and stoichiometry calculated using ITC data for interactions between E. coli LPS and HDPs. *Data was fitted using an independent model, except for PMAP-36 and E. coli O111 where a multiple sites model was used. n.d. = not defined which indicates no binding occurred. Shown is an average of two experiments.*

	***E. coli* O111**		***E. coli* K-12**	
	Kd	**n**	**Kd**	**n**
CATH-2	2.55E-06	0.66	1.15E-06	0.35
PMAP-36	2.50E-08 6.54E-08	0.11 1.56	n.d.	n.d.
PR-39	3.68E-07	0.34	n.d.	n.d.
PMAP-23	1.83E-06	1.18	3.35E-07	1.16

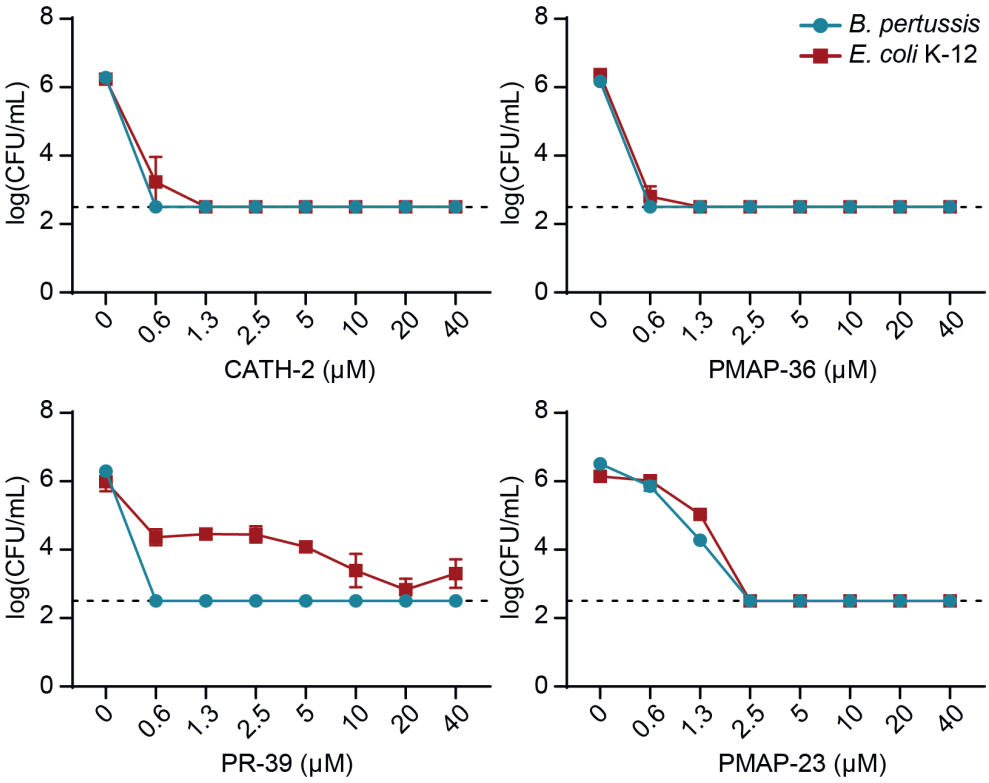

Figure 4: Determination of MBC values for B. pertussis and E. coli K-12. *Surviving bacterial colonies were detected after incubation with HDPs for 3 h in Verwey medium (n=3). Shown is the mean with SEM, dashed line shows the detection limit.*

Influence of acyl chain composition in affinity of host defense peptides for LPS

Since differences were observed in HDP effectivity between *B. pertussis* and *E. coli* K-12 for PR-39, the effect of acyl chain composition on LPS binding by HDPs was assessed using ITC. Stoichiometries were substantially different between *B. pertussis* and *E. coli* K-12 LPS binding **(Table 3)**, indicating that the acyl chain composition has a larger effect on HDP binding mechanism compared to the presence of an O-antigen. However, CATH-2 and PMAP-23 showed exothermic binding to *B. pertussis* LPS, similar as binding to *E. coli* K-12 LPS **(Fig. S2)**. Additionally, Kd values were comparable for CATH-2 and PMAP-23 binding to both LPS structures **(Table 3)**. PR-39 and PMAP-36 both interacted with *B. pertussis* LPS but not *E. coli* K-12 LPS, where PR-39 was also more efficient in killing *B. pertussis*. This indicates that a stronger interaction between PR-39 and LPS results in more efficient killing by PR-39.

Table 3: Dissociation constants and stoichiometry calculated using ITC data for interactions between B. pertussis LPS and HDPs, compared to E. coli K-12 data. *Data was fitted using an independent model, except for PMAP-36 and B. pertussis where a multiple sites model was used. n.d. = not defined which indicates no binding occurred. Shown is an average of two experiments.*

	B. pertussis		***E. coli* K-12**	
	Kd	**n**	**Kd**	**n**
CATH-2	3.38E-06	1.34	1.15E-06	0.35
PMAP-36	1.10E-07	1.50	n.d.	n.d.
	3.11E-07	0.33		
PR-39	9.95E-06	0.80	n.d.	n.d.
PMAP-23	2.71E-07	0.40	3.35E-07	1.16

Antibacterial mechanisms of CATH-2, PMAP-36, PMAP-23 and PR-39

To obtain more insight into the LPS binding mechanism of the different HDPs, a competition LPS binding assay with dansyl-labelled polymyxin B was performed as an additional method to determine relative affinities of HDPs for LPS. This revealed that membrane active peptides CATH-2 and PMAP-36 bound *E. coli* O111 tightly and that dansyl-labelled polymyxin B could not displace these peptides. In contrast, dansyl-polymyxin B could compete with PR-39 and PMAP-23 binding to *E. coli* O111 LPS **(Fig. 5)**. Similar trends were observed for *E. coli* K-12 and *B. pertussis*, where CATH-2 and PMAP-36 were also the strongest binders.

To further characterize the antibacterial mechanism of these peptides, kinetics of bacterial membrane permeabilization were investigated. To distinguish between permeabilization of the inner and the outer membrane, an *E. coli* strain expressing GFP in the cytoplasm and mCherry in the periplasm was studied. Bacteria were incubated for 30 minutes with increasing concentrations of CATH-2, PMAP-36, PR-39 and PMAP-23, also in the presence of Sytox in the medium. In this set-up, release of mCherry indicates outer membrane permeabilization, influx of Sytox indicates small perturbations of the inner mebrane, and release of GFP shows large inner membrane disruption. These experiments revealed that for both CATH-2 and PMAP-36 Sytox influx was observed before mCherry leakage. After a short delay also GFP leakage was observed, indicating small pores were formed first, followed by larger disruptions in the outer membrane and finally the inner membrane **(Fig. 6)**. Remarkably, at 10 µM and higher, the side scatter was observed to increase which indicates morphological changes in the bacteria by CATH-2 and PMAP-36. For PR-39 no Sytox influx, mCherry or GFP outflow was observed, confirming that this peptide does not affect the bacterial membrane. PMAP-23 showed Sytox influx at higher concentrations, indicating small pores were formed when concentrations were sufficiently high.

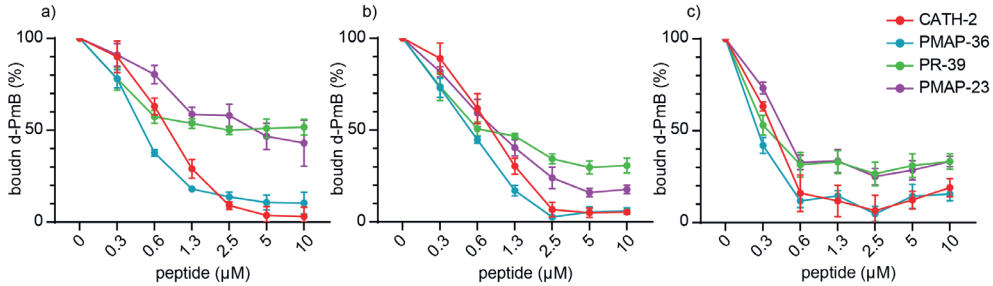

Figure 5: Competition assay with dansyl-labelled polymyxin B (d-PmB). *(a) E. coli O111, (b) E. coli K-12 or (c) B. pertussis LPS (15 µg/mL) was incubated with increasing concentrations of HDP. d-PmB (8 µM) was added afterwards and the amount of bound d-PmB was measured with the fluorescent signal at 490 nm. Shown is the mean of three independent experiments with SEM.*

In addition, membrane damage was assessed over time by exposing bacteria to 5 µM of CATH-2 and PMAP-36 or 20 µM of PR-39 and PMAP-23. This showed that within ten minutes 80% of bacterial membranes were lysed by CATH-2 and PMAP-36.

No membrane damage was observed over time by PR-39, confirming the results from the titration experiments. PMAP-23 showed a steady increase of Sytox influx, with 80% of bacterial membranes showing small pore formation after 45 minutes. Some leakage of mCherry and GFP was also observed after 45 minutes, indicating PMAP-23 is also capable of forming larger pores in both the inner and outer membrane. This affected bacterial morphology, since side scatter was also observed to increase over time by 20 µM of PMAP-23.

Discussion

In this study correlation between eradication of Gram-negative bacteria by host defense peptides and binding affinity of these HDPs for the corresponding LPS structure was investigated. Many HDPs exert their antibacterial activity through membrane active mechanisms and first encounter LPS in their interaction with Gram-negative bacteria [33]. This interaction could either facilitate pore formation or passage through the membrane, for intracellular active peptides, or could retain HDPs in the membrane, limiting their ability to reach potential targets. By correlating binding affinities of CATH-2, PMAP-36, PR-39 and PMAP-23 with killing capabilities, this study tried to distinguish between the two hypotheses.

A fine balance should exist between LPS binding by HDPs, to initially associate with the bacterial membrane, and LPS release by HDPs, to transverse through the membrane if needed. Dissociation constants (Kds) determined with ITC varied in the order of 10^{-6} and 10^{-8}, where a smaller Kd indicates stronger binding. PMAP-36 has the smallest Kds for all

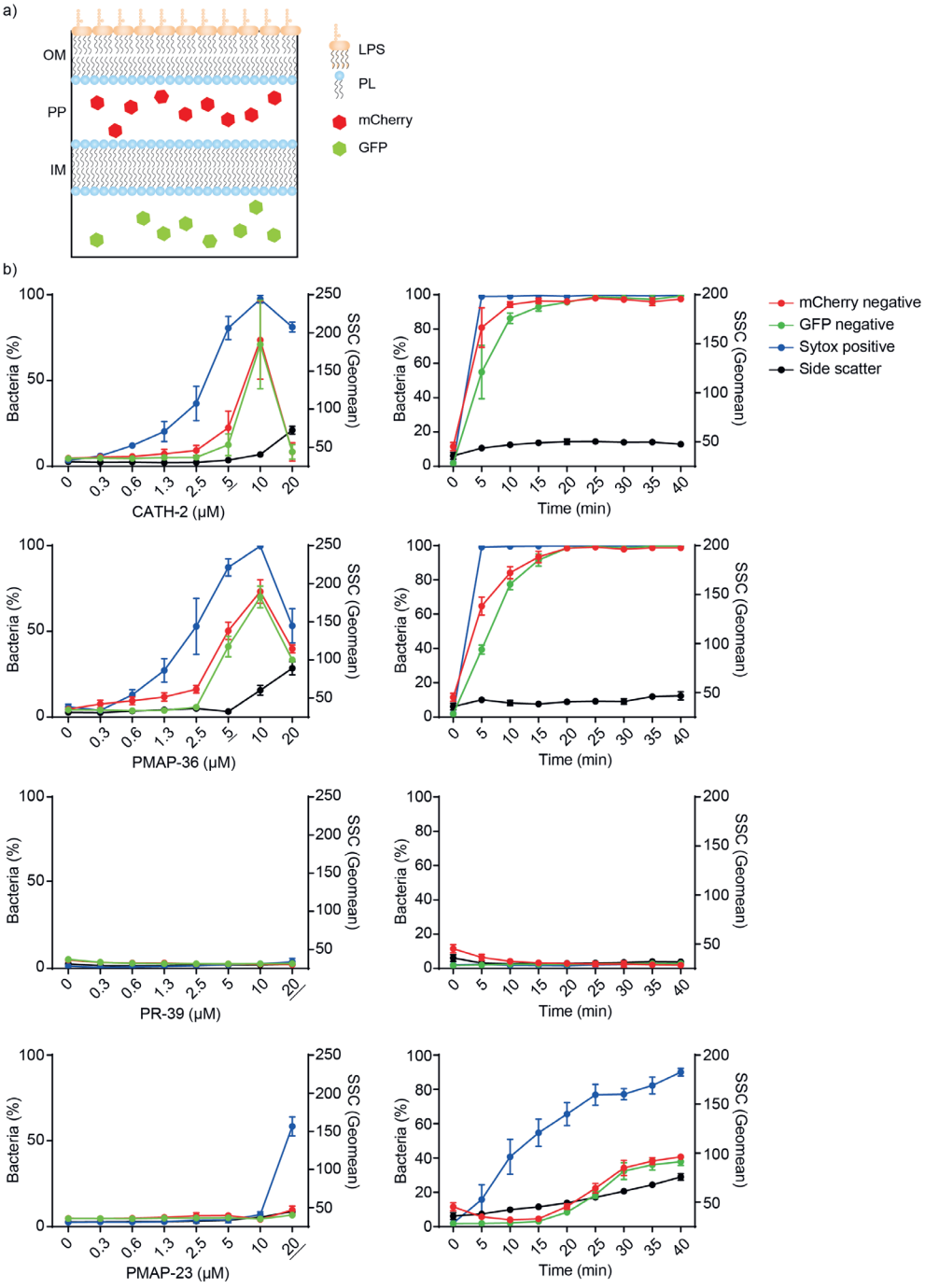
a)
OM
PP
IM
LPS
PL
mCherry
GFP
b)
mCherry negative
GFP negative
Sytox positive
Side scatter
Bacteria (%)
SSC (Geomean)
CATH-2 (μM)
PMAP-36 (μM)
PR-39 (μM)
PMAP-23 (μM)
Time (min)

Figure 6: Bacterial membrane leakage assay demonstrates antibacterial mechanisms of CATH-2, PMAP-36, PR-39 and PMAP-23. *(a) An E. coli strain expressing mCherry in the periplasm (PP) and Green Fluorescent Protein (GFP) in the cytoplasm was used to assess outer membrane (OM) and inner membrane (IM) damage. LPS, lipopolysaccharide; PL, phospholipid. (b) Increasing concentrations of peptide (left) were studied and one concentration of each peptide (underlined in the concentration graph) was also assessed over time (right). Sytox influx was also measured, to observe small pore formation and side scatter to study bacterial morphology upon treatment with peptides.*

LPS structures bound by this peptide, in the order of 10^{-7} to 10^{-8}, indicating PMAP-36 binds the bacterial membrane very tightly and probably exerts its antibacterial mechanism there. PR-39 and PMAP-23 have Kds in a similar order of magnitude (10^{-6} to 10^{-7}) and this indicates these HDPs are able to dissociate from the bacterial membrane and reach intracellular targets, but dissociation might require more time. CATH-2 has the highest Kds, in the order of 10^{-6}, indicating strong, but balanced binding with LPS and probably is the quickest HDP to reach intracellular targets.

When LPS-binding was assessed using ITC for CATH-2, PMAP-36, PR-39 and PMAP-23, also differences in binding mode between the different HDPs were observed. CATH-2 and PMAP-23 showed exothermic binding, while PMAP-36 and PR-39 showed endothermic binding **(Fig. 3)**. Furthermore, PMAP-36 showed a biphasic binding pattern. The modes of interaction were identical, regardless of peptides were interacting with *B. pertussis* or *E. coli* LPS **(Fig. 3, Fig. S2)**. The mode of interaction does reveal some information about interactions sites. Enthalpic reactions often rely on hydrogen bonds and electrostatic interactions, while entropic reactions revolve around hydrophobic interactions [34]. Exothermic interactions are most often enthalpy driven and therefore CATH-2 and PMAP-23 probably rely on electrostatic interactions for binding LPS, while the endothermic, entropy driven interactions of PR-39 and PMAP-36 rely more on hydrophobic interactions. This is confirmed by the measured enthalpy (ΔH) values from ITC, showing positive values for LPS binding of PMAP-36 and PR-39 and negative values for CATH-2 and PMAP-23.

When assessing the effect of differences in LPS structures on HDP binding and bacterial killing, the presence of O-antigen and differences in acyl chain composition were studied. It was observed that these components influenced each studied peptide differently, confirming each peptide acts via a different mechanism and probably also different binding sites on the LPS. Nevertheless, *E. coli* K-12 was consistently more resistant to most HDPs than *E. coli* O111, with the exception of PMAP-23 and pBD-2 **(Fig. 2, Fig. S1)**. This could indicate that lacking an O-antigen might protect bacteria against HDPs, but it must be noted that these strains could also be different in other ways than only LPS structure. PMAP-36 and PR-39 did not bind to *E. coli* K-12 LPS, lacking an O-antigen, but did bind to *E. coli* O111 LPS, containing an O-antigen. This suggests that the O-antigen aids in binding and subsequent killing by these two peptides, confirming their binding relies more on

hydrophobic interactions. CATH-2 MBCs were very similar for the two *E. coli* strains and also LPS-binding did not seem to be influenced by the presence or absence of an O-antigen. Despite MBCs of PMAP-23 being equal for both *E. coli* strains, PMAP-23 bound stronger to *E. coli* K-12, lacking an O-antigen and being more hydrophobic compared to *E. coli* O111, confirming PMAP-23 mediates binding through electrostatic interactions. Stoichiometries were comparable for HDPs binding to *E. coli* O111 and *E. coli* K-12, while Kds differed, indicating the presence of an O-antigen influences binding affinity more than binding mechanism of HDPs.

To assess the influence of acyl chain composition, *B. pertussis* was compared with *E. coli* K-12, both containing rough LPS. Track dilution assays showed that MBCs were not affected for either CATH-2 and PMAP-23. In addition, ITC showed similar binding affinities between these HDPs and both LPS structures, indicating that LPS-binding of these HDPs is not influenced by the acyl chain composition of LPS. Despite CATH-2 has been shown to exhibit a membrane-active antibacterial mechanism, these data suggests this mechanism is independent of LPS binding. PMAP-36 and PR-39 did not bind to *E. coli* K-12 LPS, as described above, but did bind to *B. pertussis* LPS. MBCs for PMAP-36 were equal, but for PR-39 the MBC for *B. pertussis* was 0.6 μM while it could not always kill *E. coli* K-12 even at 40 μM. Together this suggests that stronger LPS-binding enhances bacterial killing by PR-39. Stoichiometries were not comparable between *B. pertussis* and *E. coli* LPS for the different peptides, indicating that HDPs might interact differently with the different LPS structures and that this is more influenced by acyl chain composition than the O-antigen.

CATH-2 and PMAP-36 have been shown before to have a membrane-active antibacterial mechanism, but also have been shown to have intracellular targets [20,21]. CATH-2 and PMAP-36 indeed both showed very rapid complete lysis of *E. coli* membranes **(Fig. 6)**, within 20 minutes, but at higher concentrations a decrease of protein outflow was observed. This could perhaps be due to clustering of proteins intracellularly, as demonstrated before [21]. Both CATH-2 and PMAP-36 showed binding with *B. pertussis* and *E. coli* LPS. For CATH-2 binding affinities for all three LPS structures were comparable, as were the MBCs. This indicates that LPS-binding by CATH-2 is not influenced by presence of an O-antigen or acyl chain composition and that LPS-binding does not correlate with antibacterial activity of CATH-2. For PMAP-36 a biphasic binding was observed, but only for *B. pertussis* and *E. coli* O111. No binding was observed for *E. coli* K-12. However, MBCs for *B. pertussis* and *E. coli* K-12 were equal despite the difference in LPS binding. This indicates that antibacterial activity for PMAP-36 is not correlated with LPS affinity. Perhaps both CATH-2 and PMAP-36 have strong affinities for the anionic phospholipids in the bacterial membrane and do not rely on interactions with LPS for their antibacterial mechanisms.

PMAP-23 is a dual active peptide, with both membrane and intracellular antibacterial activities [26,27,35], but described before not to interact with *Salmonella* Minnesota LPS [25], which we could confirm (data not shown). Remarkably, PMAP-23 showed binding to all LPS species tested in this study, with very similar Kd values. Only binding to *E. coli* O111 LPS was slightly weaker, probably due to the presence of the hydrophilic O-antigen. In line with this, similar MBCs against both *E.coli* strains were observed. This indicates that PMAP-23 mediates LPS-binding through hydrophobic interactions and is unaffected by acyl chain variations. Furthermore, differences in LPS structure did not have great effects on PMAP-23 LPS-binding and function. Since bactericidal concentrations of PMAP-23 were quite high for all species tested, this peptide might induce bacterial membrane damage via the carpet model [26,35]. Indeed, only at higher concentrations of PMAP-23 (20 µM) was membrane damage observed in *E. coli* **(Fig. 6)**.

And lastly, PR-39, a mostly intracellular acting peptide [22,23] and highly bactericidal **(Fig. 2, Fig. 4)**, was confirmed to not affect the bacterial membrane in *E. coli* **(Fig. 6)**. Even though PR-39 did not induce membrane damage, it was shown to interact with LPS in this study, and for PR-39 stronger LPS-binding correlates with more efficient killing, suggesting that LPS-binding can aid intracellularly acting HDPs. LPS binding may be a way to increase the concentration of peptides at the bacterial surface to ensure a more efficient translocation across the bacterial membrane.

To further investigate LPS binding by these four peptides, a competition-based assay with dansyl-labelled polymyxin B was used. Polymyxin B is a peptide antibiotic shown before to bind to LPS and dansyl-labelled polymyxin B was developed as tool, since fluorescence at 485 nm markedly increases when it binds to LPS [36–38]. In this study, LPS was first incubated with peptide and afterwards, dansyl-labelled polymyxin B was added to assess whether peptides could be expelled from the complex with LPS or if there were still open binding sites left. Observed trends correspond to data obtained with the ITC, where CATH-2 and PMAP-36 bind the strongest to any species of LPS and PMAP-23 and PR-39 bind less **(Fig. 5)**. It could also indicate that PR-39 and PMAP-23 occupy different binding sites than dansyl-labelled polymyxin B. Between PMAP-23 and PR-39, it seemed that PMAP-23 binds a little stronger, corresponding to data obtained with ITC. However, percentage-wise PMAP-23 and PR-39 seemed to interact stronger with *E. coli* K-12 LPS compared to *E. coli* O111 LPS, which contradicts ITC results. This might be because the competition assay relies on binding between dansyl-polymyxin B and the corresponding LPS. Fluorescent signals of dansyl-polymyxin B binding to only LPS were indeed different between LPS species, indicating already different binding strengths between the different LPS species and dansyl-polymyxin B. Furthermore, 15 µg/mL of LPS was used in the competition assay, which is below the critical micelle concentration (CMC) for *E. coli* O111 LPS, ensuring all LPS is soluble [39]. For ITC a concentration of 0.5 mg/mL LPS was used, well above the

CMC, leading to micellar LPS, which will affect binding but represents a membrane more closely. Therefore, ITC results and dansyl-polymyxin B binding results are difficult to compare. Nevertheless, both assays show similar binding trends between the peptides, though not between different LPS species.

Concluding remarks

In this study the bactericidal activities of several HDPs were correlated with LPS-binding properties with the aim to provide further insight into antibacterial mechanisms of HDPs. The presence of an O-antigen affected binding affinities between LPS and HDPs and acyl chain composition showed an effect on stoichiometries of binding between LPS and HDPs. However, no clear-cut correlation between LPS-binding and bactericidal activity was found in this study. CATH-2 and PMAP-36 showed strong bactericidal activities regardless of LPS-binding affinities. PMAP-36 binding to LPS was enhanced by the presence of an O-antigen, indicating this peptide mediates binding through hydrophobic interactions. PMAP-23 binding to LPS was decreased by the presence of an O-antigen, indicating that this peptide mediates binding through hydrophilic interactions. However, MBCs did not correlate with LPS-binding affinities. Only for PR-39, an intracellular active peptide not inducing any membrane damage, a stronger LPS binding did correlate with an increased peptide activity.

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References

[1] M. Zasloff, Antimicrobial peptides of multicellular organisms, Nature. 415 (2002) 389–395. doi:10.1038/415389a.

[2] L. Zhang, R.L. Gallo, Antimicrobial Peptides, Springer Singapore, Singapore, 2019. doi:10.1007/978-981-13-3588-4.

[3] K. Matsuzaki, Control of cell selectivity of antimicrobial peptides., Biochim. Biophys. Acta. 1788 (2009) 1687–92. doi:10.1016/j.bbamem.2008.09.013.

[4] E.T. Rietschel, T. Kirikae, F.U. Schade, U. Mamat, G. Schmidt, H. Loppnow, A.J. Ulmer, U. Zähringer, U. Seydel, F. Di Padova, M. Schreier, H. Brade, Bacterial endotoxin: molecular relationships of structure to activity and function, FASEB J. 8 (1994) 217–225. doi:10.1096/fasebj.8.2.8119492.

[5] Kenneth Todar, Bacterial Endotoxin, Todar's Online Textb. Bacteriol. (2012). http://textbookofbacteriology.net/endotoxin.html (accessed October 24, 2019).

[6] O. Holst, A.J. Ulmer, H. Brade, H.-D. Flad, E.T. Rietschel, Biochemistry and cell biology of bacterial endotoxins, FEMS Immunol. Med. Microbiol. 16 (1996) 83–104. doi:10.1111/j.1574-695X.1996.tb00126.x.

[7] Y. Rosenfeld, Y. Shai, Lipopolysaccharide (Endotoxin)-host defense antibacterial peptides interactions: Role in bacterial resistance and prevention of sepsis, Biochim. Biophys. Acta - Biomembr. 1758 (2006) 1513–1522. doi:10.1016/j.bbamem.2006.05.017.

[8] O. Holst, S. Muller-Loennies, B. Lindner, H. Brade, Chemical structure of the lipid A of Escherichia coli J-5, Eur. J. Biochem. 214 (1993) 695–701. doi:10.1111/j.1432-1033.1993.tb17970.x.

[9] R. Stenutz, A. Weintraub, G. Widmalm, The structures of Escherichia coli O-polysaccharide antigens, FEMS Microbiol. Rev. 30 (2006) 382–403. doi:10.1111/j.1574-6976.2006.00016.x.

[10] L. Kenne, B. Lindberg, E. Söderholm, D.R. Bundle, D.W. Griffith, Structural studies of the O-antigens from Salmonella greenside and Salmonella adelaide, Carbohydr. Res. 111 (1983) 289–296. doi:10.1016/0008-6215(83)88313-X.

[11] R.D. Edstrom, E.C. Heath, The Biosynthesis of Cell Wall Lipopolysaccharide in Escherichia coli, J. Biol. Chem. 242 (1967) 4125–4133. doi:10.1016/s0021-9258(19)43278-x.

[12] S. Müller-Loennies, B. Lindner, H. Brade, Structural Analysis of Oligosaccharides from Lipopolysaccharide (LPS) of Escherichia coli K12 Strain W3100 Reveals a Link between Inner and Outer Core LPS Biosynthesis, J. Biol. Chem. 278 (2003)

34090–34101. doi:10.1074/jbc.M303985200.

[13] K. Amor, D.E. Heinrichs, E. Frirdich, K. Ziebell, R.P. Johnson, C. Whitfield, Distribution of core oligosaccharide types in lipopolysaccharides from Escherichia coli, Infect. Immun. 68 (2000) 1116–1124. doi:10.1128/IAI.68.3.1116-1124.2000.

[14] A. Ebbensgaard, H. Mordhorst, F.M. Aarestrup, E.B. Hansen, The role of outer membrane proteins and lipopolysaccharides for the sensitivity of escherichia coli to antimicrobial peptides, Front. Microbiol. 9 (2018) 2153. doi:10.3389/fmicb.2018.02153.

[15] M. Caroff, J.-R. Brisson, A. Martin, D. Karibian, Structure of the Bordetella pertussis 1414 endotoxin, FEBS Lett. 477 (2000) 8–14. doi:10.1016/S0014-5793(00)01720-8.

[16] A. Preston, B.O. Petersen, J. Duus, J. Kubler-Kielb, G. Ben-Menachem, J. Li, E. Vinogradov, Complete structures of Bordetella bronchiseptica and Bordetella parapertussis lipopolysaccharides, J. Biol. Chem. 281 (2006) 18135–18144. doi:10.1074/jbc.M513904200.

[17] M. Caroff, L. Aussel, H. Zarrouk, A. Martin, J.C. Richards, H. Thérisod, M.B. Perry, D. Karibian, Structural variability and originality of the Bordetella endotoxins, J. Endotoxin Res. 7 (2001) 63–68. doi:10.1179/096805101101532567.

[18] M.D. Balhuizen, A. van Dijk, J.W.A. Jansen, C.H.A. van de Lest, E.J.A. Veldhuizen, H.P. Haagsman, Outer Membrane Vesicles Protect Gram-Negative Bacteria against Host Defense Peptides, MSphere. 6 (2021) e00523-21. doi:10.1128/mSphere.00523-21.

[19] B.O. Murray, R.A. Dawson, L.M. Alsharaf, J. Anne Winter, Protective effects of Helicobacter pylori membrane vesicles against stress and antimicrobial agents, Microbiology. 166 (2020) 751–758. doi:10.1099/mic.0.000934.

[20] M.R. Scheenstra, M. van den Belt, J.L.M. Tjeerdsma-van Bokhoven, V.A.F. Schneider, S.R. Ordonez, A. van Dijk, E.J.A. Veldhuizen, H.P. Haagsman, Cathelicidins PMAP-36, LL-37 and CATH-2 are similar peptides with different modes of action, Sci. Rep. 9 (2019) 4780. doi:10.1038/s41598-019-41246-6.

[21] V.A.F. Schneider, M. Coorens, S.R. Ordonez, J.L.M. Tjeerdsma-Van Bokhoven, G. Posthuma, A. Van Dijk, H.P. Haagsman, E.J.A. Veldhuizen, Imaging the antimicrobial mechanism(s) of cathelicidin-2, Sci. Rep. 6 (2016) 1–11. doi:10.1038/srep32948.

[22] B. Agerberth, H. Gunne, J. Odeberg, P. Kogner, H.G. Boman, G.H. Gudmundsson, PR-39, a proline-rich peptide antibiotic from pig, and FALL-39, a tentative human counterpart, in: Vet. Immunol. Immunopathol., Elsevier, 1996: pp. 127–131. doi:10.1016/S0165-2427(96)05676-0.

[23] H.G. Boman, B. Agerberth, A. Boman, Mechanisms of action on Escherichia coli of cecropin P1 and PR-39, two antibacterial peptides from pig intestine, Infect. Immun. 61 (1993) 2978–2984. doi:10.1128/iai.61.7.2978-2984.1993.

[24] E.J.A. Veldhuizen, V.A.F. Schneider, H. Agustiandari, A. Van Dijk, J.L.M. Tjeerdsma-van Bokhoven, F.J. Bikker, H.P. Haagsman, Antimicrobial and immunomodulatory activities of PR-39 derived peptides, PLoS One. 9 (2014) e95939. doi:10.1371/journal.pone.0095939.

[25] E.J.A. Veldhuizen, M.R. Scheenstra, J.L.M. Tjeerdsma-van Bokhoven, M. Coorens, V.A.F. Schneider, F.J. Bikker, A. van Dijk, H.P. Haagsman, Antimicrobial and Immunomodulatory Activity of PMAP-23 Derived Peptides, Protein Pept. Lett. 24 (2017) 1–36. doi:10.2174/0929866524666170428150925.

[26] B. Orioni, G. Bocchinfuso, J.Y. Kim, A. Palleschi, G. Grande, S. Bobone, Y. Park, J. Il Kim, K. soo Hahm, L. Stella, Membrane perturbation by the antimicrobial peptide PMAP-23: A fluorescence and molecular dynamics study, Biochim. Biophys. Acta - Biomembr. 1788 (2009) 1523–1533. doi:10.1016/j.bbamem.2009.04.013.

[27] S. Kim, D.G. Lee, PMAP-23 triggers cell death by nitric oxide-induced redox imbalance in Escherichia coli, Biochim. Biophys. Acta - Gen. Subj. 1863 (2019) 1187–1195. doi:10.1016/J.BBAGEN.2019.04.014.

[28] Y. Sang, M. Teresa Ortega, K. Rune, W. Xiau, G. Zhang, J.L. Soulages, G.H. Lushington, J. Fang, T.D. Williams, F. Blecha, T. Melgarejo, Canine cathelicidin (K9CATH): Gene cloning, expression, and biochemical activity of a novel pro-myeloid antimicrobial peptide, Dev. Comp. Immunol. 31 (2007) 1278–1296. doi:10.1016/J.DCI.2007.03.007.

[29] E.J.A. Veldhuizen, M. Rijnders, E.A. Claassen, A. van Dijk, H.P. Haagsman, Porcine β-defensin 2 displays broad antimicrobial activity against pathogenic intestinal bacteria, Mol. Immunol. 45 (2008) 386–394. doi:10.1016/J.MOLIMM.2007.06.001.

[30] A.J. King, G. Berbers, H.F.L.M. Van Oirschot, P. Hoogerhout, K. Knipping, F.R. Mooi, Role of the polymorphic region 1 of the Bordetella pertussis protein pertactin in immunity, Microbiology. 147 (2001) 2885–2895. doi:10.1099/00221287-147-11-2885.

[31] W.F. Verwey, E.H. Thiele, D.N. Sage, L.F. Schuchardt, A simplified liquid culture medium for the growth of Hemophilus pertussis, J. Bacteriol. 58 (1949) 127–134. doi:10.1128/jb.58.2.127-134.1949.

[32] D.A. Heesterbeek, B.W. Bardoel, E.S. Parsons, I. Bennett, M. Ruyken, D.J. Doorduijn, R.D. Gorham, E.T. Berends, A.L. Pyne, B.W. Hoogenboom, S.H. Rooijakkers, Bacterial killing by complement requires membrane attack complex

formation via surface-bound C5 convertases, EMBO J. 38 (2019) e99852. doi:10.15252/embj.201899852.

[33] N. Mookherjee, M.A. Anderson, H.P. Haagsman, D.J. Davidson, Antimicrobial host defence peptides: functions and clinical potential, Nat. Rev. Drug Discov. 19 (2020) 311–332. doi:10.1038/s41573-019-0058-8.

[34] C.T. Choma, Characterizing Protein-Protein Interactions by ITC, TA Instruments. (2005) 1–6.

[35] D. Roversi, V. Luca, S. Aureli, Y. Park, M.L. Mangoni, L. Stella, How many antimicrobial peptide molecules kill a bacterium? The case of PMAP-23, ACS Chem. Biol. 9 (2014) 2003–2007. doi:10.1021/cb500426r.

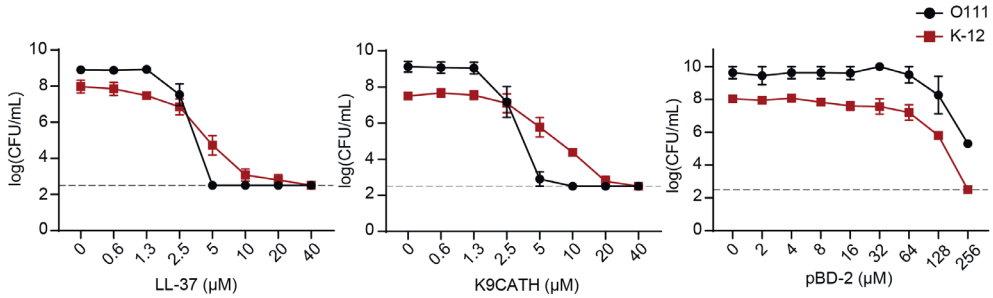
[36] A. Van Dijk, M. Van Eldik, E.J.A. Veldhuizen, H.L.M. Tjeerdsma-Van Bokhoven, M.R. De Zoete, F.J. Bikker, H.P. Haagsman, Immunomodulatory and anti-inflammatory activities of chicken cathelicidin-2 derived peptides, PLoS One. 11 (2016) 1–16. doi:10.1371/journal.pone.0147919.

[37] R. Moore, N.C. Bates, R.E.W. Hancock, Interaction of polycationic antibiotics with Pseudomonas aeruginosa Lipopolysaccharide and lipid A studied by using dansyl-polymyxin, Antimicrob. Agents Chemother. 29 (1986) 496–500. doi:10.1128/AAC.29.3.496.

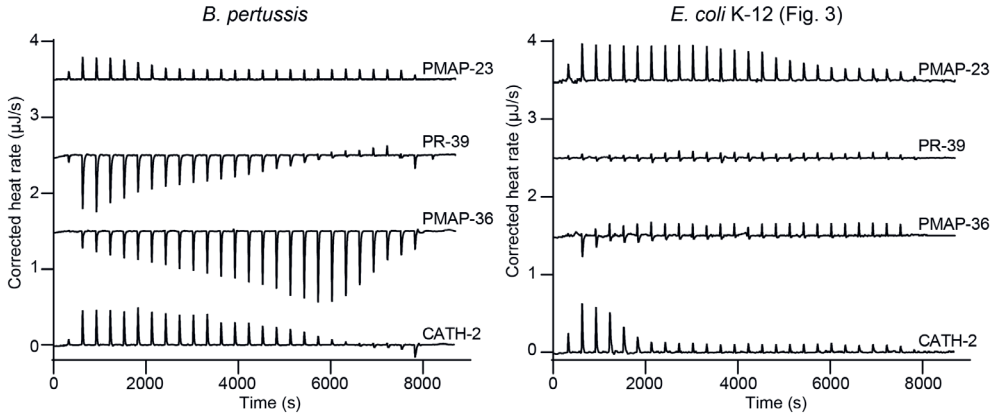
[38] N. Akhoundsadegh, C.R. Belanger, R.E.W. Hancock, Outer Membrane Interaction Kinetics of New Polymyxin B Analogs in Gram-Negative Bacilli, Antimicrob. Agents Chemother. 63 (2019) e00935-19. doi:10.1128/AAC.00935-19.

[39] C.A. Aurell, A.O. Wistrom, Critical aggregation concentrations of gram-negative bacterial lipopolysaccharides (LPS), Biochem. Biophys. Res. Commun. 253 (1998) 119–123. doi:10.1006/bbrc.1998.9773.

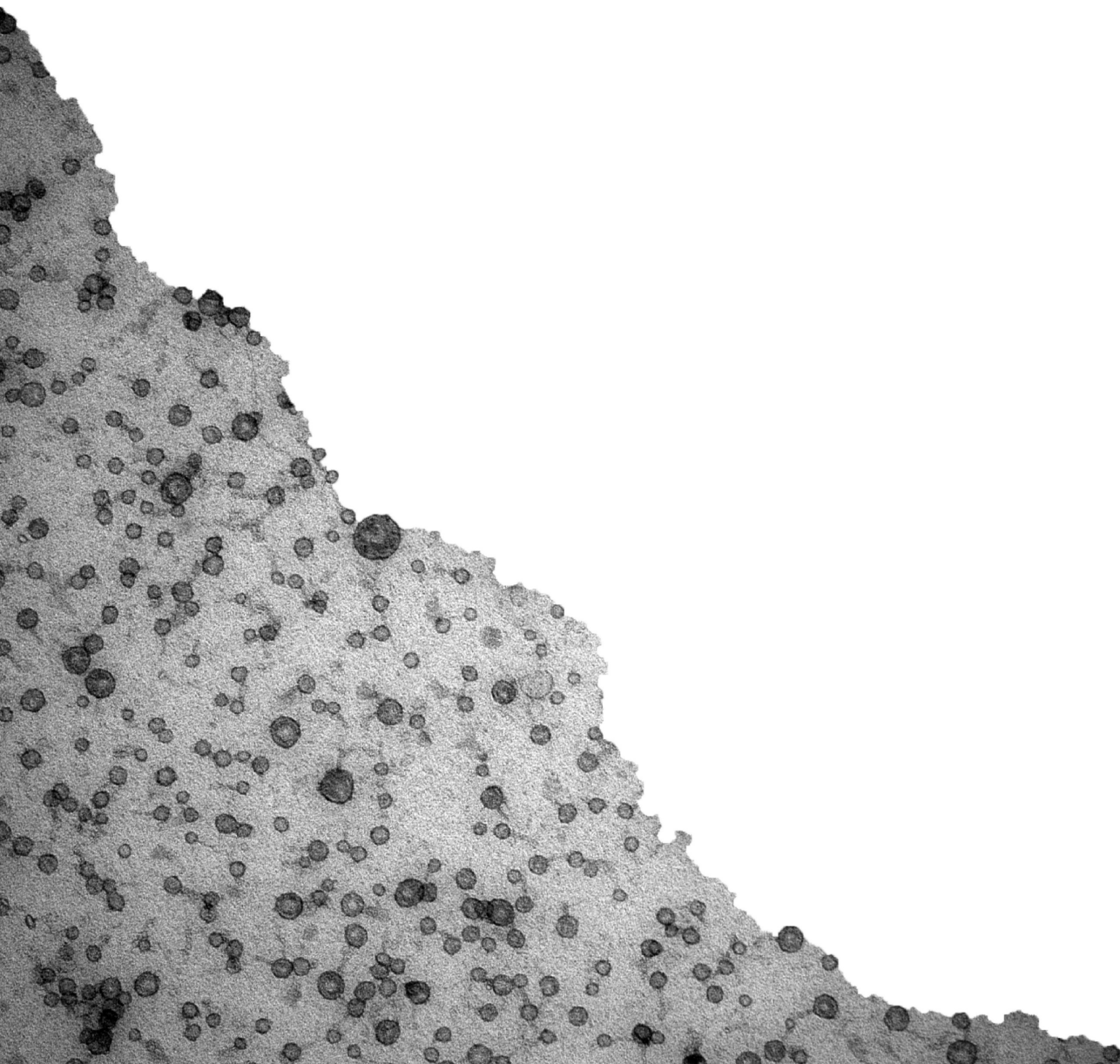
Supplementary Figures



Supplementary Figure 1: Determinations of MBC values of LL-37, K9CATH and pBD-2 for two E. coli strains. *Surviving bacterial colonies were detected after incubation with HDPs for 3 h in MHB (n=3). Shown is the mean with SEM, dashed line shows the detection limit.*



Supplementary Figure 2: ITC spectra of the interaction between LPS of B. pertussis and peptides. *CATH-2, PMAP-36, PR-39 or PMAP-23 (200 μM) was titrated into B. pertussis LPS solution (0.5 mg/mL) and heat rates were recorded. Data of HDP binding to E. coli K-12 LPS from Figure 3 has been inserted for comparison. Shown is a representative of two measurements.*



Chapter 5

PMAP-36 Reduces the Innate Immune Response induced by *Bordetella bronchiseptica*-derived Outer Membrane Vesicles

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Abstract

Host defense peptides (HDPs), such as cathelicidins, are small, cationic, amphipathic peptides and represent an important part of the innate immune system. Most cathelicidins, including the porcine PMAP-36, are membrane active and disrupt the bacterial membrane. For example, a chicken cathelicidin, CATH-2, has been previously shown to disrupt both *Escherichia coli* membranes and to release, at sub-lethal concentrations, outer membrane vesicles (OMVs). Since OMVs are considered promising vaccine candidates, we sought to investigate the effect of sub-bactericidal concentrations of PMAP-36 on both OMV release by a porcine strain of *Bordetella bronchiseptica* and on the modulation of immune responses to OMVs. PMAP-36 treatment of bacteria resulted in a slight increase in OMV release. The characteristics of PMAP-36-induced OMVs were compared with those of spontaneously released OMVs and OMVs induced by heat treatment. The stability of both PMAP-36- and heat-induced OMVs was decreased compared to spontaneous OMVs, as shown by dynamic light scattering. Furthermore, treatment of bacteria with PMAP-36 or heat resulted in an increase in negatively charged phospholipids in the resulting OMVs. A large increase in lysophospholipid content was observed in heat-induced OMVs, which was at least partially due to the activity of the outer-membrane phospholipase A (OMPLA). Although PMAP-36 was detected in OMVs isolated from PMAP-36-treated bacteria, the immune response of porcine bone-marrow-derived macrophages to these OMVs was similar as those against spontaneous or heat-induced OMVs. Therefore, the effect of PMAP-36 addition after OMV isolation was investigated. This did decrease cytokine expression of OMV-stimulated macrophages. These results indicate that PMAP-36 is a promising molecule to attenuate undesirable immune responses, for instance in vaccines.

Introduction

Outer membrane vesicles (OMVs) are spherical particles, 20-300 nm in size, that are naturally produced by all Gram-negative bacteria [1,2]. OMVs represent the outer membrane (OM) of the Gram-negative bacterium and comprise a large number and wide variety of surface-exposed antigens. This makes OMVs promising in vaccine development as has already been shown for *Neisseria meningitidis* and *Bordetella pertussis* [3–12]. Furthermore, the lipopolysaccharide (LPS) of OMVs acts as endogenous adjuvant, which is an additional advantage. However, large amounts of LPS can also cause the host immune system to overreact, evoking adverse effects against vaccine formulations. Injection with *E. coli* LPS has been shown to increase body temperature and heart rate, as well as white blood cell counts in healthy human volunteers [13]. Currently, LPS can be removed by detergent treatment, but thereby also important antigens, such as lipoproteins, are removed. Furthermore, production of large quantities of spontaneously released OMVs (sOMVs) is challenging. Current methods to induce OMV release, e.g. genetic modification and/or isolation through detergent treatment, may significantly alter OMV properties. A method to induce OMV release and maintain a native composition could be exposure of bacteria to natural stress, for instance heat [14–16] or Host Defense Peptides (HDPs).

HDPs are small, cationic, amphipathic peptides, naturally found in many species. They are produced by several types of cells, such as neutrophils and epithelial cells, and form an important part of the innate immune system [17–21]. One class of HDPs, cathelicidins, includes the porcine PMAP-36. This HDP is α-helical with a hinge region at the C-terminus containing a cysteine residue which allows the peptide to dimerize. These dimers are vital for the peptide's ability to interact with and neutralize LPS [22]. Thus far, several models for the interaction between HDPs and bacterial membranes have been proposed, all leading to membrane permeabilization and cell death [23].

Since HDPs attack the bacterial membrane, their mechanism is hypothesized to induce OMVs. OMV release is known to increase as Gram-negative bacteria respond to stressors. For instance, OMV release is increased when misfolded outer-membrane proteins accumulate in the periplasm through induction of the σ^E stress response [24]. Additionally, OMV release is upregulated by environmental stress, such as antibiotic exposure [25–27]. However, while vesicle release of *E. coli* has been observed at sub-bactericidal concentrations of CATH-2 [28], the effect of HDPs on OMV release has yet to be quantified.

In this study, we investigated the effects of sublethal concentrations of a very potent porcine HDP, PMAP-36, on OMV release in a porcine bacterium, *B. bronchiseptica*, with the goal to increase release of OMVs that are suited for vaccine usage. We compared PMAP-36-induced OMVs (pOMVs) with heat-induced OMVs (hOMVs) and sOMVs regarding size,

morphology, stability and lipid composition. Furthermore, we investigated the effect of OMVs on expression of cell-surface markers and cytokines by porcine bone marrow-derived M1 macrophages (pBMDM1). To modulate these immune responses, the immunomodulatory effect of PMAP-36 addition to isolated OMVs was also investigated.

Materials and methods

Peptide synthesis

PMAP-36 was synthesized by Fmoc-chemistry at China Peptides (CPC scientific, Sunnyvale, CA, USA). PMAP-36 was purified by reverse phase high-performance liquid chromatography to a purity of >95%. The sequence of the peptide is as follows: Ac-GRFRRLRKKTRKRLKKIGKVLKWIPPIVGSIPLGCG [22].

Bacterial strains and growth conditions

A clinical isolate of *B. bronchiseptica* from pig (BB-P19) provided by the Veterinary Microbiological Diagnostic Center ((VMDC), Division of Infectious Diseases & Immunology, Faculty of Veterinary Medicine, Utrecht University) was used throughout this study. For lipidomic analysis, also the canine isolate BB-D09 (from VMDC) and a *pldA* mutant derivative of this strain were used. The strains were grown on Difco™ Bordet-Gengou (BG) agar plates (Becton, Dickinson and Company, Sparks, USA), containing 1% glycerol (Merck, Darmstadt, Germany) and supplemented with 15% (v/v) defibrinated sheep blood (Oxoid Ltd, Basingstoke, Hampshire, UK). Liquid cultures were grown in Verwey medium [29] (pH 7.4) containing 0.1% (w/v) starch from potato (S2004, Sigma-Aldrich, St. Louis, MO, USA), 0.02% (w/v) KCl, 0.05% (w/v) KH_2PO_4, 0.01% (w/v) $MgCl_2 \bullet 6\ H_2O$ (all from Merck), 0.002% (w/v) nicotinic acid (Sigma-Aldrich), 1.4% (w/v) Bacto™ casamino acids (Becton, Dickinson and Company), and 0.001% (w/v) L-glutathione reduced (Sigma-Aldrich).

OMV isolation

To obtain OMVs from *B. bronchiseptica*, bacteria were grown overnight to an OD_{590} of approximately 1.5. Before OMV isolation was initiated, bacteria were incubated for one hour either at 56°C or with 0.5 µM of PMAP-36 at 37°C. Subsequently, bacterial cells were removed by centrifugation for 30 min at 4700 x g. The supernatant was passed through a 0.45 µm Whatman filter (GE Healthcare, Chicago, Illinois, USA) and centrifuged at 40,000 rpm for 2 h at 4°C (Ti-70 rotor, Beckman coulter, Brea, California, USA). The supernatant was decanted, and the transparent pellet was dissolved in 2 mM Tris-HCl (pH 7.5, Sigma-Aldrich) in a volume corresponding to 2% of the bacterial culture.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

Acrylamide gels (14%) were prepared as previously described [30]. For localization studies, samples were first treated with proteinase K (50 µg/mL, Thermo Fisher Scientific, Osterode am Harz, Germany) for 1 h at 37°C and then with phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich) for 30 min at room temperature. For analysis of protein content, OMVs were diluted in 2x concentrated sample buffer containing 5% v/v β-mercaptoethanol (Sigma-Aldrich), incubated for 10 min at 95°C, and 20 µL were loaded on gel. Gels were run for 30 min at 50 V and then another 60 min at 150 V. Gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 (Serva, Heidelberg, Germany) in 50:40:10 UltraPure water (MQ): methanol: acetic acid (Sigma-Aldrich, Honeywell, Charlotte, North Carolina, USA) and destained overnight in 80:10:10 MQ: methanol: acetic acid. For staining LPS with silver [31], samples were diluted in 2x sample buffer as described above. Subsequently, samples were treated with proteinase K (50 µg/mL) for 1 h at 60°C, and 12 µL were loaded on gel. After running, gels were rinsed with MQ for 5 min. Next, gels were fixed for 1 h with 40:10:50 isopropanol: acetic acid: MQ (Honeywell and Sigma-Aldrich) and oxidized with a fresh solution of 0.7% periodic acid (Sigma-Aldrich) for 10 min. The gel was then rinsed four times with MQ for 15 min and stained with 20% $AgNO_3$ (Merck) in 0.1 M NaOH and 0.0025% ammonia (both from Sigma-Aldrich). The gel was rinsed a further three times with MQ and developed for approximately 2 min with 0.005% (w/v) citric acid (Sigma-Aldrich) in 0.000185% (v/v) formaldehyde (Sigma-Aldrich). Gels were imaged with a Universal Hood III (Biorad, Hercules, California, USA).

Bicinchoninic acid (BCA) assay

Total protein concentration of isolated OMVs was determined using the Pierce BCA assay (Thermo Fisher Scientific). All samples were corrected for the signal of Verwey medium which was taken along during OMV isolation. In short, 25 µL of sample, supplemented with 2% SDS (Invitrogen, Carlsbad, California, USA), were incubated with 200 µL of working reagent at 37°C for 2 h. Absorbance was measured at 562 nm with FLUOstar Omega (BMG Labtech, Ortenberg, Germany). Bovine serum albumin (BSA, Sigma-Aldrich) was used as reference.

FM4-64 assay

Total lipid concentration of isolated OMVs was determined using the membrane-inserting fluorescent dye FM4-64 (Invitrogen). Samples (25 µL) were incubated with 200 µL FM4-64 (2.25 µg/mL) at 37°C for 10 min. Samples were excited at 485 nm and fluorescence was measured at 670 nm with the FLUOstar Omega.

Dynamic light scattering (DLS)

Samples for DLS were diluted 10-fold in 2 mM Tris-HCl unless stated otherwise. Samples were measured in micro-volume cuvettes (Sarstedt, Nümbrecht, Germany) on a Zetasizer nano (Malvern Panalytical, Malvern, UK) with a scatter angle of 173°. The standard polystyrene latex was used with a refractive index of 1.590 and absorbance of 0.010. Water was used as solvent (viscosity of 0.8872, refractive index of 1.330). Three measurements of 10-100 samplings were performed at 25°C unless stated otherwise. For the temperature gradient, steps of 5°C from 25-50°C were measured. Samples were equilibrated for 2 min and measured for 5 min.

Lipidomics

OMV pellets were obtained as described above but dissolved in phosphate-buffered saline (PBS, Thermo Fisher Scientific) instead of Tris-HCl. Lipids from OMVs were extracted using the method described by Bligh and Dyer [32]. Lipid extracts were dried under N_2, dissolved in 100 µL of chloroform and methanol (1:1), and injected (10 µL) into a hydrophilic interaction liquid chromatography column (2.6 µm HILIC 100 Å, 50x4.6 mm, Phenomenex, CA). Lipid classes were separated by gradient elution on an Infinity II 1290 UPLC (Agilent, CA) at a flow rate of 1 mL/min. A mixture of acetonitrile and acetone (9:1, v/v) was used as solvent A, while solvent B consisted of a mixture of acetonitrile, H_2O (7:3, v/v) with 50 mM ammonium formate. Both A and B contained 0.1% formic acid (v/v). Gradient elution was done as follows (time in min, % B): (0, 0), (1, 50), (3, 50), (3.1, 100), (4, 100). No re-equilibration of the column was necessary between successive samples. The column effluent was connected to a heated electrospray ionization source of an Orbitrap Fusion mass spectrometer (Thermo Scientific, MA) operated at -3600 V in the negative ionization mode. The vaporizer and ion transfer tube were set at a temperature of 275°C and 380°C, respectively. Full scan measurements (MS1) in the mass range from 450 to 1150 amu were collected at a resolution of 120.000. Parallelized data-dependent MS2 experiments were done with HCD fragmentation set at 30 V, using the dual-stage linear ion trap to generate up to 30 spectra per second. Data processing was based on the package 'XCMS' for peak recognition and integration [33]. Lipid classes were identified based on retention time and molecular species were then matched against an *in silico* generated lipid database. This database was constructed based on observed and theoretical fatty acyls and phospholipids, as well as theoretical ornithine lipids and is available at http://www.lipidomics.nl/resources. Mass accuracy of annotated lipids was typically below 2 ppm.

Electron microscopy (EM)

For negative staining of OMVs, a protocol was provided by the Cell Microscopy Center (CMC, University Medical Center, Utrecht). In short, copper grids were carbon activated, incubated with 10 µL vesicle solution for 10-30 min and washed three times with PBS. The solution was fixed on the grids using 1% glutaraldehyde (Sigma-Aldrich) in PBS for 10 min and washed two times with PBS and subsequently four times with MQ. The grids were then briefly rinsed with methylcellulose/uranyl acetate (pH 4, provided by the CMC) and incubated for 5 min with methylcellulose/uranyl acetate (pH 4) on ice. Grids were looped out of the solution and air dried. Samples were imaged on a Tecnai-12 electron microscope (FEI, Hillsboro, Oregon, USA).

Porcine primary macrophages

Primary cells isolated from pig bone marrow were differentiated into M1 macrophages using porcine granulocyte-macrophage colony-stimulating factor (GM-CSF) as previously described [34]. In short, the bone marrow cells were thawed, seeded at 50,000 cells per well, and cultured in 0.1% GM-CSF (Biorad) in Roswell Park Memorial Institute +/+ medium (RPMI, Thermo Fisher Scientific). At day 3 of the culture, cells were supplemented with 50 µL of 0.1% GM-CSF. At day 6, the differentiated macrophages were stimulated for 24 h with isolated OMVs (0.5 µg/mL protein) or purified LPS (10 ng/mL, kindly provided by J. Pérez Ortega, Utrecht University). Thereafter, cell markers and cytokines were measured using fluorescence-activated cell sorting (FACS, Becton, Dickinson and Company) and enzyme-linked immunosorbent assay (ELISA), respectively. For FACS, antibodies against the following surface markers and their dilutions were: Swc3α-PE (1:4000, Invitrogen, Carlsbad, California, USA), recombinant CTLA-4-MuIg-APC (1:1000, Ancell, Stillwater, Minnesota, USA) which binds to porcine CD80/86, CD163-FITC (1:1000), and human CD14-PB (1:100, both from Biorad). For cytokine detection, corresponding ELISA kits were used (R&D Systems, Minneapolis, Minnesota, USA).

Results

Dose determination of PMAP-36 treatment

To determine whether PMAP-36 stimulates OMV release, *B. bronchiseptica* strain BB-P19 was incubated with PMAP-36 at concentrations varying from 0-5 µM. The maximum concentration tested, 5 µM, is 4-fold below the minimum bactericidal concentration for an overnight culture (MBC, data not shown). First, OMV release was studied by analyzing the protein content of isolated OMV fractions using SDS-PAGE, which revealed an increase in protein concentration with increasing PMAP-36 concentrations **(Fig. 1a)**. Remarkably,

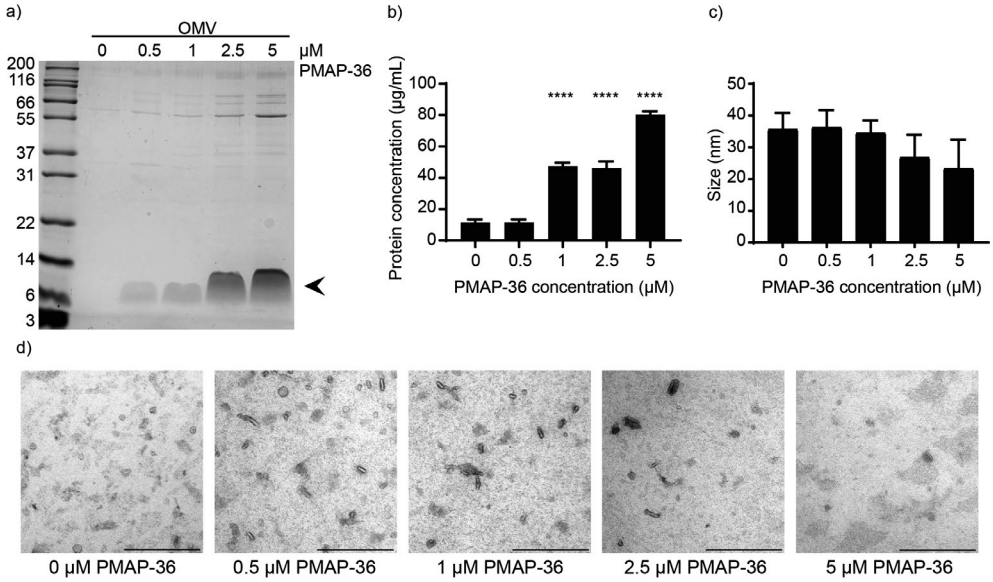

Figure 1: Effect of PMAP-36 on OMV release of B. bronchiseptica. *(a) Increasing concentrations of PMAP-36 were supplemented during OMV induction and isolated OMVs were analyzed with SDS-PAGE. Black arrow points to PMAP-36 dimers present in isolated OMVs. (b) Protein concentrations of isolated OMVs were quantified using the BCA assay. Shown is the mean of three measurements with SD. Significant differences are indicated by ****p<0.0001, obtained using a one-way ANOVA with a post-hoc Dunnett test, compared to 0 µM PMAP-36. (c) Size of isolated OMVs was determined using DLS. Shown is the mean of three measurements with SD. (d) Isolated OMVs were visually inspected using EM. Bars represent 200 nm. Shown is a representative image of three individual experiments.*

PMAP-36 was observed in the isolated OMV fractions around 7 kDa, presumably representing the dimeric form of the peptide despite the presence of β-mercaptoethanol in the sample buffer. An increase in protein concentration in the isolated OMV fraction was confirmed quantitatively using a BCA assay **(Fig. 1b)**. An increase in protein concentration with increasing PMAP-36 concentrations suggests an increase in OMV release. However, at higher concentrations, the mere increase of PMAP-36 present in the OMVs could also be reflected in a higher protein signal in the BCA assay. Furthermore, a higher protein concentration does not necessarily correspond to an increase in intact OMVs, but can also be caused by soluble proteins. Therefore, the integrity of the released OMVs was investigated by EM visualization and size measurements by DLS. It was observed that at the highest concentrations of PMAP-36, the average size of OMVs tended to decrease **(Fig. 1c)**. This result could indicate that OMVs are disintegrating at high PMAP-36 concentrations. The electron micrographs also suggested that less OMVs were present in the samples at 2.5 and 5 µM PMAP-36, probably due to disintegration of OMVs. Furthermore, gray patches were observed in the background at 5 µM of PMAP-36, indicating protein from the sample had dried and was stained **(Fig. 1d)**. This result suggests

that OMVs are disrupted at high PMAP-36 concentrations and release their protein content. Furthermore, DLS and EM showed that 1 µM of PMAP-36 was the maximum safe concentration to induce OMVs. Therefore, to prevent any OMV disruption, a safe concentration of 0.5 µM of PMAP-36 was used in further experiments.

To investigate the localization of PMAP-36, inside or outside the OMV, proteinase K digestion was applied followed by analysis of the protein patterns using SDS-PAGE. The PMAP-36 band of around 7 kDa was lost in the OMV samples, indicating PMAP-36 was accessible for proteinase K **(Fig. S1)**. This suggests that PMAP-36 is on the outside of the OMV. Remarkably, PMAP-36 digestion was incomplete in the control condition, i.e. when pure peptide was digested with proteinase K. This could be due to aggregation, therefore rendering PMAP-36 less accessible for digestion.

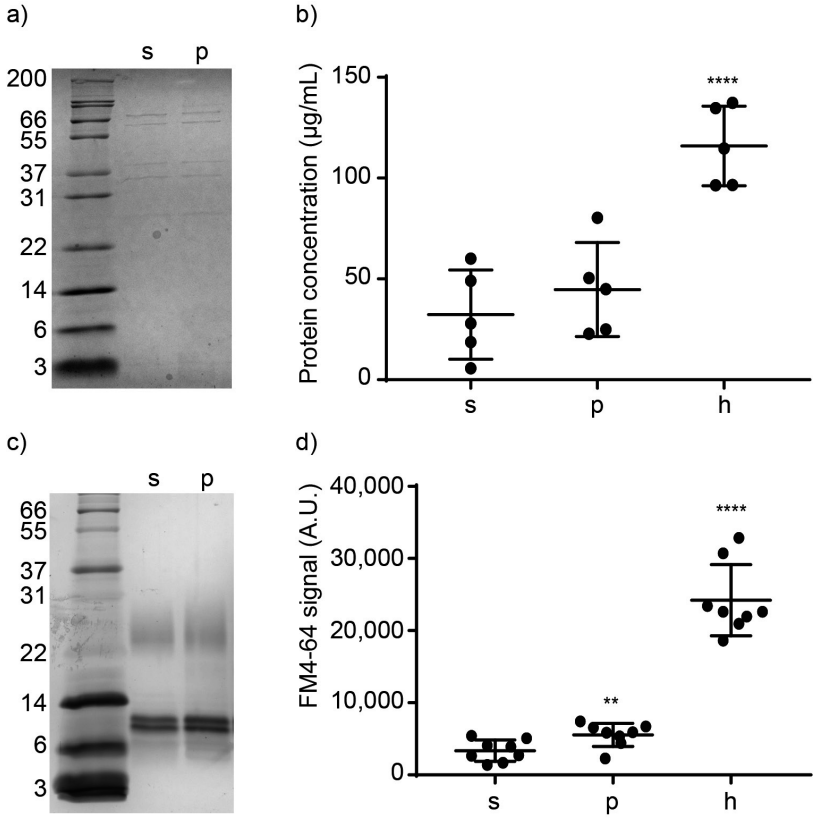

Figure 2: Protein and lipid quantification of induced OMVs. *(a-b) Protein content and concentrations of isolated OMVs were determined using (a) SDS-PAGE and quantified using (b) BCA assay (n=5). (c) LPS content of isolated OMVs was visualized using silver staining on SDS-PAGE and (d) total lipid concentration was quantified using FM4-64 lipid dye (n=8). s = sOMVs, p = pOMVs, h = hOMVs. Significant differences compared to sOMVs are indicated by **p<0.01, ****p<0.0001, obtained using a one-way ANOVA with a post-hoc Dunnett test. Gels are representative of three individual experiments.*

Protein quantification of isolated OMVs

The isolated vesicles were inspected using SDS-PAGE to visualize differences in OMV release. Protein patterns on SDS-PAGE were identical for sOMVs and pOMVs **(Fig. 2a)**. Visually, no differences in quantity could be observed. Therefore, the protein concentration in the isolated OMVs was quantified using a BCA assay. Since heat-treatment was previously shown to induce OMVs in *B. pertussis* and in a canine strain of *B. bronchiseptica* [16], it was taken along as control treatment. PMAP-36 treatment showed a slight, but not significant, increase in the protein concentration of isolated OMVs (1.4x), whereas heat treatment did show a significant increase in protein concentration of isolated OMVs (3.6x) **(Fig. 2b)**.

Lipid quantification of isolated OMVs

In addition to protein quantification, OMV release was also determined by analysis of lipid quantities. LPS was visualized by staining with silver **(Fig. 2c)**. Two bands for the lipid A plus core sugar moiety were visible around 10 kDa, with the higher band caused by PagP activity, which has been shown to transfer a 16 carbon acyl chain from phospholipids to *B. bronchiseptica* LPS [35]. Also O-antigen-containing LPS was observed around 25 kDa [36]. A slight increase in LPS quantity was observed for pOMVs. Subsequently, lipid concentrations were compared using the FM4-64 lipid dye, revealing a significant 1.7-fold increase for pOMVs and a 6.5-fold increase for hOMVs relative to sOMVs **(Fig. 2d)**.

Morphological characterization of isolated OMVs

Previously, OMVs have been described as 20-300 nm spherical blebs of the OM [37,38]. *B. bronchiseptica* OMVs were observed to be at the small end of this spectrum in this study. The size of OMVs, measured with DLS, ranged from 20-40 nm **(Fig. 3a)**. EM analysis revealed even smaller OMVs than those shown by DLS measurements, with vesicles as small as 15 nm **(Fig. 3b)**. Neither PMAP-36 nor heat treatment affected OMV size. However, in pOMV samples, tubular structures were observed with EM **(insert Fig. 3b)**. Presumably, PMAP-36 interacts with the OMV membrane and affects their shape.

Thermal stability of isolated OMVs

To determine the thermal stability of isolated OMVs, the size and counts of OMVs were measured with DLS while being subjected to a temperature gradient. Temperature was raised from 25°C to 50°C with 5°C steps and the OMVs from different induction methods were compared. sOMVs showed no differences in size with increasing temperature, suggesting they are stable at higher temperatures **(Fig. 3c, left panel)**. However, pOMVs increased in size from 35°C onwards, indicating their aggregation or fusion. hOMVs tended

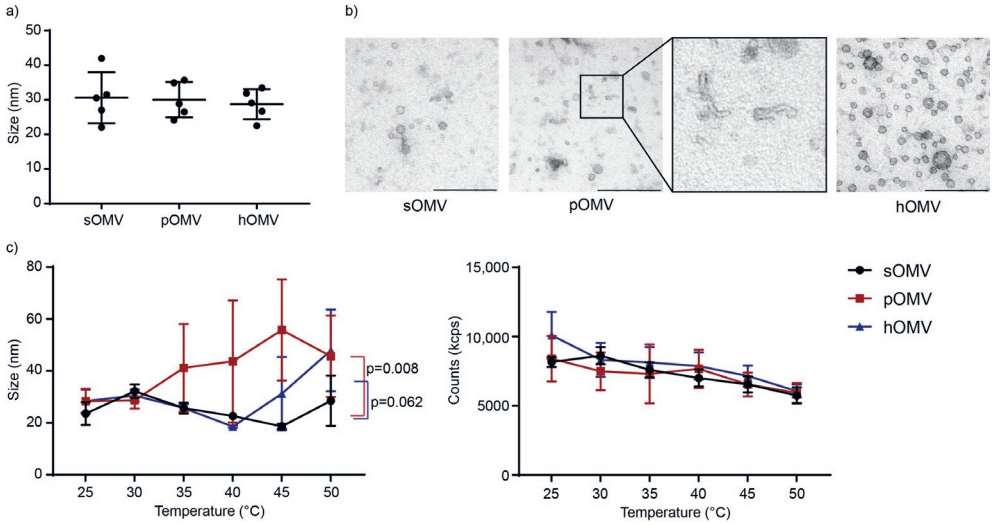

Figure 3: Morphology and stability of induced OMVs. *(a) Size of isolated OMVs was measured with DLS (n=5). (b) Isolated OMVs visualized using EM. Insert of pOMVs is four times enlarged. Shown is a representative image of three individual experiments. Bars represent 200 nm. (c) Stability of OMVs was assessed by size (left panel) and count (right panel) variations over a temperature gradient (n=3). Shown is the mean with SEM. Statistic differences compared to sOMVs were calculated using a linear mixed-model analysis with a post-hoc Dunnett test.*

to increase in size from 45°C onwards, being more stable than pOMVs, but slightly less stable than sOMVs. Counts decreased for all OMV types, likely due to sedimentation during the measurement **(Fig. 3c, right panel)**.

Lipidomic analysis of isolated OMVs

The lipid classes in isolated OMVs were investigated to explain changes in morphology and stability. Differences in phospholipid composition of the different OMV preparations were clearly observed by mass spectrometric analysis. Cardiolipin was detected in the OMVs but was excluded from further analysis as it could not be reliably quantified due to interfering compounds in the OMV isolates. Of the phospholipid classes, phosphatidylglycerol (PG) was increased in OMVs obtained after treatment of bacteria with either PMAP-36 or heat **(Fig. 4a)**. Furthermore, the lysophospholipid content in OMVs was increased after heat treatment, while the phosphatidylethanolamine (PE) content was reduced, consistent with its conversion into lyso-PE **(Fig. 4a)**. When compared to the lipid composition of whole cells (WC), lysophospholipids appeared exclusively present in OMVs **(Fig. 4b)**. The increase in lysophospholipids in hOMVs could be due to enzymatic hydrolysis by outer-membrane phospholipase A (OMPLA), which is encoded by the *pldA* gene. To test this possibility, we analyzed lipid species of OMVs isolated from a *pldA* mutant in a canine strain of *B. bronchiseptica*, BB-D09, since we were unable to introduce the mutation into BB-P19. The OMVs of the wild-type canine strain showed differences in lipid composition relative to

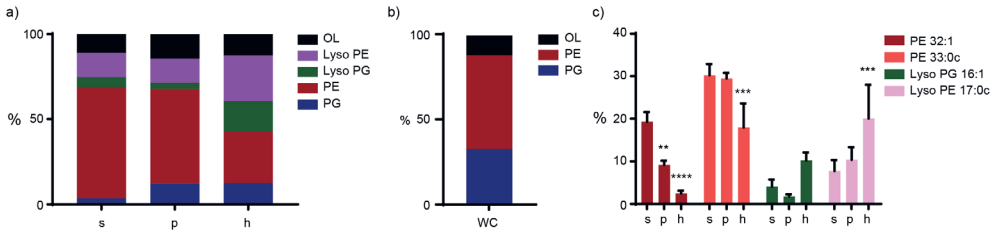

Figure 4: Lipidomic analysis of isolated OMVs. *Isolated OMVs were subjected to mass spectrometry to determine lipid composition (n=3). (a) Total lipid composition of OMVs and (b) of whole cells. (c) Four most affected lipid species. s = sOMVs, p = pOMVs, h = hOMVs, WC = Whole Cells. Significant differences compared to sOMVs are indicated by **p<0.01, ***p<0.001, ****p<0.0001, obtained using a paired two-way ANOVA with a post-hoc Dunnett test.*

those of the porcine strain, such as the relatively higher abundance of PG **(Fig. S2)**. However, it was still considered a good model to investigate the influence of OMPLA. The *pldA* mutant was also subjected to heat or peptide treatment before OMV isolation and the lipidomic analysis of the OMVs indicated that although lysophospholipids were still detectable **(Fig. S2)**, their abundance is strongly diminished in the *pldA* mutant. These data indicate that the majority of the lysophospholipids produced upon heat treatment results from OMPLA activity. The remaining portion of lysophospholipids, present in both sOMVs and hOMVs, might be the result of enzymes such as the acyltransferase PagP [35,39].

Around 90% of the lipids could be identified as belonging to only thirteen lipid species **(Fig. S3)**. PE was found to be the most prominent lipid class, specifically with 16:0, 16:1 or 17:0c fatty acid chains, where the 'c' indicates a cyclopropane moiety in the acyl chain. Concordantly, lysoPE 16:0, 16:1 and 17:0c were the most prominent lysophospholipids. *B. bronchiseptica* was shown before to contain mostly 16:0 and 17:0c fatty acid chains, but 16:1 appeared to be less prominent in previous datasets [40,41]. In our study, 16:0 and 16:1 seem to be present in comparable amounts. The most affected lipid species in OMVs obtained after either peptide or heat treatment were PE 32:1, PE 33:0c, lysoPG 16:1 and lysoPE 17:0c **(Fig. 4c)**. PE 33:0c was found to decrease only upon heat treatment, while PE 32:1 decreased upon both PMAP-36 and heat treatment. Furthermore, lysoPE 17:0c was found to increase in hOMVs. Presumably, lysoPE 17:0c is the product of enzymatic conversion of its diacyl counterpart, for instance by the acyltransferase activity of PagP. Additionally, lysoPG 16:1 increased in the hOMVs and showed a decreasing trend in pOMVs **(Fig. 4c)**.

Next to phospholipids, another class of polar lipids was identified in the lipid extract. Ultrahigh resolution, accurate mass tandem mass spectrometry demonstrated notable amounts of ornithine lipids (OL) in OMVs **(Fig. S4)**. This lipid class has previously been described to be present in Bordetella pertussis [40]. Furthermore, this lipid contains two fatty acids that can vary, mostly being two 16:0 acyl chains (approximately 8-10%) or one

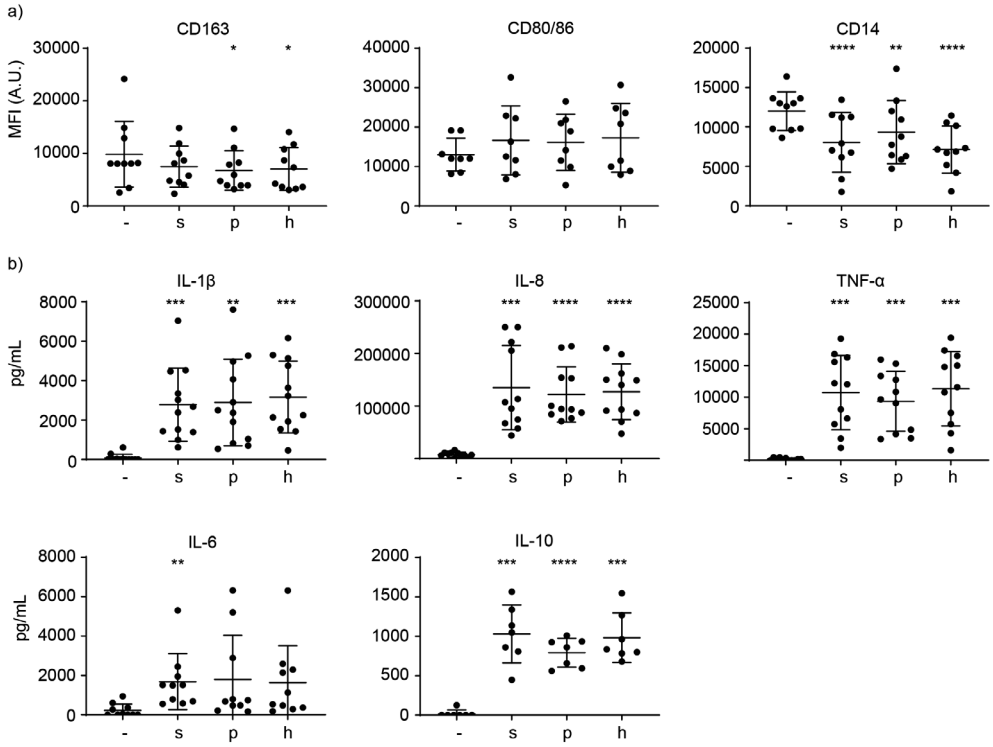

Figure 5: pBMDM1 response to induced OMVs. *pBMDM1 macrophages were stimulated with isolated OMVs and activation was measured by determining (a) cell-surface markers using FACS and (b) cytokines using ELISA. MFI = mean fluorescence intensity. - = no stimulation, s = sOMVs, p = pOMVs, h = hOMVs (n=7-12). Significant differences compared to the non-stimulated control are indicated by *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, obtained using a repeated measures one-way ANOVA with Geisser-Greenhouse correction and post-hoc Dunnett test.*

16:0 and one 14:0 (approximately 2-4%) **(Fig. S3)**. Strikingly, the relative amounts of these lipids in the OMVs were not influenced by either treatment.

Immune response of pBMDM1 on OMVs induced by different methods

pBMDM1 were used to investigate immune responses induced by OMVs. After 24 h stimulation with OMVs, cells were gated on the myeloid marker SWC3α and pBMDM1 activation was determined by measuring cell-surface markers CD163, CD80/86 and CD14 using FACS. The expression of CD163 and CD80/86 was marginally affected after stimulation of pBMDM1s with OMVs, whilst CD14 expression was significantly decreased **(Fig. 5a)**. However, expression of all markers was similar after stimulation with sOMVs, pOMVs or hOMVs. Macrophage activation was further determined by measuring the release of cytokines TNF-α, IL-1β, IL-6, IL-8 and IL-10 using ELISA. Cytokine release was induced by OMVs but again no differences were observed between different OMVs

(Fig. 5b). These results suggest that although all OMVs could potently induce immune responses, these were not affected by the induction method used for OMV release.

Immune response of pBMDM1 to OMVs supplemented with PMAP-36

The lack of effect of PMAP-36 on immune responses in peptide-induced OMVs could be explained by possible inaccessibility or a too low concentration of PMAP-36 in OMVs after isolation to neutralize LPS. Therefore, free PMAP-36 was added to isolated OMVs and the subsequent immunomodulation of pBMDM1 responses was studied. Expression of cell-surface markers CD163 and CD80/86 showed no differences upon stimulation with OMVs and PMAP-36, whilst CD14 slightly increased but only significantly for stimulation with hOMVs and PMAP-36 **(Fig. S5)**, indicating neutralization of the LPS-induced CD14 decrease observed earlier. Furthermore, cytokine release showed a decrease in pro-inflammatory IL-1β, IL-6, IL-8 and TNF-α, as well as in anti-inflammatory IL-10 secretion upon PMAP-36 addition to OMVs **(Fig. 6)**.

PMAP-36 has previously been shown to interact with *E. coli* LPS and neutralize subsequent macrophage responses [42]. To investigate whether this mechanism holds true for PMAP-36 and *B. bronchiseptica* LPS, experiments were performed using purified *B. bronchiseptica* LPS. Cell-surface marker expression was not affected by addition of PMAP-36. However, addition of PMAP-36 did result in a decreasing trend in LPS-induced cytokine release of pBMDM1 **(Fig. S6)**. The decrease was significant for TNF-α and IL-10, suggesting that the immune suppression of PMAP-36 was inhibiting macrophage activation in general, since both pro- and anti-inflammatory cytokine expression was decreased by PMAP-36.

Discussion

In this study, a sub-lethal concentration of PMAP-36 was applied to *B. bronchiseptica* to induce OMV release. The pOMVs were extensively assessed to investigate their properties and qualities. Lipid quantification showed that PMAP-36 treatment significantly increased OMV release **(Fig. 2d)**. This increase is consistent with the literature, which shows that stress induces release of OMVs [14–16,24,26,37,43]. Previously, it has been demonstrated that antibiotics increased OMV release in *E. coli*. However, the effect varied from a 2- to 4-fold induction for tetracycline, ampicillin, and ceftriaxone, up to a 10-fold induction by polymyxin B [44]. Additionally, in *Pseudomonas aeruginosa*, treatment with D-cycloserine and polymyxin B both demonstrated a 6 to 9-fold increase in OMV release [45]. The stress induction by PMAP-36 might be explained by its interactions with membranes. Cationic HDPs interact electrostatically with the negatively charged bacterial membrane. In *B. bronchiseptica* however, the phosphate groups on LPS appeared to be shielded to a large extent by glucosamines [46], possibly explaining the small effect of PMAP-36 on OMV

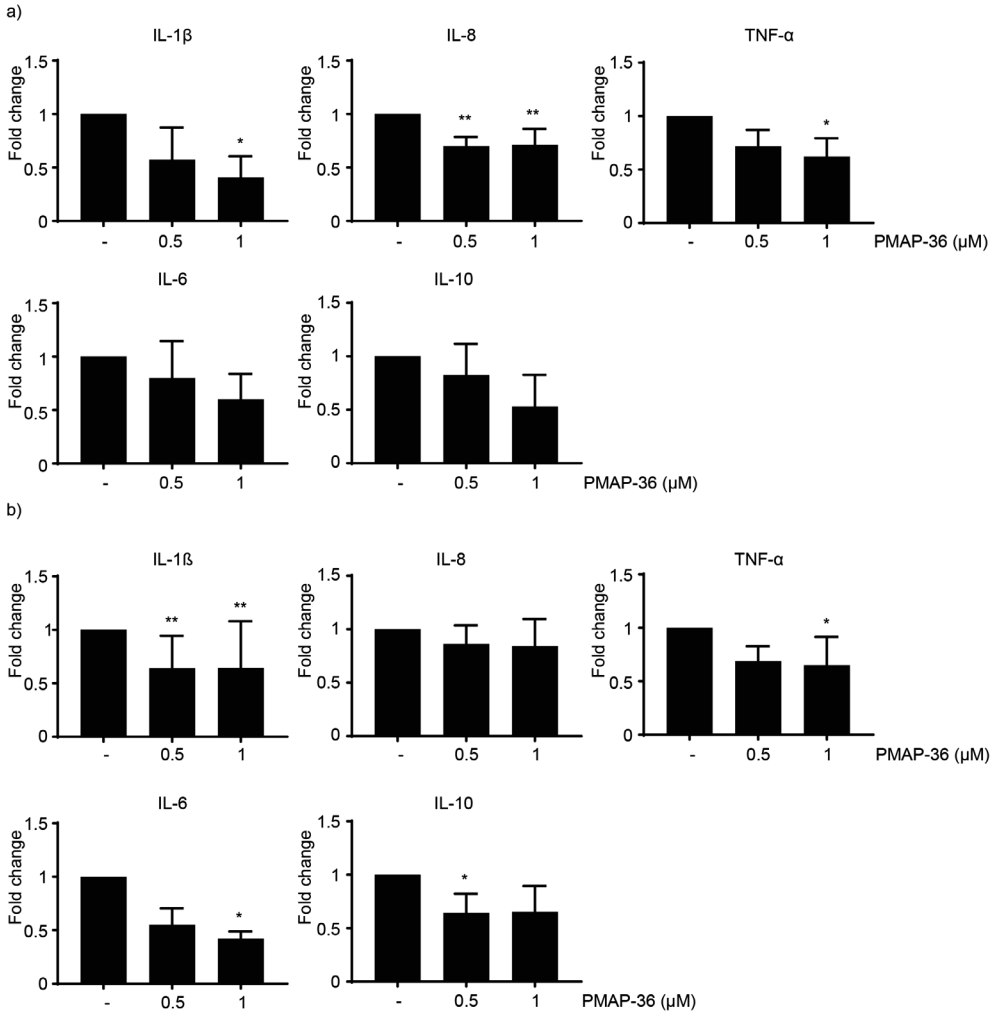

Figure 6: PMAP-36 modulation of pBMDM1 cytokine expression in response to OMVs. *pBMDM1 macrophages were stimulated with (a) sOMVs or (b) hOMVs in the presence of different concentrations of PMAP-36. Cytokine secretion was determined by ELISA. Values were converted to fold change with respect to the results obtained with OMVs not supplemented with PMAP-36. Shown is the mean with SD. Significant differences are indicated by *p<0.05, **p<0.01, obtained by using a linear mixed-model analysis on the raw data with a post-hoc Dunnett test (n=3-7).*

formation. Additionally, because HDPs contain a hydrophobic region, the hydrophobic core of the membrane can be reached and high concentrations of HDPs can lead to membrane permeabilization, for which several models have been suggested [21,23]. Before HDP-induced membrane permeabilization occurs, OMVs could already be formed due to the bulging effect of HDPs on the membrane. OMV release in response to HDPs

could be a defense mechanism, with bacteria either using OMVs to remove HDP-affected membranes or using them as decoy membrane for the HDPs [44].

PMAP-36 indeed interacted with the bacterial membrane, since it was found in the OMV fraction. Proteinase K digestion revealed PMAP-36 to be, at least partially, on the outside of the OMV **(Fig. S1)**. Remarkably, PMAP-36 digestion by proteinase K in the control condition, i.e. pure PMAP-36 without OMVs, was incomplete. A possible explanation is that PMAP-36 in pure solution aggregates, rendering large parts of the peptide inaccessible for proteinase K, while the peptide on OMVs is prevented from aggregation, rendering the peptide accessible. This suggests that PMAP-36 is not deeply inserted into the OMV membrane. PMAP-36 induction showed an increasing trend for protein concentration and a significant increase in lipid quantities in the isolated OMV fraction, suggesting PMAP-36 treatment enhances OMV release **(Fig. 2)**. It needs to be taken into account that both assays could be influenced by the presence of PMAP-36 in the OMVs. PMAP-36 gives a signal in the BCA assay, being partly responsible for the increase in protein concentration. Furthermore, the presence of PMAP-36 could potentially hamper or promote insertion of FM4-64 into the OMV membrane, influencing the quantification. In any case, heat treatment was observed to increase OMV release to a larger extent than PMAP-36 treatment, making it more suitable method for production of large amounts of OMVs [16]. However, stress might alter OMV properties, and stress-induced OMVs must therefore be characterized further to confirm their potential for vaccine applications.

Occasionally, pOMVs appeared cylindrical in EM studies **(Fig. 3)**. The morphology of synthetic vesicles has previously been described to be influenced by peptides [47]. The use of PMAP-36 for OMV release also affected the stability of resulting OMVs. OMVs induced by PMAP-36 stress started to aggregate around 35°C. Similarly, hOMVs tended to aggregate around 45°C, but size differences were not significant. In both cases, variation was considerable, probably due to the fact that not all OMVs aggregated at a similar temperature or time point. At 40°C, for example, part of the OMV population might still have their original size, while another part has already aggregated. To decrease heterogeneity, temperature incubations could be prolonged in time. However, sOMVs, pOMVs and hOMVs all showed a slight decrease in counts during the measurement, probably due to sedimentation, so this could influence the measurement if time would be too prolonged. Remarkably, counts of pOMVs and hOMVs did not decrease quicker than those of sOMVs, which one would expect when particles aggregate. Perhaps, larger particles are counted more frequently and therefore this was not observed. Since PMAP-36 was observed to be present in the isolated OMVs, it could be inserted and thereby destabilize the membrane. However, the stability of sOMVs to which 0.5 µM PMAP-36 was added did not decrease with increasing temperatures (data not shown). Furthermore, EM showed no morphological differences of the sOMVs after PMAP-36 addition (data not

shown). Possibly, PMAP-36 is unable to insert at that concentration into OMVs after their isolation.

To further explain differences observed in stability, lipid species in isolated OMVs were identified using mass spectrometry **(Fig. 4)**. sOMVs contained mostly PE **(Fig. S3)**, in agreement with previous studies of the *B. bronchiseptica* total cell lipidome. These previous studies are extended by our lipidomic characterization of OMVs, demonstrating the presences of specifically PE 33:0c (30.2%) and PE 32:1 (19.2%). This is in agreement with the reported preference of *B. bronchiseptica* for 16:0, 16:1 and 17:0c fatty acids [40,41]. PE is a lipid with a relatively small head group, preferring structures with a negative curvature, and it could make up the inner monolayer of the OMV. In pOMVs, the amount of PG species, which are negatively charged, was enriched relative to that in sOMVs. This can be linked to the interaction between negatively charged PG and positively charged PMAP-36. During OMV formation, phospholipid concentration in the outer leaflet increases [48]. The bacteria could use negatively charged OMVs to dispose already bound PMAP-36 or to act as decoy for free PMAP-36. The PG species in the pOMVs had identical fatty acid composition compared to the most abundant PE species in sOMVs, in accordance with the preference of *B. bronchiseptica* for these acyl chains **(Fig. S3)**. Upon heat treatment on the other hand, there was a relative increase in lysophospholipids at the cost of diacyl phospholipids. Both OMPLA and PagP are enzymes in the OM capable of enzymatic conversion of phospholipids. OMPLA functions primarily as phospholipase A1 and is capable of removing an acyl chain, but also has phospholipase A2 and 1-acyl- and 2-acyllysophospholipase activity [49]. PagP is an acyltransferase and not only specifically removes a 16:0 acyl chain from phospholipids, but transfers this acyl chain to LPS [35]. The relative increase of lysoPE 17:0c can be linked to the relative decrease of PE 33:0c, where a 16:0 fatty acid is lost, probably due to PagP transferring this acyl chain to LPS. Remarkably, the decrease of PE 32:1 did not result in an increase in lysoPE 16:0 or lysoPE 16:1, as one would expect. This could be due the activity of OMPLA, potentially converting PE into mere acyl chains and a glycerophosphoryldiester. Furthermore, the lysoPG content of hOMVs was increased relative to that in sOMVs, while diacyl PG didn't decrease. It needs to be taken into account that a decrease of a specific lipid in the OMV lipidome can also be the result of retention of that lipid in the bacterial OM and vice versa. Lipidomic analysis of the OMVs of a canine *pldA* mutant strain revealed that OMPLA-mediated enzymatic hydrolysis was the main process responsible for hydrolysis upon heat treatment. However, some production of lysophospholipids was still observed at a lower temperature, even in the *pldA* mutant. The active site of both OMPLA and PagP is situated in the outer leaflet of the bilayer, leading to lysophospholipids to also end up in the outer leaflet of OMVs. Since lysophospholipids only contain a single fatty acid, they tend to form structures with a high positive curvature, such as micelles [50]. Therefore, they are very well suited to make up

the outer leaflet of a bilayer. However, lysophospholipids could decrease OMV stability. Furthermore, interactions between OMVs and immune molecules or cells could be influenced by the presence of lysophospholipids, since they have detergent-like activities.

Despite the different properties of the differently induced OMVs, immune responses did not differ **(Fig. 5)**. In previous studies, immune responses to *E. coli* LPS were shown to decrease by addition of PMAP-36, due to an interaction between the peptide and LPS [42]. This led to the hypothesis that LPS in pOMVs would also be neutralized by the peptide, resulting in a decreased immune response. However, this was not observed. The three OMV preparations showed no difference in macrophage activation, suggesting the available PMAP-36 in pOMVs is unable to neutralize LPS. A possible explanation is that PMAP-36 is trapped by the OMVs and incapable of interacting with released LPS to neutralize TLR4 responses. Accordingly, free PMAP-36 was able to decrease cytokine release of pBMDM1 stimulated with pure *B. bronchiseptica* LPS **(Fig. S6)**. To investigate whether PMAP-36 is inaccessible in pOMVs, sOMVs were supplemented with increasing concentrations of PMAP-36 before stimulation of macrophages. Whereas stimulation with hOMVs alone decreased CD14 surface expression **(Fig. 5a)**, addition of PMAP-36 to hOMVs was shown to negate this decrease **(Fig. S5)**. Furthermore, addition of PMAP-36 decreased cytokine release of OMV-stimulated pBMDM1 **(Fig. 6)**. This shows that PMAP-36 is able to neutralize LPS in OMVs and to decrease immune responses. An alternative explanation for the lack of modulation by PMAP-36 present in pOMVs is that the PMAP-36 concentration present in these OMVs is plainly too low, but this is rather impossible in our hands to detect and compare. Apart from PMAP-36, also another, synthetic peptide was previously shown to reduce OMV-induced immune responses [51] suggesting that peptides could be promising immunomodulatory molecules in vesicle-based vaccines. Notably, a decreasing trend was observed for both pro- and anti-inflammatory cytokines upon supplementation of OMVs with PMAP-36 **(Fig. 6)**. This suggests that the immune suppression by PMAP-36 is not specifically reducing either pro- or anti-inflammatory processes. Higher concentrations of PMAP-36 appeared to be toxic to macrophages and could therefore, unfortunately, not be tested (data not shown). Investigation of the immune modulatory properties of a range of HDPs might identify a less toxic peptide, specifically modulating the immune response.

For the human cathelicidin LL-37, it is well known that it has multiple immunomodulatory functions, including the ability to neutralize LPS-induced macrophage activation [52–54]. Several other cathelicidins, such as CATH-2 and PMAP-36, were also shown to reduce TNF-α release from LPS-stimulated stimulated macrophages [42]. Moreover, synthetically designed peptides have proven to be very promising immunomodulators. Innate Defense Regulator peptides (IDRs) were shown to induce chemokine secretion while simultaneously reducing LPS-induced cytokine secretion *in vitro* and, likewise,

inflammation *in vivo* [55,56]. However, these peptides decrease secretion of both pro- and anti-inflammatory cytokines, which might not be necessary for an optimal immune response. When a different balance is needed between pro- and anti-inflammatory responses, lowering both is not appropriate. Therefore, screening larger sets of HDPs for their immunomodulatory properties might reveal some that skew the balance between pro- and anti-inflammatory responses which could be useful for vaccine applications. In OMV-based vaccines for instance, only pro-inflammatory responses have to be decreased. While for *B. pertussis* OMV-based vaccines are already favorable compared to whole-cell vaccines with respect to reactogenicity [8], LPS is still present, which might cause undesirable side effects. HDPs might be promising molecules to address this issue, since they specifically neutralize LPS. Currently, the supplementation of vaccines with HDPs has already shown successful results, in both a bacterial subunit vaccine and a viral vaccine [57]. Some well-known HDPs, such as indolicidin, LL-37 and BMAP-27, as well as derivatives thereof were used to enhance pro-inflammatory properties [58]. This shows the potential of HDPs as excipient molecules and their ability to be adjusted to obtain optimal immune modulation.

Concluding Remarks

We have investigated the effect of PMAP-36 on OMV release. OMV release was only slightly induced by bacterial stimulation with PMAP-36. Furthermore, we have investigated the subsequent immune response of isolated OMVs and the effect of PMAP-36 addition thereon. Innate immune responses to OMVs were effectively decreased after addition of PMAP-36. This indicates that HDPs are promising excipient for vaccine applications. Investigating the immunomodulatory properties of other HDPs might result in an ideal adjuvant for OMV-based vaccines.

Acknowledgements

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References

[1] C. Schwechheimer, M.J. Kuehn, Outer-membrane vesicles from Gram-negative bacteria: Biogenesis and functions, Nat. Rev. Microbiol. 13 (2015) 605–619. doi:10.1038/nrmicro3525.

[2] B.L. Deatherage, J.C. Lara, T. Bergsbaken, S.L.R. Barrett, S. Lara, B.T. Cookson, Biogenesis of bacterial membrane vesicles, Mol. Microbiol. 72 (2009) 1395–1407. doi:10.1111/j.1365-2958.2009.06731.x.

[3] J. Holst, P. Oster, R. Arnold, M. Tatley, L. Næss, I. Aaberge, Y. Galloway, A. McNicholas, J. O'Hallahan, E. Rosenqvist, S. Black, Vaccines against meningococcal serogroup B disease containing outer membrane vesicles (OMV): Lessons from past programs and implications for the future, Hum. Vaccin. Immunother. 9 (2013) 1241–1253. doi:10.4161/hv.24129.

[4] J. Holst, D. Martin, R. Arnold, C.C. Huergo, P. Oster, J. O'Hallahan, E. Rosenqvist, Properties and clinical performance of vaccines containing outer membrane vesicles from Neisseria meningitidis, Vaccine. 27 (2009) B3–B12. doi:10.1016/j.vaccine.2009.04.071.

[5] D.M. Granoff, Review of Meningococcal Group B Vaccines, Clin. Infect. Dis. 50 (2010) S54–S65. doi:10.1086/648966.

[6] P.B. Keiser, S. Biggs-Cicatelli, E.E. Moran, D.H. Schmiel, V.B. Pinto, R.E. Burden, L.B. Miller, J.E. Moon, R.A. Bowden, J.F. Cummings, W.D. Zollinger, A phase 1 study of a meningococcal native outer membrane vesicle vaccine made from a group B strain with deleted lpxL1 and synX, over-expressed factor H binding protein, two PorAs and stabilized OpcA expression, Vaccine. 29 (2011) 1413–1420. doi:10.1016/j.vaccine.2010.12.039.

[7] B. van de Waterbeemd, G. Zomer, P. Kaaijk, N. Ruiterkamp, R.H. Wijffels, G.P.J.M. van den Dobbelsteen, L.A. van der Pol, Improved Production Process for Native Outer Membrane Vesicle Vaccine against Neisseria meningitidis, PLoS One. 8 (2013) 1–11. doi:10.1371/journal.pone.0065157.

[8] R.H.M. Raeven, J. Brummelman, J.L.A. Pennings, L. Van Der Maas, W. Tilstra, K. Helm, E. Van Riet, W. Jiskoot, C.A.C.M. Van Els, W.G.H. Han, G.F.A. Kersten, B. Metz, Bordetella pertussis outer membrane vesicle vaccine confers equal efficacy in mice with milder inflammatory responses compared to a whole-cell vaccine, Sci. Rep. 6 (2016) 1–15. doi:10.1038/srep38240.

[9] R.H.M. Raeven, L. Van Der Maas, W. Tilstra, J.P. Uittenbogaard, T.H.E. Bindels, B. Kuipers, A. Van Der Ark, J.L.A. Pennings, E. Van Riet, W. Jiskoot, G.F.A. Kersten, B. Metz, Immunoproteomic Profiling of Bordetella pertussis Outer Membrane Vesicle Vaccine Reveals Broad and Balanced Humoral Immunogenicity, J.

Proteome Res. 14 (2015) 2929–2942. doi:10.1021/acs.jproteome.5b00258.

[10] M.E. Gaillard, D. Bottero, A. Errea, M. Ormazábal, M.E. Zurita, G. Moreno, M. Rumbo, C. Castuma, E. Bartel, D. Flores, P. van der Ley, A. van der Ark, D. F. Hozbor, Acellular pertussis vaccine based on outer membrane vesicles capable of conferring both long-lasting immunity and protection against different strain genotypes, Vaccine. 32 (2014) 931–937. doi:10.1016/j.vaccine.2013.12.048.

[11] R. Roberts, G. Moreno, D. Bottero, M.E. Gaillard, M. Fingermann, A. Graieb, M. Rumbo, D. Hozbor, Outer membrane vesicles as acellular vaccine against pertussis, Vaccine. 26 (2008) 4639–4646. doi:10.1016/j.vaccine.2008.07.004.

[12] D.F. Hozbor, Outer membrane vesicles: an attractive candidate for pertussis vaccines, Expert Rev. Vaccines. 16 (2017) 193–196. doi:10.1080/14760584.2017.1276832.

[13] S. Copeland, H.S. Warren, S.F. Lowry, S.E. Calvano, D. Remick, Acute Inflammatory Response to Endotoxin in Mice and Humans, Clin. Vaccine Immunol. 12 (2005) 60–67. doi:10.1128/cdli.12.1.60-67.2005.

[14] M. von Bergen, F. Steiniger, T. Baumgarten, L.Y. Wick, H.J. Heipieper, J. Seifert, S. Sperling, Membrane Vesicle Formation as a Multiple-Stress Response Mechanism Enhances Pseudomonas putida DOT-T1E Cell Surface Hydrophobicity and Biofilm Formation, Appl. Environ. Microbiol. 78 (2012) 6217–6224. doi:10.1128/aem.01525-12.

[15] C. Eberlein, T. Baumgarten, S. Starke, H.J. Heipieper, Immediate response mechanisms of Gram-negative solvent-tolerant bacteria to cope with environmental stress: cis-trans isomerization of unsaturated fatty acids and outer membrane vesicle secretion, Appl. Microbiol. Biotechnol. 102 (2018) 2583–2593. doi:10.1007/s00253-018-8832-9.

[16] E.F. de Jonge, M.D. Balhuizen, R. van Boxtel, J. Wu, H.P. Haagsman, J. Tommassen, Heat shock enhances outer-membrane vesicle release in Bordetella spp., Curr. Res. Microb. Sci. 2 (2021) 100009. doi:10.1016/j.crmicr.2020.100009.

[17] L. Hazlett, M. Wu, Defensins in innate immunity, Cell Tissue Res. 343 (2011) 175–188. doi:10.1007/s00441-010-1022-4.

[18] M. Zasloff, Antimicrobial peptides of multicellular organisms, Nature. 415 (2002) 389–395. doi:10.1038/415389a.

[19] M. Zanetti, Cathelicidins, multifunctional peptides of the innate immunity, J. Leukoc. Biol. 75 (2004) 39–48. doi:10.1189/jlb.0403147.

[20] R. van Harten, E. van Woudenbergh, A. van Dijk, H. Haagsman, Cathelicidins: Immunomodulatory Antimicrobials, Vaccines. 6 (2018) 63.

doi:10.3390/vaccines6030063.

[21] N. Mookherjee, M.A. Anderson, H.P. Haagsman, D.J. Davidson, Antimicrobial host defence peptides: functions and clinical potential, Nat. Rev. Drug Discov. 19 (2020) 311–332. doi:10.1038/s41573-019-0058-8.

[22] M.R. Scheenstra, M. van den Belt, J.L.M. Tjeerdsma-van Bokhoven, V.A.F. Schneider, S.R. Ordonez, A. van Dijk, E.J.A. Veldhuizen, H.P. Haagsman, Cathelicidins PMAP-36, LL-37 and CATH-2 are similar peptides with different modes of action, Sci. Rep. 9 (2019) 4780. doi:10.1038/s41598-019-41246-6.

[23] L. Zhang, R.L. Gallo, Antimicrobial Peptides, Springer Singapore, Singapore, 2019. doi:10.1007/978-981-13-3588-4.

[24] C. Schwechheimer, M.J. Kuehn, Synthetic effect between envelope stress and lack of outer membrane vesicle production in escherichia coli, J. Bacteriol. 195 (2013) 4161–4173. doi:10.1128/JB.02192-12.

[25] A.J. McBroom, A.P. Johnson, S. Vemulapalli, M.J. Kuehn, Outer membrane vesicle production by Escherichia coli is independent of membrane instability, J. Bacteriol. 188 (2006) 5385–5392. doi:10.1128/JB.00498-06.

[26] K.W. Chan, C. Shone, J.R. Hesp, Antibiotics and iron-limiting conditions and their effect on the production and composition of outer membrane vesicles secreted from clinical isolates of extraintestinal pathogenic E. coli, Proteomics - Clin. Appl. 11 (2017) 1600091. doi:10.1002/prca.201600091.

[27] S. Devos, W. Van Putte, J. Vitse, G. Van Driessche, S. Stremersch, W. Van Den Broek, K. Raemdonck, K. Braeckmans, H. Stahlberg, M. Kudryashev, S.N. Savvides, B. Devreese, Membrane vesicle secretion and prophage induction in multidrug-resistant Stenotrophomonas maltophilia in response to ciprofloxacin stress, Environ. Microbiol. 19 (2017) 3930–3937. doi:10.1111/1462-2920.13793.

[28] V.A.F. Schneider, M. Coorens, S.R. Ordonez, J.L.M. Tjeerdsma-Van Bokhoven, G. Posthuma, A. Van Dijk, H.P. Haagsman, E.J.A. Veldhuizen, Imaging the antimicrobial mechanism(s) of cathelicidin-2, Sci. Rep. 6 (2016) 1–11. doi:10.1038/srep32948.

[29] W.F. Verwey, E.H. Thiele, D.N. Sage, L.F. Schuchardt, A simplified liquid culture medium for the growth of Hemophilus pertussis, J. Bacteriol. 58 (1949) 127–134. doi:10.1128/jb.58.2.127-134.1949.

[30] Biorad, General tips for handcasting, (2014). http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6201.pdf (accessed May 1, 2019).

[31] C.M. Tsai, C.E. Frasch, A sensitive silver stain for detecting lipopolysaccharides in

polyacrylamide gels, Anal. Biochem. 119 (1982) 115–119. doi:10.1016/0003-2697(82)90673-X.

[32] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification., Can. J. Biochem. Physiol. 37 (1959) 911–917. doi:10.1139/059-099.

[33] C.A. Smith, E.J. Want, G. O'Maille, R. Abagyan, G. Siuzdak, XCMS: Processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification, Anal. Chem. 78 (2006) 779–787. doi:10.1021/ac051437y.

[34] J. Gao, M.R. Scheenstra, A. van Dijk, E.J.A. Veldhuizen, H.P. Haagsman, A new and efficient culture method for porcine bone marrow-derived M1- and M2-polarized macrophages, Vet. Immunol. Immunopathol. 200 (2018) 7–15. doi:10.1016/j.vetimm.2018.04.002.

[35] A. Preston, E. Maxim, E. Toland, E.J. Pishko, E.T. Harvill, M. Caroff, D.J. Maskell, Bordetella bronchiseptica PagP is a Bvg-regulated lipid A palmitoyl transferase that is required for persistent colonization of the mouse respiratory tract, Mol. Microbiol. 48 (2003) 725–736. doi:10.1046/j.1365-2958.2003.03484.x.

[36] M.S. Peppler, Two physically and serologically distinct lipopolysaccharide profiles in strains of Bordetella pertussis and their phenotype variants, Infect. Immun. 43 (1984) 224–232. doi:10.1128/iai.43.1.224-232.1984.

[37] A.J. McBroom, M.J. Kuehn, Release of outer membrane vesicles by Gram-negative bacteria is a novel envelope stress response, Mol. Microbiol. 63 (2007) 545–558. doi:10.1111/j.1365-2958.2006.05522.x.

[38] A. Kulp, M.J. Kuehn, Biological Functions and Biogenesis of Secreted Bacterial Outer Membrane Vesicles, Annu. Rev. Microbiol. 64 (2010) 163–184. doi:10.1146/annurev.micro.091208.073413.

[39] P.M. Hwang, W.Y. Choy, E.I. Lo, L. Chen, J.D. Forman-Kay, C.R.H. Raetz, G.G. Privé, R.E. Bishop, L.E. Kay, Solution structure and dynamics of the outer membrane enzyme PagP by NMR, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 13560–13565. doi:10.1073/pnas.212344499.

[40] Y. Kawai, A. Moribayashi, Characteristic lipids of Bordetella pertussis: simple fatty acid composition, hydroxy fatty acids, and an ornithine-containing lipid, J. Bacteriol. 151 (1982) 996–1005. doi:10.1128/jb.151.2.996-1005.1982.

[41] S. Dees, S. Thanabalasundrum, C.W. Moss, D.G. Hollis, R.E. Weaver, Cellular fatty acid composition of group IVe, a nonsaccharolytic organism from clinical sources, J. Clin. Microbiol. 11 (1980) 664–668. doi:10.1128/jcm.11.6.664-668.1980.

[42] M. Coorens, V.A.F. Schneider, A.M. de Groot, A. van Dijk, M. Meijerink, J.M.

Wells, M.R. Scheenstra, E.J.A. Veldhuizen, H.P. Haagsman, Cathelicidins Inhibit Escherichia coli –Induced TLR2 and TLR4 Activation in a Viability-Dependent Manner, J. Immunol. 199 (2017) 1418–1428. doi:10.4049/jimmunol.1602164.

[43] H.M. Kulkarni, R. Nagaraj, M. V Jagannadham, Protective role of E. coli outer membrane vesicles against antibiotics, Microbiol. Res. 181 (2015) 1–7. doi:10.1016/j.micres.2015.07.008.

[44] A.J. Manning, M.J. Kuehn, Contribution of bacterial outer membrane vesicles to innate bacterial defense, BMC Microbiol. 11 (2011) 1–14. doi:10.1186/1471-2180-11-258.

[45] I.A. MacDonald, M.J. Kuehna, Stress-induced outer membrane vesicle production by Pseudomonas aeruginosa, J. Bacteriol. 195 (2013) 2971–2981. doi:10.1128/JB.02267-12.

[46] J. Pérez-Ortega, R.M. Van Harten, R. Van Boxtel, M. Plisnier, M. Louckx, D. Ingels, H.P. Haagsman, J. Tommassen, Reduction of endotoxicity in Bordetella bronchiseptica by lipid A engineering: Characterization of lpxL1 and pagP mutants, Virulence. 12 (2021) 1452–1468. doi:10.1080/21505594.2021.1929037.

[47] G. Polido, X. Shi, D. Xu, C. Guo, R. Thai, J.P. Patterson, N.C. Gianneschi, T.M. Suchyna, F. Sachs, G.P. Holland, Investigating the interaction of Grammostola rosea venom peptides and model lipid bilayers with solid-state NMR and electron microscopy techniques, Biochim. Biophys. Acta - Biomembr. 1861 (2019) 151–160. doi:10.1016/j.bbamem.2018.08.004.

[48] S. Roier, F.G. Zingl, F. Cakar, S. Durakovic, P. Kohl, T.O. Eichmann, L. Klug, B. Gadermaier, K. Weinzerl, R. Prassl, A. Lass, G. Daum, J. Reidl, M.F. Feldman, S. Schild, A novel mechanism for the biogenesis of outer membrane vesicles in Gram-negative bacteria, Nat. Commun. 7 (2016). doi:10.1038/ncomms10515.

[49] A.J.G. Horrevoets, T.M. Hackeng, H.M. Verheij, R. Dijkman, G.H. de Haas, Kinetic Characterization of Escherichia coli Outer Membrane Phospholipase A Using Mixed Detergent–Lipid Micelles, Biochemistry. 28 (1989) 1139–1147. doi:10.1021/bi00429a031.

[50] N. Fuller, R.P. Rand, The influence of lysolipids on the spontaneous curvature and bending elasticity of phospholipid membranes, Biophys. J. 81 (2001) 243–254. doi:10.1016/S0006-3495(01)75695-0.

[51] A. Pfalzgraff, W. Correa, L. Heinbockel, A.B. Schromm, C. Lübow, N. Gisch, G. Martinez-de-Tejada, K. Brandenburg, G. Weindl, LPS-neutralizing peptides reduce outer membrane vesicle-induced inflammatory responses, Biochim. Biophys. Acta - Mol. Cell Biol. Lipids. 1864 (2019) 1503–1513. doi:10.1016/j.bbalip.2019.05.018.

[52] M.G. Scott, D.J. Davidson, M.R. Gold, D. Bowdish, R.E.W. Hancock, The human antimicrobial peptide LL-37 is a multifunctional modulator of innate immune responses., J. Immunol. 169 (2002) 3883–91. doi:10.4049/jimmunol.169.7.3883.

[53] Y. Lai, R.L. Gallo, AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense, Trends Immunol. 30 (2009) 131–141. doi:10.1016/j.it.2008.12.003.

[54] D. Vandamme, B. Landuyt, W. Luyten, L. Schoofs, A comprehensive summary of LL-37, the factoctum human cathelicidin peptide, Cell. Immunol. 280 (2012) 22–35. doi:10.1016/j.cellimm.2012.11.009.

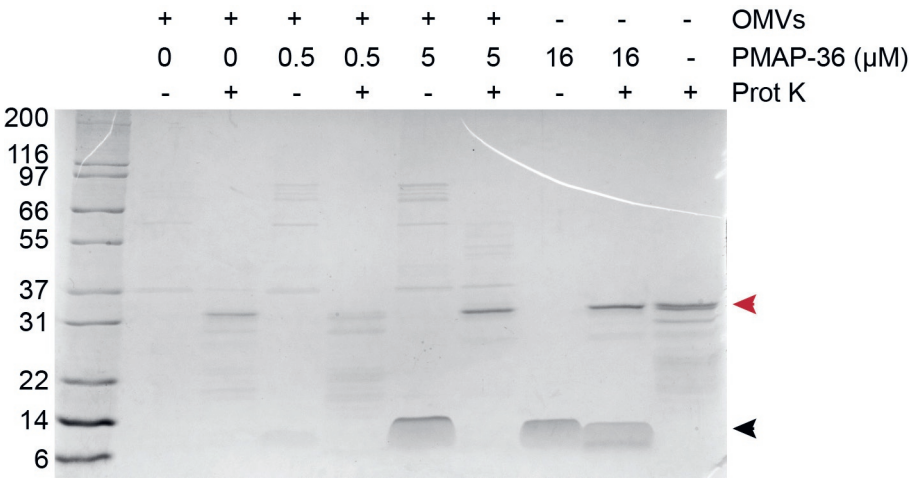
[55] F. Niyonsaba, L. Madera, N. Afacan, K. Okumura, H. Ogawa, R.E.W. Hancock, The innate defense regulator peptides IDR-HH2, IDR-1002, and IDR-1018 modulate human neutrophil functions, J. Leukoc. Biol. 94 (2013) 159–170. doi:10.1189/jlb.1012497.

[56] K.C. Wuerth, R. Falsafi, R.E.W. Hancock, Synthetic host defense peptide IDR-1002 reduces inflammation in Pseudomonas aeruginosa lung infection, (2017). doi:10.1371/journal.pone.0187565.

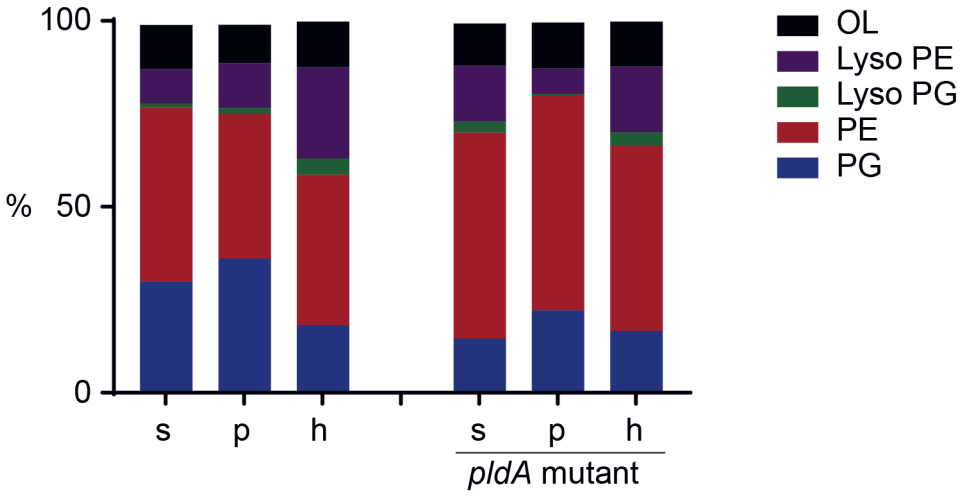
[57] R. Garg, L. Babiuk, S. van Drunen Littel-van den Hurk, V. Gerdts, A novel combination adjuvant platform for human and animal vaccines, Vaccine. 35 (2017) 4486–4489. doi:10.1016/j.vaccine.2017.05.067.

[58] S. van den Hurk, V. Gerdts, A. Potter, L. Babiuk, R. Hancock, S. Halperin, J. Kovacs-Nolan, G. Mutwiri, S. Lee, M.-N. Kweon, J. Kindrachuk, M. Elliott, COMBINATION ADJUVANT FORMULATION, US 9.408,908 B2, 2016. https://patents.google.com/patent/US9408908B2/en (accessed January 7, 2020).

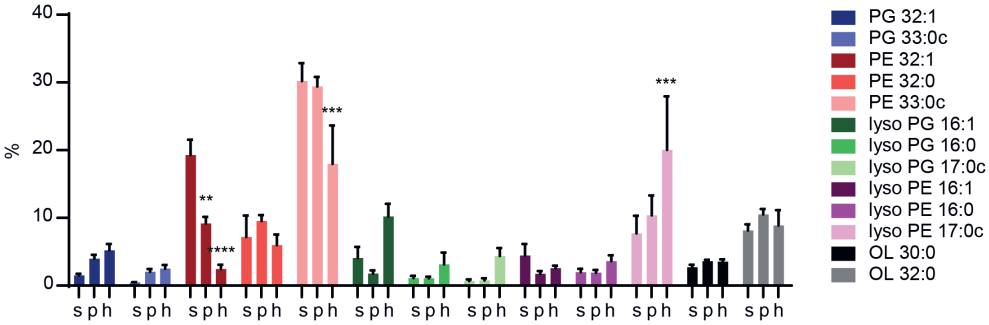
Supplementary Figures



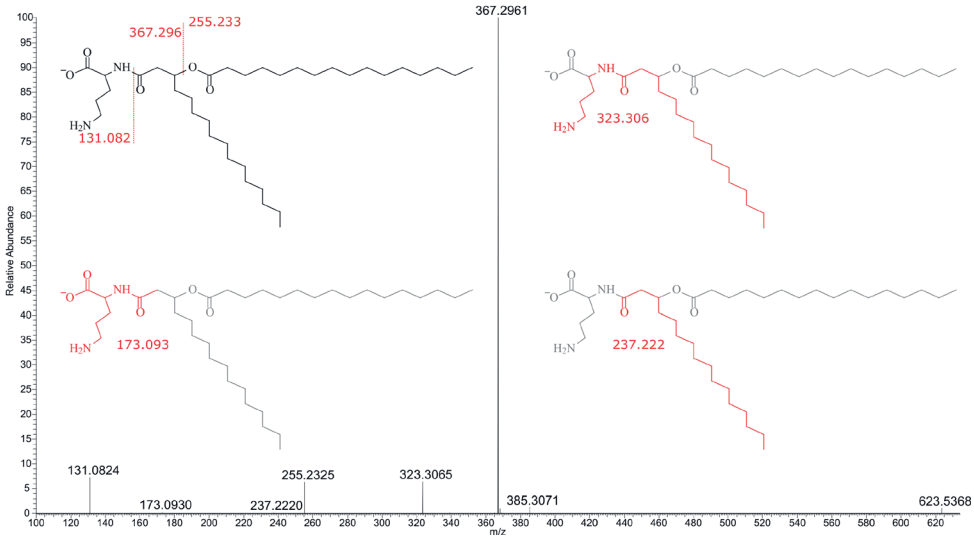
Supplementary Figure 1: Localization of PMAP-36 in the isolated OMVs. *pOMVs were subjected to proteinase K treatment and the integrity of PMAP-36 was assessed using SDS-PAGE. Red and black arrows indicate respectively proteinase K and PMAP-36 on the gel. The first six lanes contain pOMVs isolated with different PMAP-36 concentrations. The next two lanes contain pure PMAP-36 as control.*



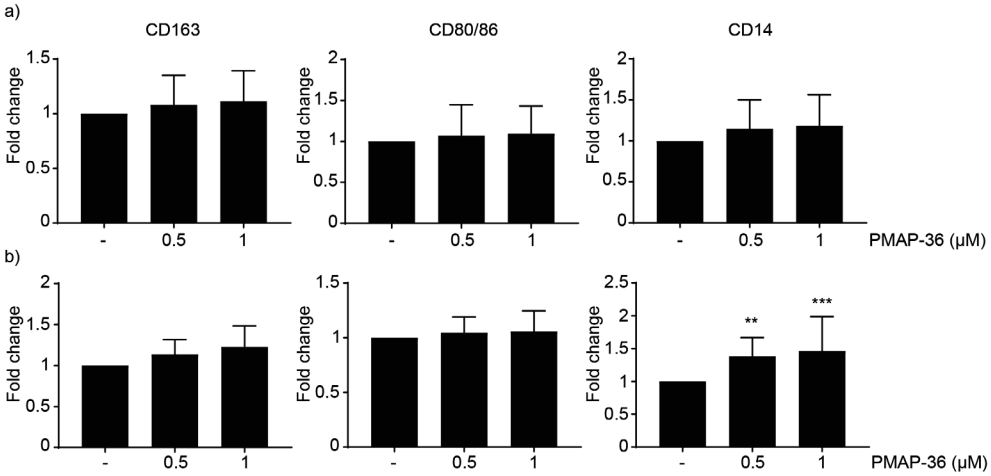
Supplementary Figure 2: Lipidomic analysis of OMVs produced by B. bronchiseptica strain BB-D09 and its pldA mutant derivative. *B. bronchiseptica was subjected to different treatments and phospholipid composition of isolated OMVs was determined using mass spectrometry (n=3). Cardiolipin was detected but could not be quantified. s = sOMVs, p = pOMVs, h = hOMVs.*



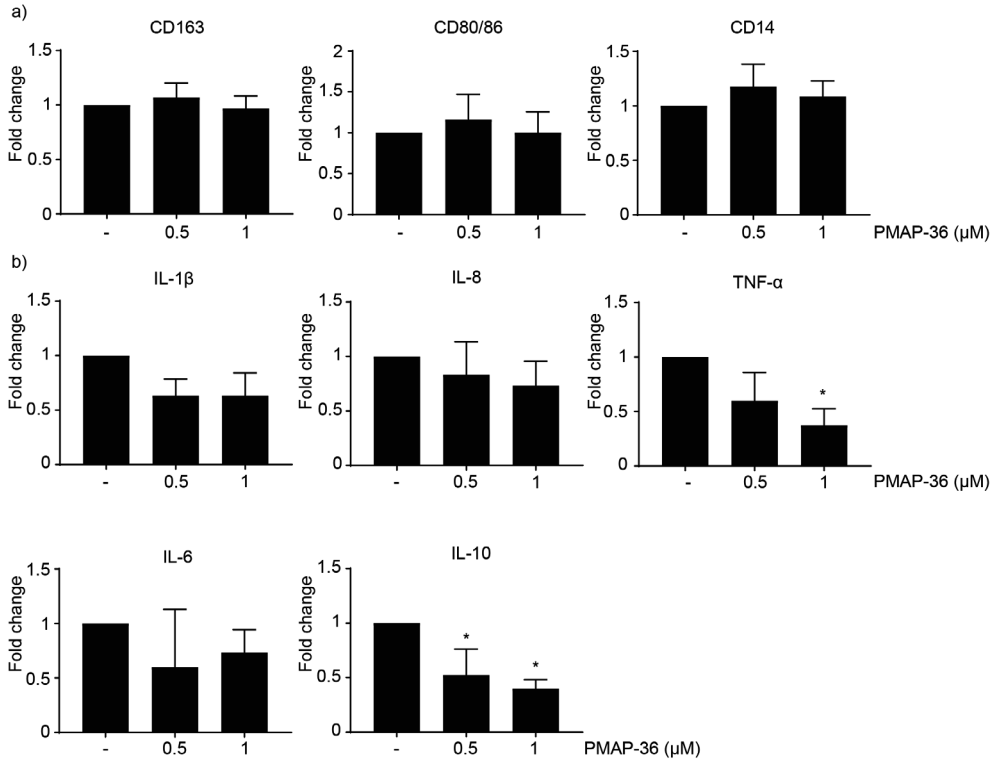
Supplementary Figure 3: Most prominent lipid species in B. bronchiseptica BB-P19 OMVs. *B. bronchiseptica was subjected to different treatments and phospholipid composition of OMVs was determined using mass spectrometry. Depicted are the phospholipid classes that account together for approximately 90% of the total phospholipids. s = sOMVs, p = pOMVs, h = hOMVs. Significant differences compared to sOMVs are indicated by **p<0.01, ***p<0.001, ****p<0.0001, obtained using a paired two-way ANOVA with a post-hoc Dunnett test.*



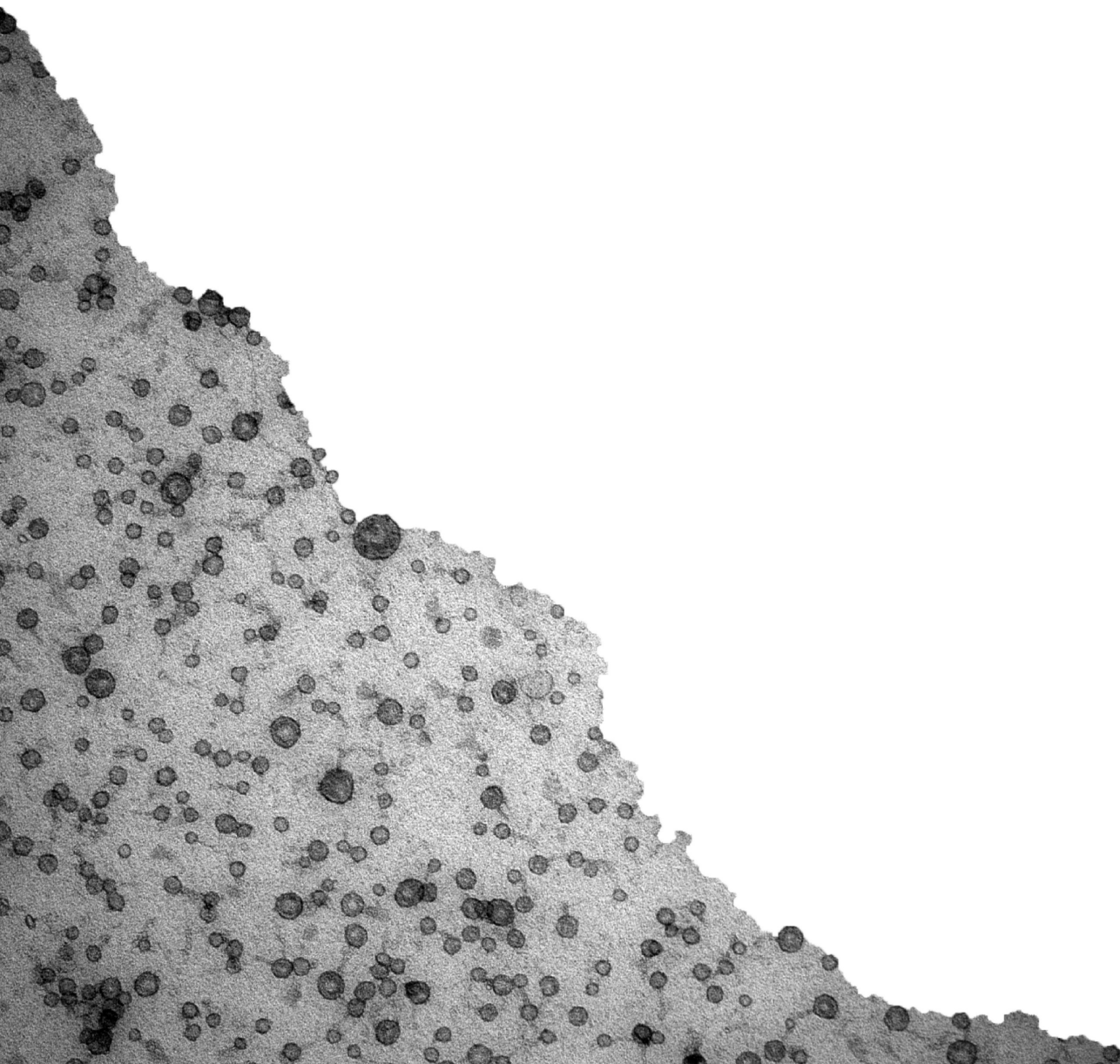
Supplementary Figure 4: Ultra-high resolution accurate mass MS2 spectrum demonstrating the presence of ornithine lipid in OMVs. *Structures matching m/z values are displayed in red, with the full structure displayed on the top left, corresponding to the precursor ion at m/z 623.5368.*



Supplementary Figure 5: PMAP-36 modulation of pBMDM1 cell-surface markers in response to OMVs. *pBMDM1 macrophages were stimulated with (a) sOMVs or (b) hOMVs in the presence of different concentrations of PMAP-36. Cell-surface markers CD163, CD80/86 and CD14 were measured using FACS. Values were converted to fold change compared to the values for OMVs without supplemented PMAP-36. Significant differences are indicated by *p<0.05, **p<0.01, ***p<0.001, obtained by using a linear mixed-model analysis on the raw data with a post-hoc Dunnett test (n=8).*



Supplementary Figure 6: PMAP-36 modulation of pBMDM1 response to LPS of B. bronchiseptica. *Porcine BMDM1 macrophages were stimulated with LPS isolated from B. bronchiseptica supplemented with different concentrations of PMAP-36. Activation was measured by (a) cell surface markers and (b) cytokines. Values were converted to fold changes compared to LPS without supplemented PMAP-36. Significant differences are indicated by *p<0.05, obtained by using a linear mixed-model analysis on the raw data with a post-hoc Dunnett test (n=3-6).*



Chapter 6

Modulation of Outer Membrane Vesicle-based Immune Responses by Cathelicidins

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MANUSCRIPT IN PREPARATION

Abstract

Antibiotic resistance is increasing and one strategy to prevent resistance development is the use of bacterial vaccines. For Gram-negative bacteria, natural outer membrane vesicles (OMVs) could be used for vaccine development. These vesicular structures are naturally produced by all Gram-negative bacteria and contain several antigens in their native environment. However, despite that the presence of lipopolysaccharide (LPS) may aid as intrinsic adjuvant, there is a risk that it may also cause undesired immune responses. Therefore, molecules to dampen LPS-induced toll-like receptor (TLR) 4 activation may be needed. Here host defense peptides (HDPs), like cathelicidins, can play an important role. They have been shown before to interact with LPS and thereby neutralize LPS-induced TLR4 activation. However, there is currently no knowledge about neutralization in an OMV-based setting. Therefore, in this paper the immune modulating capacity of HDPs was investigated after macrophage stimulation with either spontaneous or heat-induced OMVs. This revealed that the cathelicidins LL-37, CATH-2, PMAP-36 and K9CATH were able to modulate immune responses. Interestingly, immune modulation by these cathelicidins was different for spontaneous compared to heat-induced OMVs. Interaction studies revealed that the mode of binding of cathelicidins to OMVs slightly differed between OMV classes. Furthermore, TLR screening revealed that TLR2, 4, 5 and 9 were involved in stimulation of macrophages by OMVs, with TLR4-mediated activation being the most important pathway. Uptake of OMVs did not play a major role in macrophage activation. Taken together, this study shows how OMVs can activate macrophages and how cathelicidins may modulate these immune responses.

Introduction

Nowadays we are well aware of the fact that antibiotic resistance is increasing and becoming problematic [1]. Strategies to combat resistance include development of new antibiotic compounds or bacterial vaccines [2]. A current and effective method for development of Gram-negative bacterial vaccines is the use of outer membrane vesicles (OMVs). These 20-300 nm large spherical structures are released by all Gram-negative species and are similar in composition as the bacterial surface [3,4]. They contain multiple surface-antigens and are non-replicative, which makes them promising particles for vaccine usage [5–8]. OMV-based vaccines have been proven to be very successful in clonal outbreaks of *Neisseria meningitidis* [9]. An OMV-based vaccine is also very promising to overcome waning immunity in current *Bordetella pertussis* vaccines, as OMVs have shown to confer protection in mice studies [10]. Furthermore, OMVs of periodontal pathogens were shown to activate the NF-κB pathway and induce cytokine release in macrophages [11]. Despite these successes and increased knowledge about immunomodulation by OMVs [12,13], some issues may preclude the wide-spread use of OMV-based vaccines. One drawback is that the presence of LPS not only functions as intrinsic adjuvant but may also result in a high degree of reactogenicity and adverse effects. Furthermore, not all OMV-based vaccines may evoke a desired Th1/Th17 response.

Well known immunomodulatory molecules are host defense peptides (HDPs). Cathelicidins are a sub-class of HDPs and were first discovered for their antimicrobial effects [14–16]. Nevertheless, they were also shown to have many immunomodulatory functions, reviewed elsewhere [17,18]. Most relevant in the context of an OMV-based vaccine is their ability to neutralize LPS-induced TLR4 activation. A cathelicidin screen revealed that LL-37, CRAMP, K9CATH, PMAP-36 and several chicken cathelicidins reduced LPS-induced TLR4 activation [19]. Furthermore, CATH-2 was shown to reduce not only TLR4 activation, but also TLR2 activation [20]. However, no knowledge exists about LPS-neutralization of natural HDPs in an OMV-based setting. To date, only one synthetic anti-endotoxin peptide was studied and shown to reduce interleukin 1β (IL-1β) and tumor necrosis factor (TNF) release in macrophages after OMV stimulation [21].

The immunomodulatory capacity of a range of cathelicidins was investigated in an OMV-based setting. The Gram-negative bacterium *Bordetella bronchiseptica* was used as a model system, which causes atrophic rhinitis in pigs and kennel cough in dogs [22]. There is a need for a new generation vaccine against *B. bronchiseptica* [23], as well as for the human pathogen *B. pertussis*, which causes whooping cough [24–26]. For HDPs it was decided to not limit the choice by originating species, since it was shown that modulation by HDPs is not species specific [19,27,28]. Therefore, LL-37 from human, CATH-2 from chicken,

PMAP-36, PMAP-23 and PR-39 from pig, K9CATH from dog and synthetically designed IDR-1018 and IDR-2005 were selected to be tested.

To investigate immune responses and subsequent modulation by HDPs, a murine macrophage cell line was stimulated with *B. bronchiseptica* OMVs. Since it was shown that heat shock induced OMV release and therefore might have promising industrial application, not only spontaneous OMVs (sOMVs) were studied, but also heat-induced OMVs (hOMVs) [29,30]. Macrophages were potently activated by sOMVs and hOMVs but only a limited number of HDPs could modulate these responses. Not only TLR4 was activated by OMVs, but also TLR2, TLR5 and TLR9. Furthermore, by blocking uptake of OMVs it was shown that this had limited effect on macrophage stimulation. Concluding, this study showed the capability of LL-37, CATH-2, PMAP-36 and K9CATH to modulate OMV-based immune responses.

Materials & Methods

Peptide synthesis

PMAP-36, CATH-2, PMAP-23, PR-39, K9CATH, IDR-1018 and IDR-2005 were synthesized by Fmoc-chemistry at China Peptides (CPC scientific, Sunnyvale, CA, USA). LL-37 was synthesized by Fmoc-chemistry at the Academic Centre for Dentistry Amsterdam (Amsterdam, the Netherlands). All peptides were purified to a purity of >95% by reverse phase high-performance liquid chromatography. Sequences and characteristics of the peptides are shown in **Table 1**.

Table 1: Sequence, organism of origin, number of amino acids (No. aa) and charge of studied peptides [31–35]**.**

Peptide	***Sequence***	***Origin***	***No. aa***	***Charge***
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLR NLVPRTES	Human	37	6+
CATH-2	RFGRFLRKIRRFRPKVTITIQGSARF-NH_2	Chicken	26	8+
PMAP-36	Ac-GRFRRLRKKTRKRLKKIGKVLKWIPPIVG SIPLGCG	Porcine	36	13+
PMAP-23	RIIDLLWRVRRPQKPKFVTVWVR	Porcine	23	6+
PR-39	RRRPRPPYLPRPRPPPFFPPRLPPRIPPG FPPRFPPRFP	Porcine	39	10+
K9CATH	RLKELITTGGQKIGEKIRRIGQRIKDFFKN LQPREEKS	Canine	38	5+
IDR-1018	VRLIVAVRIWRR-NH_2	Synthetic	12	5+
IDR-2005	VRLIVRVRIWRR-NH2	Synthetic	12	6+

Bacterial growth

A clinical isolate of *B. bronchiseptica* from pig (BB-P19) (Veterinary Microbiological Diagnostic Centre (VMDC), Utrecht University) was used throughout this study, grown on Difco™ Bordet-Gengou (BG) agar plates (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA), containing 1% glycerol (Merck, Darmstadt, Germany) supplemented with 15% (v/v) defibrinated sheep blood (Oxoid Ltd, Basingstoke, Hampshire, UK). Liquid cultures were grown in Verwey medium [36] (pH 7.4) containing 0.02% (w/v) KCl, 0.05% (w/v) KH_2PO_4, 0.01% (w/v) $MgCl_2 \bullet 6\ H_2O$ (all from Merck), 0.002% (w/v) nicotinic acid (Sigma-Aldrich, Saint Louis, Missouri, USA), 1.4% (w/v) Bacto™ casamino acids (Becton, Dickinson and Company), and 0.001% (w/v) L-glutathione reduced (Sigma-Aldrich). *E. coli* ATCC 25922 was grown in lysogeny broth (LB) containing 1% yeast extract, 0.5% tryptone (both Becton, Dickinson and Company) and 1% NaCl (Merck).

OMV isolation

OMVs were isolated as described before [37]. In short, bacteria were grown overnight to an OD_{590} of approximately 1.5. Before OMV isolation was initiated, bacteria were treated for 1 h at 56°C or 37°C, for hOMVs or sOMVs, respectively. Subsequently, bacterial cells were removed by centrifugation for 30 min at 4700 x g. The supernatant was passed through a 0.45 µm Whatman filter (GE Healthcare, Chicago, Illinois, USA) and centrifuged at 40,000 rpm for 2 h at 4°C (Ti-70 rotor, Beckman coulter, Brea, California, USA). The supernatant was decanted and the transparent pellet was dissolved in 2 mM Tris-HCl (pH 7.5, Sigma-Aldrich) in a volume corresponding to 2% of the bacterial culture.

Bicinchoninic acid (BCA) assay

Total protein concentration of isolated OMVs was determined using the Pierce BCA assay (Thermo Fisher Scientific, Waltham, MA, USA). All samples were corrected for the signal of Verwey medium which was taken along during OMV isolation. In short, 25 µL of sample, supplemented with 2% SDS (Invitrogen, Carlsbad, California, USA), were incubated with 200 µL of working reagent at 37°C for 2 h. Absorbance was measured at 562 nm with FLUOstar Omega (BMG Labtech, Ortenberg, Germany). Bovine serum albumin (BSA, Sigma-Aldrich) was used as reference.

RAW cell stimulation

RAW264.7 cells were cultured in DMEM medium (Thermo Fisher Scientific) with 10% fetal calf serum (Bodinco B.V., Alkmaar, the Netherlands) at 37°C under 5.0% CO_2. For stimulation, $5x10^4$ cells were seeded per well, in a 96-wells tissue culture treated microtiter plate (Corning Incorporated, Corning, New York, USA) and incubated overnight to adhere.

Macrophages were stimulated for 24 h with 0.025 µg/mL OMVs, 10 ng/mL *B. bronchiseptica* LPS (unless stated otherwise), $1*10^6$ CFU/mL heat-killed *E. coli*, different concentrations of peptides, uptake inhibitors or combinations thereof, diluted in fresh medium. Supernatant was collected and stored at -20°C. Fillipin (Sigma-Aldrich), chlorpromazine (CPZ, Sigma-Aldrich), methyl-β-cyclodextrin (MbCD, Sigma-Aldrich), Nystatin (Merck) and ethylisopropyl amiloride (EIPA, Sigma-Aldrich) were used.

Enzyme-Linked Immuno Sorbent Assay

A sandwich ELISA was used to measure cytokine concentrations using ELISA Duoset kits (R&D systems, Minneapolis, MN, USA). A 96-wells plate (Thermo Fisher Scientific) was incubated overnight with 100 µL capture antibody (diluted as described in kits protocol). Afterwards the plate was washed three times with wash buffer (PBS 0.05% Tween20 (MP Biomedicals, Irvine, California, USA)). Washing was performed after each incubation. Next, the plate was incubated for 1 h with 100 µL block buffer at room temperature (RT) (PBS with 1% BSA (Sigma Aldrich)). Then 100 µL of either standard or sample was added and the plate was incubated for 2 h at RT. The plate was incubated with 100 µL detection antibody (diluted as described in the kits' protocol) in reagent buffer (PBS / 1% BSA (Sigma Aldrich)) for 2 h at RT. Next, 100 µL mAb Streptavidin-HRP (R&D Systems) was added to the plate and incubated for 20 min at RT. Subsequently, 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB, Invitrogen, Carlsbad, California, USA) substrate was added and the plate was incubated for 5-20 min at RT in the dark until colorimetric changes were visible. Without washing, 50 µL stop solution was added (0.05% H_2SO_4 (Sigma Aldrich)). Samples were measured at 450 nm with a correction for background absorption at 590 nm using a FLUOstar Omega microplate reader (BMG Labtech GmbH, Ortenberg, Germany).

Griess assay

The Griess assay was performed to measure NO production. Either 50 µL sodium nitrite (Sigma Aldrich) diluted in DMEM as standard or 50 µL sample was added in a 96-wells plate. Samples were incubated for 5 min with 50 µL 1% sulfanilamide and 5.1% ortho-phosphoric acid (both from Merck) at RT in the dark. Afterwards 50 µL 0.1% N- (1-Naphthyl) ethylenediamine dihydrochloride (Merck) was added and incubated for 5 min at RT in the dark. Absorbance was measured at 550 nm using a FLUOstar Omega microplate reader (BMG Labtech GmbH).

WST-1 assay

RAW264.7 cells were seeded as described above. Cells were stimulated for 24 h with different concentrations of peptides. Afterwards, the medium was replaced with 100 µL culture medium containing 10% WST-1 (Roche, Basel, Switzerland) and incubated for 15-

20 min. The absorbance at 450 nm was measured using a FLUOstar Omega microplate reader (BMG Labtech GmbH) with a background correction at 630 nm. Cell viability was calculated using the non-stimulated sample as 100% viable.

Isothermal Titration Calorimetry (ITC)

ITC measurements were performed in a Low Volume NanoITC (TA Instruments-Waters LLC, New Castle, DE, USA). OMVs were 2-fold diluted in buffer (75% 2 mM Tris pH 7.5, 25% MQ). The chamber was filled with 200 µL of OMV solution. PMAP-36 was prepared in an identical buffer to a concentration of 200 µM. PMAP-36 was titrated into the chamber, with 1.96 µL per titration with a 300 s interval. Experiments were performed at 37°C and analyzed using the NanoAnalyze software (TA instruments, Asse, Belgium).

Dynamic Light Scattering (DLS)

Samples for DLS were diluted 10-fold in 2 mM Tris pH 7.5 unless stated otherwise. Samples were measured in micro-volume cuvettes (Sarstedt, Nümbrecht, Germany) on a Zetasizer nano (Malvern Panalytical, Malvern, UK) with a scatter angle of 173°. The standard polystyrene latex was used with a refractive index of 1.590 and absorbance of 0.010. Water was used as solvent (viscosity of 0.8872, refractive index of 1.330). Three measurements of 10-100 samplings were performed. For the temperature gradient, steps of 5°C from 25-45°C were measured. Samples were equilibrated for 2 min and each temperature measurement was 5 min.

QuantiBlue assay

HEK-Blue cell line NULL1, TLR-2, TLR-3, TLR-4, TLR-5 and TLR-9 (InvivoGen, San Diego, USA) were kindly received from Andreja Novak (Section of Immunology, Division of Infectious Diseases and Immunology, Utrecht University). The cells were cultured at 37°C under 5% CO_2 in DMEM supplemented with 10% heat inactivated FCS (30 min at 56°C), 1% penicillin/streptomycin (Thermo Fisher), 100 µg/ml Zeocin, 100 µg/ml Normocin and selective antibiotics specific for each cell line: TLR-2 and TLR-4 (30 µg/mL Blasticidin, 200 µg/mL Hygromycin), TLR-3 and TLR-5 (30 µg/mL Blasticidin) and TLR-9 (10 µg/mL Blasticidin). All antibiotics were obtained from InvivoGen. For stimulation the cells were seeded in a 96-well tissue culture treated microtiter plate in a concentration of 5x104 cells/well and incubated overnight to adhere. Medium was removed and cells were incubated for 24 h with different stimulants diluted in fresh medium. After 24 h the supernatant was removed and 20 µl supernatant was mixed with 180 µl of Quanti-Blue (InvivoGen) in a clear flat-blottom immune nonsterile 96-well plate (Thermo Fisher). After 1 h the absorbance was determined at 230 nm with the FLUOstar Omega microplate reader.

Results

Spontaneous and heat-induced OMVs evoke similar immune responses

To investigate immune responses evoked by OMVs, RAW cells were dose-dependently stimulated with either sOMVs or hOMVs, whereafter cell activation was determined by measuring NO production **(Fig. S1)**. sOMVs were obtained from bacterial cultures after overnight growth, while hOMVs were obtained after treating the overnight bacterial culture for 1 h at 56°C. While the mean NO production caused by hOMVs was slightly higher than that of sOMVs, for both OMVs a concentration of 0.025 µg/mL protein was chosen to be used in further experiments.

Next, immune responses evoked by spontaneous and heat-induced OMVs were compared. RAW cells were stimulated with OMVs and subsequently NO, TNFα and IL-10 release was determined **(Fig. 1)**. These experiments clearly showed that both classes of OMVs were capable of activating RAW cells. Remarkably, responses to hOMVs were consistently higher, both in the pro-inflammatory NO production and TNFα release, as well as in the anti-inflammatory IL-10 release.

Immune modulation of HDPs is minimal

HDPs from different species were investigated for their modulation of NO production and TNFα and IL-10 release from RAW cells stimulated by either spontaneous or heat-induced OMVs. Strikingly, modulation was relatively low, compared to modulation of pure LPS-evoked immune responses. LL-37, CATH-2, PMAP-36 and K9CATH **(Fig. 2)** were able to modulate responses of the macrophages, while PMAP-23, PR-39, IDR-1018 and IDR-2005 did not show any modulation **(Fig. S2)**. LL-37 and CATH-2 showed a decrease in OMV-induced NO production and IL-10 release, but only for sOMVs. Furthermore, CATH-2 showed an increase in TNFα and IL-10 release, only for hOMVs.

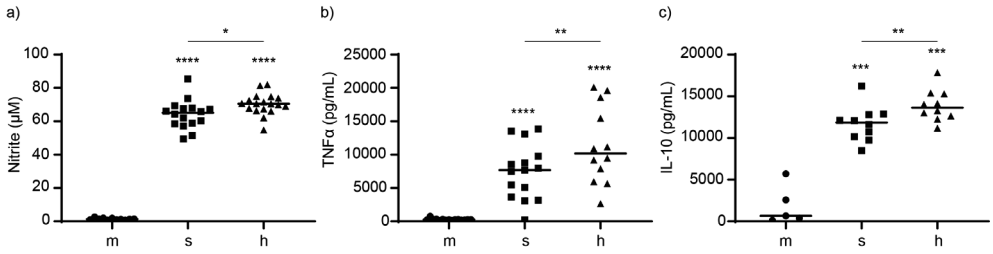

Figure 1: Activation of RAW264.7 cells upon stimulation with spontaneous and heat-induced OMVs. *RAW cells were stimulated with OMVs. (a) NO production was measured by Griess assay, (b) TNFα and (c) IL-10 using ELISA. m = medium, s = sOMVs, h = hOMVs. Results were analyzed using a paired mixed-model analysis with Geiser-Greenhouse correction and Tukey post-hoc test (n=14-18). *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, compared to medium unless depicted otherwise.*

PMAP-36 showed a decrease of NO production at high (4 μM) concentrations, but an increase in TNFα at low (0.5 μM) concentrations, both only for sOMVs. Furthermore, for hOMVs, PMAP-36 showed an increase in NO production and IL-10 release. K9CATH showed a consistent decrease of NO production and TNFα, and an increase of IL-10 production, albeit more pronounced for sOMVs than for hOMVs. Concluding, only a few cathelicidins were capable of modulating OMV-evoked immune responses and do so differently for spontaneous and heat-induced OMVs, as summarized in **Table 2**.

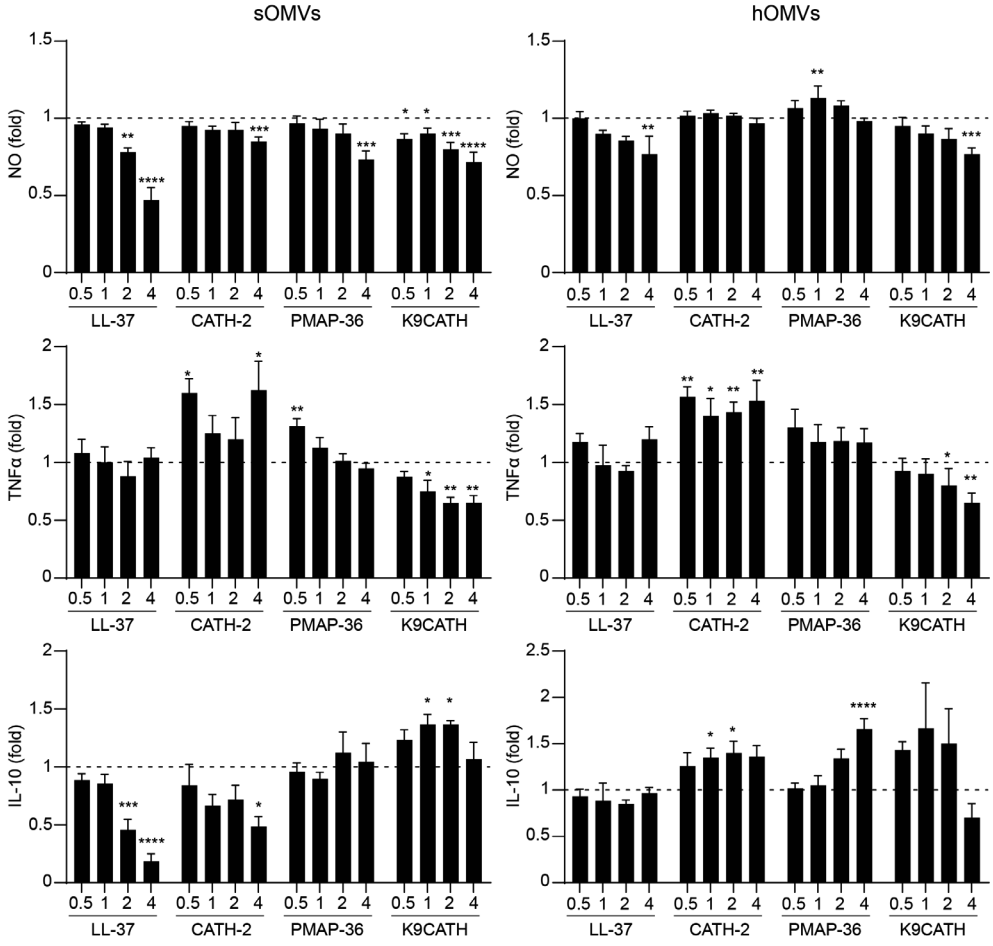

Figure 2: Modulation of RAW264.7 cell activation by cathelicidins upon stimulation with spontaneous and heat-induced OMVs. *RAW cells were stimulated with 0.025 μg/mL OMVs and a dose of LL-37, CATH-2, PMAP-36 or K9CATH (0.5, 1, 2 or 4 μM). Results are normalized to signals obtained by only OMV stimulation (depicted by the dotted line). NO production was measured by Griess assay, TNFα and IL-10 using ELISA. Results were analyzed using a repeated measures two-way ANOVA with Dunnett post-hoc test (n=3-6). *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, compared to OMVs without cathelicidin supplemented.*

Table 2: Overview of modulation of RAW264.7 macrophage activation by cathelicidins upon stimulation with OMVs. *Cathelicidins showing modulation in Figure 2 are summarized, using blue marking for downregulation and red marking for upregulation of immune responses.*

		NO	***TNFα***	***IL-10***
LL-37	*sOMV*	-		-
	hOMV	-		
CATH-2	*sOMV*	-	+	-
	hOMV		+	+
PMAP-36	*sOMV*	-	+	
	hOMV	+		+
K9CATH	*sOMV*	-	-	+
	hOMV	-	-	

To control for any responses caused by HDPs alone, metabolic activity and NO production of RAW cells was assessed after stimulation with 4 µM peptide **(Fig. S3)**. None of the HDPs affected metabolic activity or NO production. To investigate whether the lack of HDP modulation of the OMV-evoked immune response was due to a specific characteristic of *B. bronchiseptica* LPS the following experiment was done. RAW cells were stimulated with 10 ng/mL *B. bronchiseptica* LPS in the absence or presence of HDP (1 µM or 4 µM). This confirmed that indeed only LL-37, CATH-2, PMAP-36 and K9CATH were able to modulate LPS-evoked immune responses **(Fig. 3)**. NO production and TNFα release was consequently decreased and, although not significant, IL-10 release showed a decreasing trend by all four cathelicidins as well.

OMVs activate macrophages through multiple TLRs

Some cathelicidins were able to fully downregulate LPS-mediated immune responses, but not OMV-mediated immune responses. Therefore, it was investigated whether OMVs activate immune cells solely by TLR4 activation or also by other TLRs. A HEK-Blue cell system was used, expressing one specific human TLR and a reporter gene, secreted embryonic alkaline phosphatase (SEAP), which can be quantified by assessing colorimetric changes of the QuantiBlue reagent. These experiments showed that OMVs were able to activate TLR2, TLR4, TLR5, and TLR9 **(Fig. 4)**. Interestingly, sOMVs showed a higher activation of TLR5 and TLR9, compared to hOMVs. TLR3 on the other hand was not stimulated by OMVs **(Fig. S4)**.

Cathelicidins were investigated for their potential to neutralize TLR activation by OMVs and not all TLRs could be neutralized by all peptides. TLR2, activated by lipoproteins, was only neutralized by addition of LL-37 and K9CATH. TLR4, activated by LPS, was also only neutralized by LL-37 and K9CATH and slightly by 4 µM PMAP-36 in the case of hOMVs.

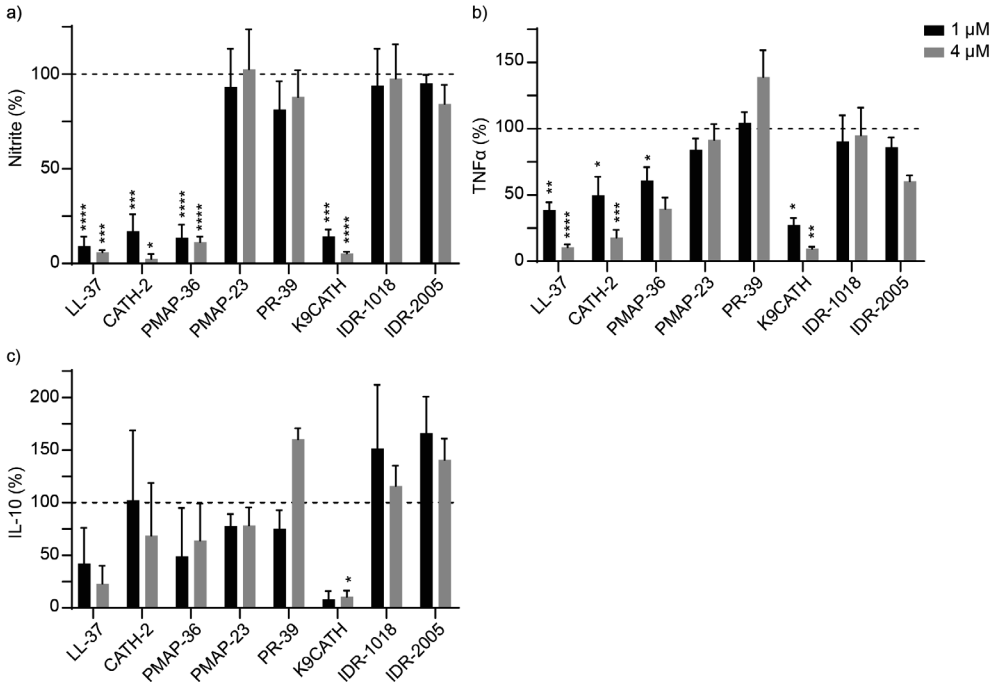

Figure 3: LPS neutralizing capability of HDPs. *RAW264.7 cells were stimulated with B. bronchiseptica LPS and a dose of HDP, stimulation with only LPS was set to 100%. (a) NO production was measured by Griess assay, (b) TNFα and (c) IL-10 using ELISA. Results were analyzed with a two-way ANOVA and Dunnett post-hoc. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, compared to OMVs without HDP supplemented.*

Next, TLR5 was also stimulated by OMVs, showing the presence of flagellae in the sample, but this could not be neutralized by any HDP tested. TLR9, activated by DNA, was mainly stimulated by sOMVs and could be downregulated slightly by LL-37, CATH-2 and PMAP-36.

Uptake of OMVs does not influence macrophage activation

Not much is known about uptake of OMVs, but the TLR9 activation suggested that OMVs or components thereof were internalized. To investigate whether uptake was necessary for OMVs to evoke an immune response, several inhibitors were added during stimulation of RAW264.7 cells. Filipin, nystatin and methyl-β-cyclodextrin (MbCD) all inhibit caveolae-dependent endocytosis, but only nystatin also interferes with lipid raft mediated uptake [38,39]. Chlorpromazine (CPZ) inhibits clathrin-mediated endocytosis, while ethylisopropyl amiloride (EIPA) blocks macropinocytosis [38,39]. Several inhibitors were tested, but only nystatin and EIPA were able to significantly reduce the OMV-induced activation, suggesting OMVs can be internalized by macrophages via different routes **(Fig. 5)**. The nitrite production was only slightly reduced, suggesting internalization of OMVs

may be only partially responsible for the immune response observed. No cytotoxic effects were observed for the other inhibitors, only some metabolic activation of macrophages was observed **(Fig. S5)**. Activation of macrophages by heat-killed *Escherichia coli* was used to control for inhibitor function, but only nystatin was able to block uptake of *E. coli* and subsequent activation of macrophages **(Fig. S5)**.

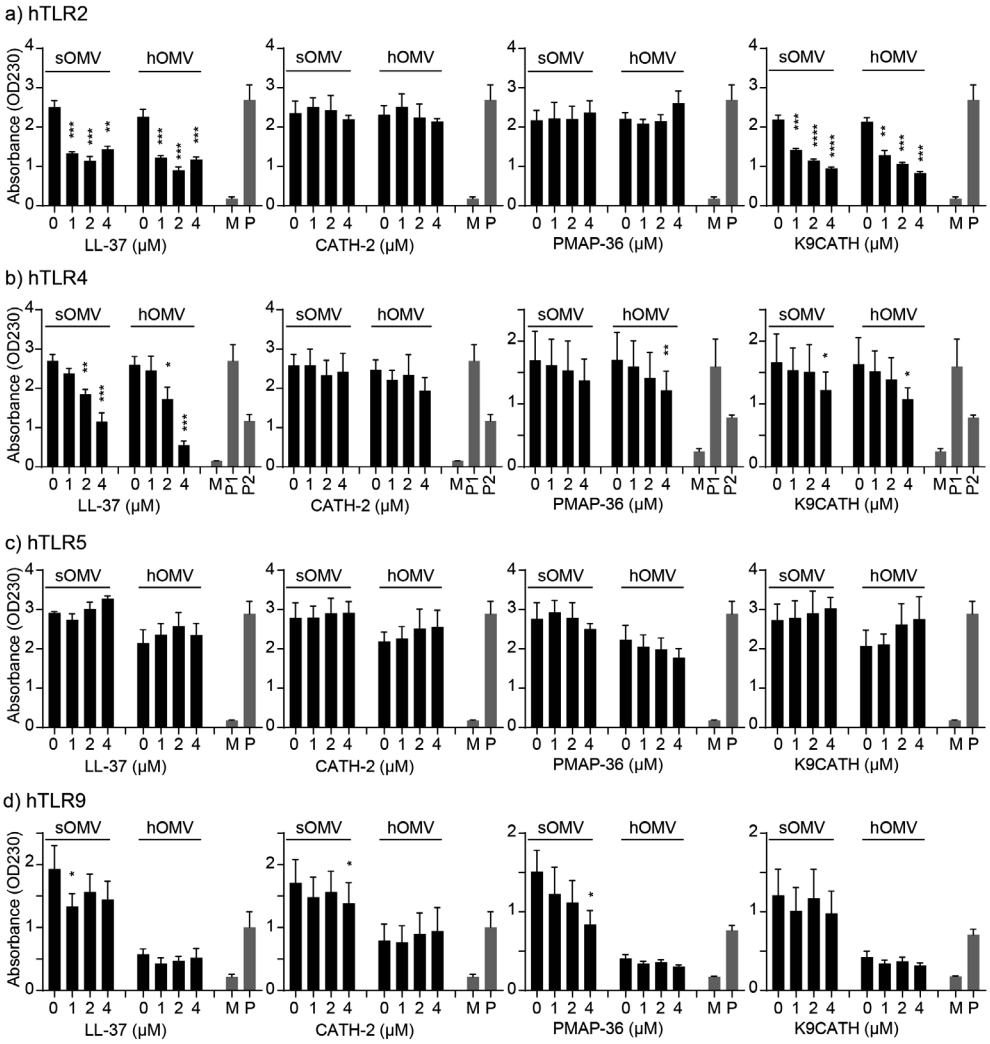

Figure 4: Screen of TLR activation in HEK-cells and modulation by cathelicidins. *HEK-Blue (a) hTLR2, (b) hTLR4, (c) hTLR5 or (d) hTLR9 were stimulated with 0.025 μg/mL sOMVs or hOMVs and a dose of peptide. (M) is DMEM-medium and (P) is a positive control, which differed per cell-line: TLR2: 100 ng/ml Pam3CSK4, TLR4: p1 = 10 ng/ml E. coli LPS, p2= 10 ng/ml B. bronchiseptica LPS, TLR5: 10 ng/ml flagellin from Salmonella Typhimurium, TLR9: 2.5 μM ODN2006. Results were analyzed using a repeated measures two-way ANOVA with Dunnett post-hoc test (n=3). *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, compared to OMVs without cathelicidin supplemented.*

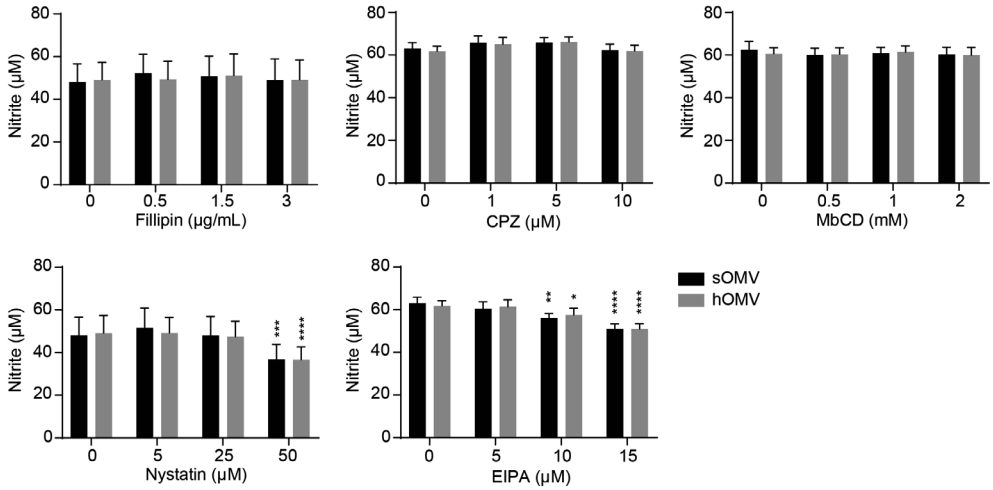

Figure 5: Effect of uptake inhibitors on macrophage activation by OMVs. *RAW264.7 cells were stimulated with 0.025 µg/mL sOMVs or hOMVs, together with a dose of Fillipin, Chlorpromazine (CPZ), methyl-β-cyclodextrin (MbCD), Nystatin or Ethylisopropyl amiloride (EIPA). Results were analyzed using a repeated measures two-way ANOVA with Dunnett post-hoc test (n=3-4). *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, compared to OMVs without inhibitor supplemented.*

Spontaneous and heat-induced OMVs interact differently with PMAP-36

Cathelicidins were shown to differentially modulate immune responses mediated by spontaneous or heat-induced OMVs. Therefore, interactions between cathelicidins and the two OMV classes were investigated. This was performed for PMAP-36, since it was shown to decrease NO production after stimulation with sOMVs but to increase NO production after stimulation with hOMVs **(Fig. 2)**. To investigate the interaction between PMAP-36 and OMVs, ITC was performed. Both classes of OMVs are involved in an endothermic reaction with PMAP-36, but the highest interaction peak locates at a different moment for spontaneous and heat-induced OMVs, indicating a slightly different interaction mechanism **(Fig. 6a)**.

To further look into the interaction and possible effect on OMV stability, a temperature gradient was applied to OMVs, either with or without PMAP-36, and size of the OMVs was measured using DLS. This revealed that PMAP-36 addition did not influence stability of hOMVs, but it did decrease stability of sOMVs **(Fig. 6b)**. This suggests that the interaction between PMAP-36 and spontaneous or heat-induced OMVs is different and this might explain the differences in modulation of immune responses evoked by spontaneous or heat-induced OMVs.

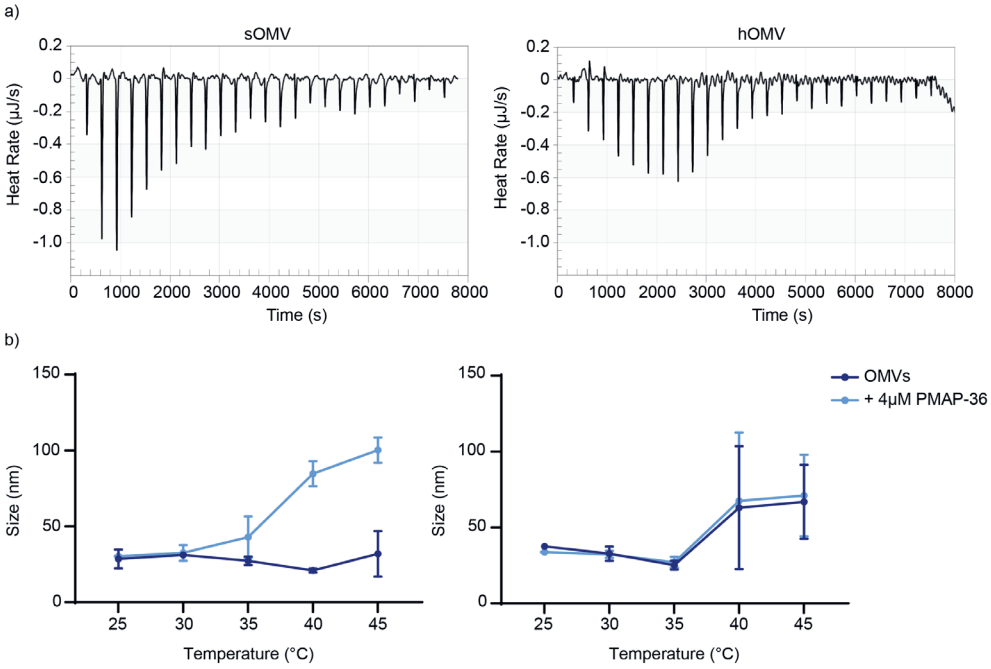

Figure 6: Interaction between PMAP-36 and spontaneous or heat-induced OMVs. *(a) ITC was used to investigate binding between 200 µM PMAP-36 and spontaneous or heat-induced OMVs (30 µg/mL protein). Spectra obtained indicate minor differences in affinity and binding mode. (b) DLS was used to observe differences in stability of sOMVs (left) or hOMVs (right) over a temperature gradient after addition of 4 µM PMAP-36. PMAP-36 did affect stability of spontaneous OMVs.*

Discussion

In this study the response of macrophages to *B. bronchiseptica* OMVs was assessed and the ability of HDPs to modulate these immune responses was investigated. Since heat treatment of bacteria has been shown to induce release of OMVs, and therefore might be economically interesting for vaccine development, hOMVs were tested alongside sOMVs in this study. For OMVs to be used in vaccines, modulation might be necessary, since immune responses evoked by OMVs or LPS might be too severe and cause unwanted side-effects. HDPs are known to efficiently neutralize LPS-evoked TLR4 activation [19] but in this study modulation by HDPs of OMVs was shown to be relatively low **(Fig. 2)**. Furthermore, it was assessed that OMVs activate not only through TLR4, but also TLR2, TLR5 and TLR9 **(Fig. 4)** indicating that OMV samples also contain other TLR agonists like lipoproteins, flagellin and DNA. Likewise, OMVs from *Shigella* and *Salmonella* have also been shown to activate TLR2, TLR4 and TLR5 [40].

When RAW264.7 macrophages were stimulated with spontaneous or heat-induced OMVs, NO production, TNFα and IL-10 secretion were all efficiently induced **(Fig. 1)**. *E. coli* OMVs

were shown before to activate RAW264.7 macrophages almost to the same extent as heat-killed bacteria [41]. However, differences were visible between sOMVs and hOMVs of *B. bronchiseptica*, where hOMVs induced more production of NO, TNFα and IL-10 despite the use of equal protein concentrations to stimulate the macrophages. This could indicate that different components are present in hOMVs, which more efficiently stimulate macrophages. Ratios of proteins and lipids were described to differ between sOMVs and hOMVs [37], where hOMVs have 2-fold more lipids compared to protein concentration, which could affect immune activation. Interestingly, when sOMVs and hOMVs were used to stimulate HEK-Blue cells, sOMVs evoked a higher activation, especially for hTLR5 and hTLR9 **(Fig. 4)**. This indicates that the origin of the TLR receptor, being mouse versus human, might also influence the response to OMVs of this strain of *B. bronchiseptica*. These effects were also observed for *Rhodobacter sphaeroides*, where it acted as agonist of TLR4 in horses and hamsters, but as antagonist in humans mice [42].

Modulation of OMV-evoked immune responses by HDPs was assessed and only four out of the eight HDPs tested were able to modulate OMV-induced immune responses; LL-37, CATH-2, PMAP-36 and K9CATH. NO production was decreased by all cathelicidins for sOMVs, while expression of TNFα was only decreased by K9CATH and IL-10 only by LL-37 and CATH-2 **(Fig. 2)**. When polymyxin B and Pep19-2.5 were studied in the context of OMV modulation, both showed a 4-fold reduction of TNFα production in THP-1 macrophages [21]. The lack of modulation can be due to components being less accessible to HDPs when presented in an OMV, as shown in **Figure 3** where some HDPs were in fact able to fully neutralize soluble LPS. Modulation of OMV-induced TLR4 activation in the HEK-Blue cells was also minimal, compared to previously described results in literature [19]. Pure *B. bronchiseptica* LPS was also tested in hTLR4 HEK-Blue cells to investigate the LPS-neutralizing potential in this cell line and this showed that K9CATH could reduce pure LPS-evoked stimulation four-fold (data not shown), while OMV-evoked stimulation could only be reduced 1.5-fold in HEK-Blue cells **(Fig. 4)**.

Additionally, OMVs were shown to stimulate multiple TLRs and not all of them can be neutralized by all cathelicidins **(Fig. 4)**. In the HEK-Blue system, LL-37 was able to neutralize TLR2-, TLR4- and TLR9-mediated activation slightly, while CATH-2 was only able to slightly neutralize TLR9-mediated activation. PMAP-36 only neutralized TLR4 and TLR9 slightly, while K9CATH neutralized TLR2 and TLR4. Modulation of TLR5 was not achieved by these peptides and has been described as limited in literature as well [43]. However, in RAW264.7 cells CATH-2 and PMAP-36 were able to neutralize LTA-induced TLR2 activation [19]. In peripheral blood mononuclear cells (PBMCs) LL-37 was also shown to decrease TLR2 and TLR4 stimulation, although TLR2 neutralization was not achieved in keratinocytes [43–45]. Furthermore, while CATH-2 showed a slight decrease of TLR9-mediated activation in this study, it was found before to enhance DNA-mediated TLR9

activation [46]. Since DNA is not freely available in the OMV preparations, CATH-2 might interact different in this study. This suggests that the origin of cell stimulated and the presentation of the components used to stimulate may affect the modulation by the HDP. The human LL-37 seems more efficient in modulating immune responses in the human TLR HEK-Blue system, while the chicken CATH-2 and porcine PMAP-36 seem more efficient in modulating immune responses in the mouse macrophage system compared with the human TLR HEK-Blue system. This could indicate HDPs not only block immune responses by interacting with the OMV, but also with the immune cell, as described for LL-37. In those studies, TNFα production was reduced when LL-37 was used to stimulate THP-1 macrophages for 30 min before or after LPS stimulation [44,47]. To distinguish between direct effects on LPS and indirect effects on the immune cells, similar sequential stimulations could be employed for other HDPs.

Since TLR9 was shown to be activated by OMVs, uptake of OMVs (or components) was investigated. Several inhibitors were added to RAW264.7 macrophages during stimulation with OMVs. Filipin, nystatin and methyl-β-cyclodextrin (MbCD) all inhibit caveolae-dependent endocytosis, but only nystatin also interferes with lipid raft mediated uptake [38,39]. Chlorpromazine (CPZ) inhibits clathrin-mediated endocytosis, while ethylisopropyl amiloride blocks macropinocytosis [38,39]. A significant decrease of OMV-induced macrophage activation was only observed in the presence of nystatin and EIPA **(Fig. 5)**. Since the other caveolae-dependent endocytosis inhibitors showed no decrease, the effect of nystatin is probably mediated by inhibition of lipid raft mediated endocytosis. EIPA blocks macropinocytosis, indicating that OMVs can be internalized via different routes. Both inhibitors only decreased macrophages activation slightly, indicating OMV-induced activation of macrophages is most prominently caused by surface receptors such as TLR4 and only slightly by internal receptors such as TLR9. OMVs are also capable of fusing with host cell membranes [38] although this might not be the preferred route for TLR9 activation. However, it must be noted that hTLR9 expression in the HEK-Blue system is artificial and not fully controlled, its location of expression is unknown for instance. Additionally, uptake of OMVs can differ between cell types. It must be noted that not all inhibitors are equally efficient in every cell type, so RAW264.7 macrophages might not respond well to the inhibitors used [48]. Alternatively, OMVs can be labelled and followed, a methodology which showed that *E. coli* OMVs are endocytosed by RAW264.7 cells [41]. OMVs of the gut commensal *Bacteroides thetaiotaomicron* were shown to be endocytosed by Caco-2 cells mainly via dynamin-dependent endocytosis [39]. Not only Gram-negative OMVs are endocytosed, also *Streptococcal* membrane vesicles were shown to be taken up by various cell types [49]. Properties of the vesicle can also influence uptake or interactions with host-cell membranes, since OMVs containing rough LPS were shown to interact with

host-cell membranes faster than OMVs containing smooth LPS [50]. All these factors have to be taken into account when studying uptake of OMVs.

Modulation of cathelicidins did not only depend on the TLR activated, but also on the OMV type used for stimulation. Heat treatment of bacteria was shown to induce more OMVs, but also modulate properties of the resulting hOMVs [37]. Generally, HDPs were less capable of modulation hOMV-induced immune responses compared to sOMV-induced immune responses. This could be caused by the difference in molecular composition and physical structure [37]. Compared to sOMVs, hOMV protein concentration increased 3.6x, while lipid concentration increased 6.5x, which results in a different lipid to protein ratio in hOMVs compared to sOMVs. Similar to that, DNA to protein ratios might differ, which could explain that hTLR9 is more stimulated by sOMVs compared to hOMVs **(Fig. 4)**, since OMV concentrations used for stimulation are based on protein concentration. Furthermore, interactions between HDPs and OMVs could be influenced, since hOMVs were shown to contain more lysophospholipids which might affect the curvature of the membrane [37]. With PMAP-36 it was shown that even though the peptide interacted with both OMV types, it only affected stability of sOMVs at higher temperatures **(Fig. 6)**. Many factors can influence OMV interactions with immune cells and HDPs and this needs to be taken into account when contemplating vaccine potential of OMVs.

Since OMVs contain many antigens in their native environment, but are non-replicative particles, they are promising for vaccine development. However, OMV yield may not be very high and therefore heat-induction of OMVs seems a promising method to obtain economically viable preparations. Immune responses are still efficiently evoked by hOMVs and antigens were still recognized by antibodies and therefore probably still immunogenic [30]. However, hOMVs are less sensitive to modulation by cathelicidins, although LL-37 and K9CATH are good candidates for modulation. Furthermore, current OMV preparations contain flagellin, which activates TLR5, and might excessively stimulate the immune system and deviate from immune responses against relevant antigens. Therefore, further purification, perhaps by size exclusion or density gradient ultracentrifugation [51,52], might be required before OMVs can be used in vaccines.

Concluding remarks

This study shows that both spontaneous and heat-induced OMVs of *B. bronchiseptica* induce immune responses in RAW264.7 macrophages and hTLR2-, hTLR4-, hTLR5- and hTLR9-mediated HEK-Blue cell line activation. Furthermore, HDPs were tested for neutralization capabilities and only LL-37, CATH-2, PMAP-36 and K9CATH were able to do so. They could not neutralize all TLR activations and modulated immune responses differently for sOMVs and hOMVs. Overall, OMVs have great potential to be used in

bacterial vaccines and HDPs are capable of balancing the resulting immune responses, but this does depend on the induction method of OMVs and host species.

Acknowledgements

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References

[1] C.L. Ventola, The antibiotic resistance crisis: part 1: causes and threats., P T. 40 (2015) 277–83.

[2] C.L. Ventola, The antibiotic resistance crisis: part 2: management strategies and new agents., P T. 40 (2015) 344–52.

[3] B.L. Deatherage, J.C. Lara, T. Bergsbaken, S.L.R. Barrett, S. Lara, B.T. Cookson, Biogenesis of bacterial membrane vesicles, Mol. Microbiol. 72 (2009) 1395–1407. doi:10.1111/j.1365-2958.2009.06731.x.

[4] C. Schwechheimer, M.J. Kuehn, Outer-membrane vesicles from Gram-negative bacteria: Biogenesis and functions, Nat. Rev. Microbiol. 13 (2015) 605–619. doi:10.1038/nrmicro3525.

[5] R. Acevedo, S. Fernández, C. Zayas, A. Acosta, M.E. Sarmiento, V.A. Ferro, E. Rosenqvist, C. Campa, D. Cardoso, L. Garcia, J.L. Perez, Bacterial outer membrane vesicles and vaccine applications, Front. Immunol. 5 (2014) 1–6. doi:10.3389/fimmu.2014.00121.

[6] L. van der Pol, M. Stork, P. van der Ley, Outer membrane vesicles as platform vaccine technology, Biotechnol. J. 10 (2015) 1689–1706. doi:10.1002/biot.201400395.

[7] M.J.H. Gerritzen, D.E. Martens, R.H. Wijffels, L. van der Pol, M. Stork, Bioengineering bacterial outer membrane vesicles as vaccine platform, Biotechnol. Adv. 35 (2017) 565–574. doi:10.1016/j.biotechadv.2017.05.003.

[8] S. Wang, J. Gao, Z. Wang, Outer membrane vesicles for vaccination and targeted drug delivery, WIREs Nanomedicine and Nanobiotechnology. 11 (2019) e1523. doi:10.1002/wnan.1523.

[9] J. Holst, P. Oster, R. Arnold, M. Tatley, L. Næss, I. Aaberge, Y. Galloway, A. McNicholas, J. O'Hallahan, E. Rosenqvist, S. Black, Vaccines against meningococcal serogroup B disease containing outer membrane vesicles (OMV): Lessons from past programs and implications for the future, Hum. Vaccin. Immunother. 9 (2013) 1241–1253. doi:10.4161/hv.24129.

[10] M.E. Gaillard, D. Bottero, A. Errea, M. Ormazábal, M.E. Zurita, G. Moreno, M. Rumbo, C. Castuma, E. Bartel, D. Flores, P. van der Ley, A. van der Ark, D. F. Hozbor, Acellular pertussis vaccine based on outer membrane vesicles capable of conferring both long-lasting immunity and protection against different strain genotypes, Vaccine. 32 (2014) 931–937. doi:10.1016/j.vaccine.2013.12.048.

[11] J.D. Cecil, N.M. O'Brien-Simpson, J.C. Lenzo, J.A. Holden, W. Singleton, A. Perez-Gonzalez, A. Mansell, E.C. Reynolds, Outer membrane vesicles prime and activate

macrophage inflammasomes and cytokine secretion in vitro and in vivo, Front. Immunol. 8 (2017) 1–22. doi:10.3389/fimmu.2017.01017.

[12] W. Cai, D.K. Kesavan, J. Wan, M.H. Abdelaziz, Z. Su, H. Xu, Bacterial outer membrane vesicles, a potential vaccine candidate in interactions with host cells based, Diagn. Pathol. 13 (2018) 1–12. doi:10.1186/s13000-018-0768-y.

[13] M. Kaparakis-Liaskos, R.L. Ferrero, Immune modulation by bacterial outer membrane vesicles, Nat. Rev. Immunol. 15 (2015) 375–387. doi:10.1038/nri3837.

[14] M. Zasloff, Antimicrobial peptides of multicellular organisms, Nature. 415 (2002) 389–395. doi:10.1038/415389a.

[15] M. Zanetti, Cathelicidins, multifunctional peptides of the innate immunity, J. Leukoc. Biol. 75 (2004) 39–48. doi:10.1189/jlb.0403147.

[16] K.A. Brogden, Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria?, Nat. Rev. Microbiol. 3 (2005) 238–250. doi:10.1038/nrmicro1098.

[17] R. van Harten, E. van Woudenbergh, A. van Dijk, H. Haagsman, Cathelicidins: Immunomodulatory Antimicrobials, Vaccines. 6 (2018) 63. doi:10.3390/vaccines6030063.

[18] N. Mookherjee, M.A. Anderson, H.P. Haagsman, D.J. Davidson, Antimicrobial host defence peptides: functions and clinical potential, Nat. Rev. Drug Discov. 19 (2020) 311–332. doi:10.1038/s41573-019-0058-8.

[19] M. Coorens, M.R. Scheenstra, E.J.A. Veldhuizen, H.P. Haagsman, Interspecies cathelicidin comparison reveals divergence in antimicrobial activity, TLR modulation, chemokine induction and regulation of phagocytosis, Sci. Rep. 7 (2017) 1–11. doi:10.1038/srep40874.

[20] M. Coorens, V.A.F. Schneider, A.M. de Groot, A. van Dijk, M. Meijerink, J.M. Wells, M.R. Scheenstra, E.J.A. Veldhuizen, H.P. Haagsman, Cathelicidins Inhibit Escherichia coli –Induced TLR2 and TLR4 Activation in a Viability-Dependent Manner, J. Immunol. 199 (2017) 1418–1428. doi:10.4049/jimmunol.1602164.

[21] A. Pfalzgraff, W. Correa, L. Heinbockel, A.B. Schromm, C. Lübow, N. Gisch, G. Martinez-de-Tejada, K. Brandenburg, G. Weindl, LPS-neutralizing peptides reduce outer membrane vesicle-induced inflammatory responses, Biochim. Biophys. Acta - Mol. Cell Biol. Lipids. 1864 (2019) 1503–1513. doi:10.1016/j.bbalip.2019.05.018.

[22] T. Magyar, T. Donkó, F. Kovács, Atrophic rhinitis vaccine composition triggers different serological profiles that do not correlate with protection, Acta Vet. Hung. 56 (2008) 27–40. doi:10.1556/AVet.56.2008.1.4.

[23] J.A. Ellis, How well do vaccines for Bordetella bronchiseptica work in dogs? A critical review of the literature 1977-2014, Vet. J. 204 (2015) 5–16. doi:10.1016/j.tvjl.2015.02.006.

[24] C. Locht, Pertussis: acellular, whole-cell, new vaccines, what to choose?, Expert Rev. Vaccines. 15 (2016) 671–673. doi:10.1586/14760584.2016.1161511.

[25] D. Jayasundara, E. Lee, S. Octavia, R. Lan, M.M. Tanaka, J.G. Wood, Emergence of pertactin-deficient pertussis strains in Australia can be explained by models of vaccine escape, Epidemics. 31 (2020) 100388. doi:10.1016/j.epidem.2020.100388.

[26] N. Burdin, L.K. Handy, S.A. Plotkin, What Is Wrong with Pertussis Vaccine Immunity? The Problem of Waning Effectiveness of Pertussis Vaccines., Cold Spring Harb. Perspect. Biol. 9 (2017) 1–18. doi:10.1101/cshperspect.a029454.

[27] A. Van Dijk, M. Van Eldik, E.J.A. Veldhuizen, H.L.M. Tjeerdsma-Van Bokhoven, M.R. De Zoete, F.J. Bikker, H.P. Haagsman, Immunomodulatory and anti-inflammatory activities of chicken cathelicidin-2 derived peptides, PLoS One. 11 (2016) 1–16. doi:10.1371/journal.pone.0147919.

[28] V.A.F. Schneider, A. van Dijk, A.M. van der Sar, M.D. Kraaij, E.J.A. Veldhuizen, H.P. Haagsman, Prophylactic administration of chicken cathelicidin-2 boosts zebrafish embryonic innate immunity, Dev. Comp. Immunol. 60 (2016) 108–114. doi:10.1016/j.dci.2016.02.023.

[29] M.D. Balhuizen, E.J.A. Veldhuizen, H.P. Haagsman, Outer Membrane Vesicle Induction and Isolation for Vaccine Development, Front. Microbiol. 12 (2021) 629090. doi:10.3389/fmicb.2021.629090.

[30] E.F. de Jonge, M.D. Balhuizen, R. van Boxtel, J. Wu, H.P. Haagsman, J. Tommassen, Heat shock enhances outer-membrane vesicle release in Bordetella spp., Curr. Res. Microb. Sci. 2 (2021) 100009. doi:10.1016/j.crmicr.2020.100009.

[31] M.R. Scheenstra, M. van den Belt, J.L.M. Tjeerdsma-van Bokhoven, V.A.F. Schneider, S.R. Ordonez, A. van Dijk, E.J.A. Veldhuizen, H.P. Haagsman, Cathelicidins PMAP-36, LL-37 and CATH-2 are similar peptides with different modes of action, Sci. Rep. 9 (2019) 4780. doi:10.1038/s41598-019-41246-6.

[32] E.J.A. Veldhuizen, M.R. Scheenstra, J.L.M. Tjeerdsma-van Bokhoven, M. Coorens, V.A.F. Schneider, F.J. Bikker, A. van Dijk, H.P. Haagsman, Antimicrobial and Immunomodulatory Activity of PMAP-23 Derived Peptides, Protein Pept. Lett. 24 (2017) 1–36. doi:10.2174/0929866524666170428150925.

[33] E.F. Haney, B. Wu, K. Lee, A.L. Hilchie, R.E.W. Hancock, Aggregation and Its Influence on the Immunomodulatory Activity of Synthetic Innate Defense Regulator Peptides, Cell Chem. Biol. 24 (2017) 969-980.e4. doi:10.1016/j.chembiol.2017.07.010.

[34] Y. Sang, M. Teresa Ortega, K. Rune, W. Xiau, G. Zhang, J.L. Soulages, G.H. Lushington, J. Fang, T.D. Williams, F. Blecha, T. Melgarejo, Canine cathelicidin (K9CATH): Gene cloning, expression, and biochemical activity of a novel pro-myeloid antimicrobial peptide, Dev. Comp. Immunol. 31 (2007) 1278–1296. doi:10.1016/J.DCI.2007.03.007.

[35] E.J.A. Veldhuizen, V.A.F. Schneider, H. Agustiandari, A. Van Dijk, J.L.M. Tjeerdsma-van Bokhoven, F.J. Bikker, H.P. Haagsman, Antimicrobial and immunomodulatory activities of PR-39 derived peptides, PLoS One. 9 (2014) e95939. doi:10.1371/journal.pone.0095939.

[36] W.F. Verwey, E.H. Thiele, D.N. Sage, L.F. Schuchardt, A simplified liquid culture medium for the growth of Hemophilus pertussis, J. Bacteriol. 58 (1949) 127–134. doi:10.1128/jb.58.2.127-134.1949.

[37] M.D. Balhuizen, C.M. Versluis, R.M. van Harten, E.F. de Jonge, J.F. Brouwers, C.H.A. van de Lest, E.J.A. Veldhuizen, J. Tommassen, H.P. Haagsman, PMAP-36 reduces the innate immune response induced by Bordetella bronchiseptica-derived outer membrane vesicles, Curr. Res. Microb. Sci. 2 (2021) 100010. doi:10.1016/j.crmicr.2020.100010.

[38] E.J. O'Donoghue, A.M. Krachler, Mechanisms of outer membrane vesicle entry into host cells, Cell. Microbiol. 18 (2016) 1508–1517. doi:10.1111/cmi.12655.

[39] E.J. Jones, C. Booth, S. Fonseca, A. Parker, K. Cross, A. Miquel-Clopés, I. Hautefort, U. Mayer, T. Wileman, R. Stentz, S.R. Carding, The Uptake, Trafficking, and Biodistribution of Bacteroides thetaiotaomicron Generated Outer Membrane Vesicles, Front. Microbiol. 11 (2020) 1–14. doi:10.3389/fmicb.2020.00057.

[40] F. Mancini, O. Rossi, F. Necchi, F. Micoli, OMV Vaccines and the Role of TLR Agonists in Immune Response, Int. J. Mol. Sci. 21 (2020) 4416. doi:10.3390/ijms21124416.

[41] R. Hu, H. Lin, J. Li, Y. Zhao, M. Wang, X. Sun, Y. Min, Y. Gao, M. Yang, Probiotic Escherichia coli Nissle 1917-derived outer membrane vesicles enhance immunomodulation and antimicrobial activity in RAW264.7 macrophages, BMC Microbiol. 20 (2020) 268. doi:10.1186/s12866-020-01953-x.

[42] D. Werling, O.C. Jann, V. Offord, E.J. Glass, T.J. Coffey, Variation matters: TLR structure and species-specific pathogen recognition, Trends Immunol. 30 (2009) 124–130. doi:10.1016/j.it.2008.12.001.

[43] M.R. Scheenstra, R.M. van Harten, E.J.A. Veldhuizen, H.P. Haagsman, M. Coorens, Cathelicidins Modulate TLR-Activation and Inflammation, Front. Immunol. 11 (2020) 1137. doi:10.3389/fimmu.2020.01137.

[44] N. Mookherjee, K.L. Brown, D.M.E. Bowdish, S. Doria, R. Falsafi, K. Hokamp, F.M. Roche, R. Mu, G.H. Doho, J. Pistolic, J.-P. Powers, J. Bryan, F.S.L. Brinkman, R.E.W. Hancock, Modulation of the TLR-Mediated Inflammatory Response by the Endogenous Human Host Defense Peptide LL-37, J. Immunol. 176 (2006) 2455–2464. doi:10.4049/jimmunol.176.4.2455.

[45] M.A. Alford, B. Baquir, F.L. Santana, E.F. Haney, R.E.W. Hancock, Cathelicidin Host Defense Peptides and Inflammatory Signaling: Striking a Balance, Front. Microbiol. 11 (2020) 1–18. doi:10.3389/fmicb.2020.01902.

[46] M. Coorens, A. van Dijk, F. Bikker, E.J.A. Veldhuizen, H.P. Haagsman, Importance of Endosomal Cathelicidin Degradation To Enhance DNA-Induced Chicken Macrophage Activation., J. Immunol. 195 (2015) 3970–7. doi:10.4049/jimmunol.1501242.

[47] N. Mookherjee, H.L. Wilson, S. Doria, Y. Popowych, R. Falsafi, J.J. Yu, Y. Li, S. Veatch, F.M. Roche, K.L. Brown, F.S.L. Brinkman, K. Hokamp, A. Potter, L.A. Babiuk, P.J. Griebel, R.E.W. Hancock, Bovine and human cathelicidin cationic host defense peptides similarly suppress transcriptional responses to bacterial lipopolysaccharide, J. Leukoc. Biol. 80 (2006) 1563–1574. doi:10.1189/jlb.0106048.

[48] D. Vercauteren, R.E. Vandenbroucke, A.T. Jones, J. Rejman, J. Demeester, S.C. De Smedt, N.N. Sanders, K. Braeckmans, The use of inhibitors to study endocytic pathways of gene carriers: Optimization and pitfalls, Mol. Ther. 18 (2010) 561–569. doi:10.1038/mt.2009.281.

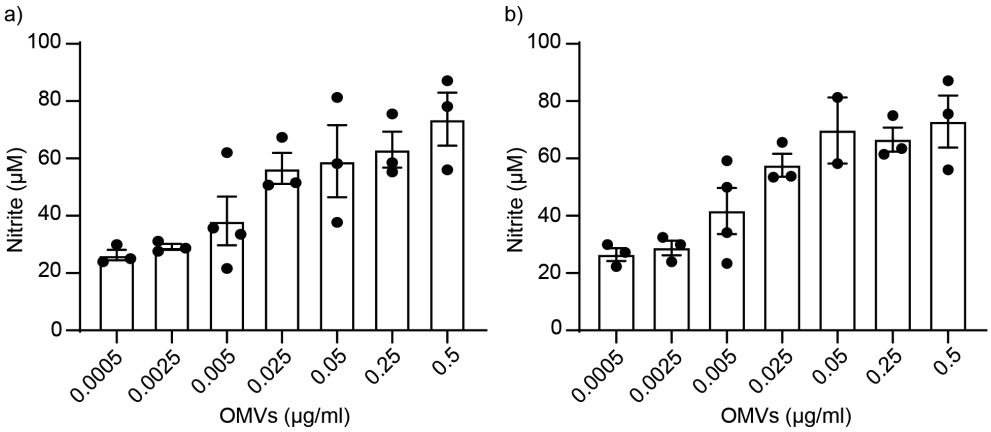
[49] M. Mehanny, M. Koch, C.M. Lehr, G. Fuhrmann, Streptococcal Extracellular Membrane Vesicles Are Rapidly Internalized by Immune Cells and Alter Their Cytokine Release, Front. Immunol. 11 (2020) 80. doi:10.3389/fimmu.2020.00080.

[50] D. Jefferies, S. Khalid, To infect or not to infect: molecular determinants of bacterial outer membrane vesicle internalization by host membranes, J. Mol. Biol. 432 (2020) 1251–1264. doi:10.1016/j.jmb.2020.01.008.

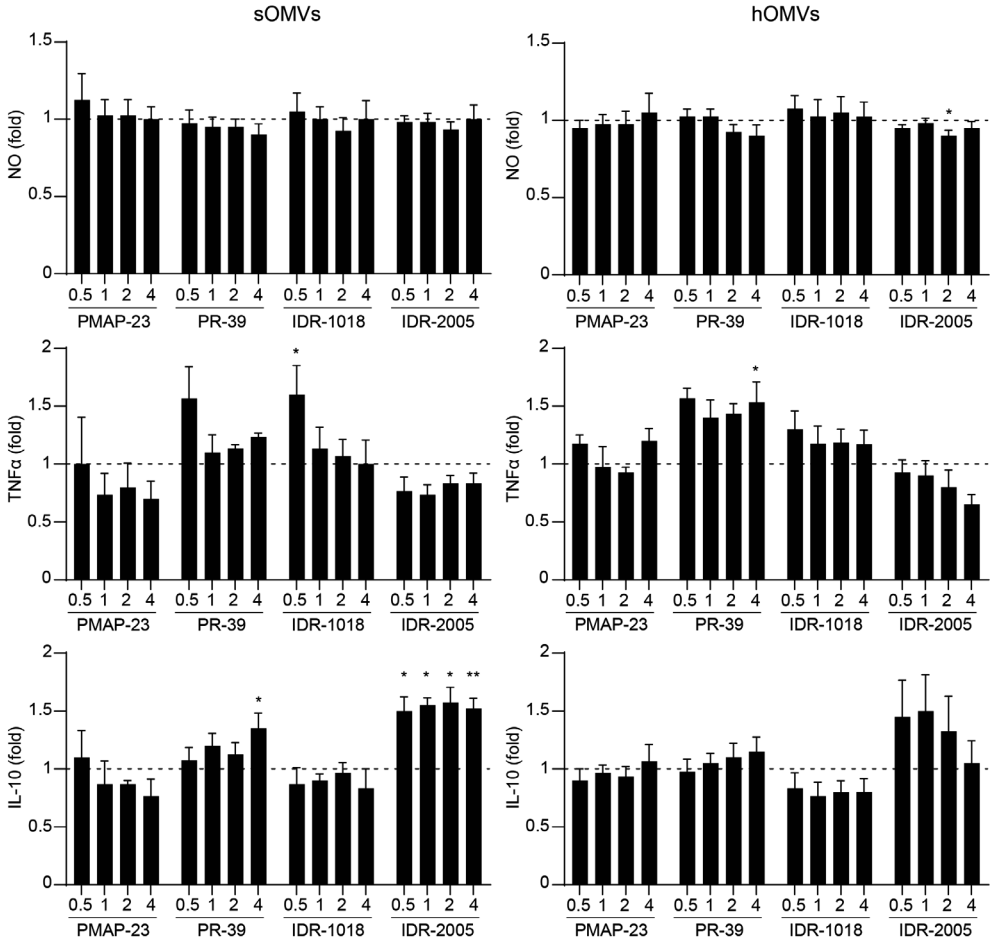
[51] J. Klimentová, J. Stulík, Methods of isolation and purification of outer membrane vesicles from gram-negative bacteria, Microbiol. Res. 170 (2015) 1–9. doi:10.1016/j.micres.2014.09.006.

[52] G. Qing, N. Gong, X. Chen, J. Chen, H. Zhang, Y. Wang, R. Wang, S. Zhang, Z. Zhang, X. Zhao, Y. Luo, X.-J. Liang, Natural and engineered bacterial outer membrane vesicles, Biophys. Reports. 5 (2019) 184–198. doi:10.1007/s41048-019-00095-6.

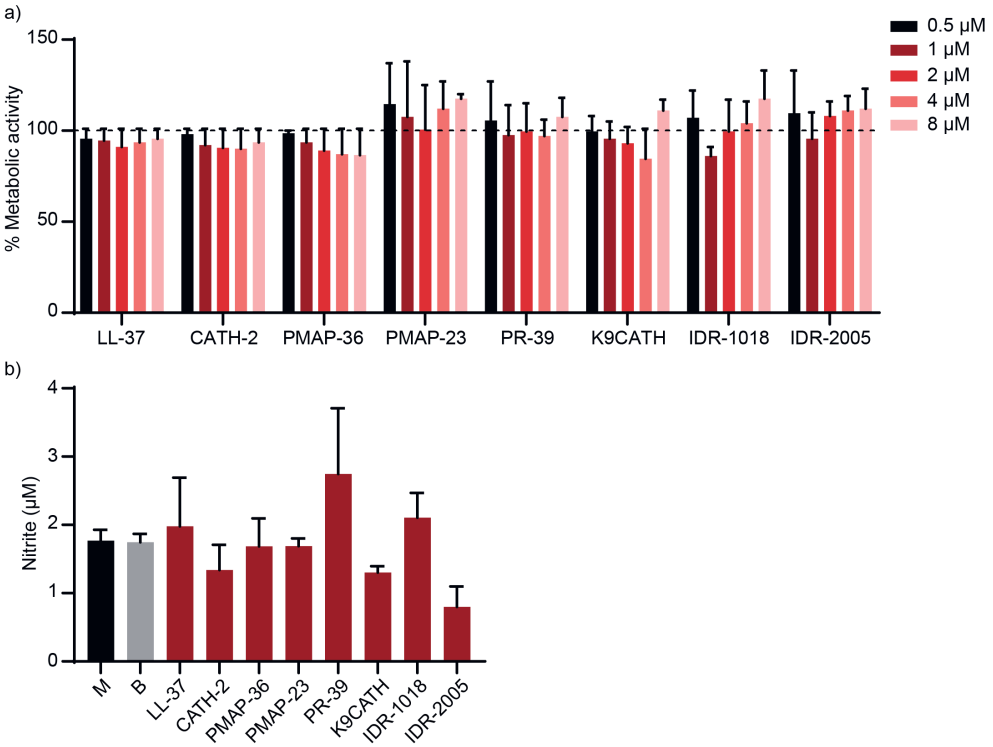
Supplementary Figures



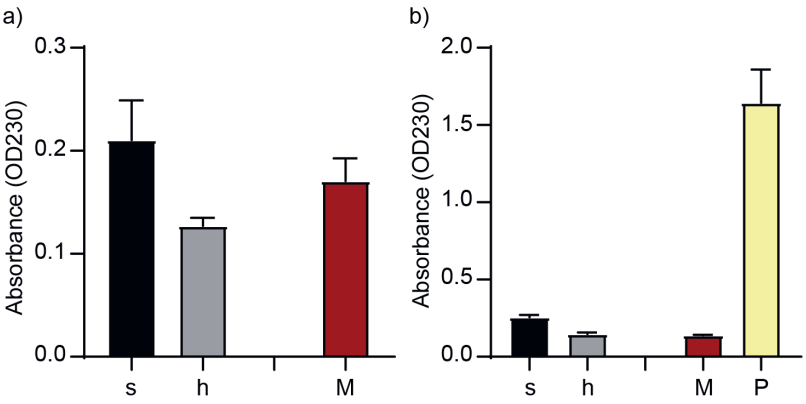
Supplementary Figure 1: OMV dose-dependent NO production by RAW264.7 cells. *Cells were stimulated with serial dilutions of (a) spontaneous and (b) heat-induced B. bronchiseptica OMVs (n=3-4). A concentration of OMVs of 0.025 µg/mL protein was chosen for further experiments.*



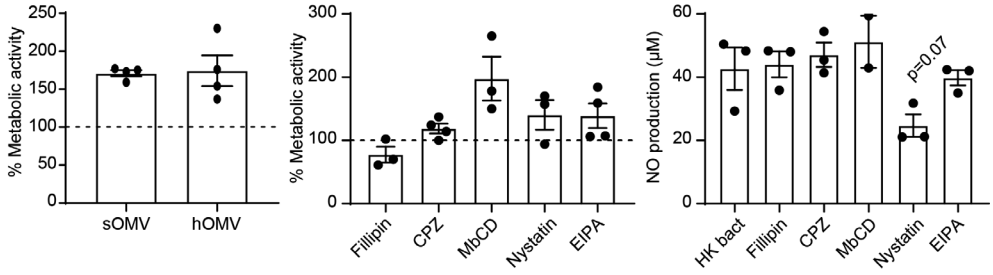
Supplementary Figure 2: Modulation by PMAP-23, PR-39, IDR-1018 and IDR-2005 of RAW264.7 cell activation upon stimulation with spontaneous or heat-induced OMVs. *Cells were stimulated with OMVs and a dose of PMAP-23, PR-39, IDR-1018 or IDR-2005 (0.5, 1, 2 or 4 μM). Results are normalized to signals obtained by only OMV stimulation (depicted by the dotted line). NO production was measured by Griess assay, TNFα and IL-10 using ELISA. No dose-dependent modulation was observed for these four HDPs. Results were analyzed using a repeated measures ANOVA with Dunnett post-hoc test (n=3-6). *=p<0.05, **=p<0.01, compared to OMVs without HDP supplemented.*



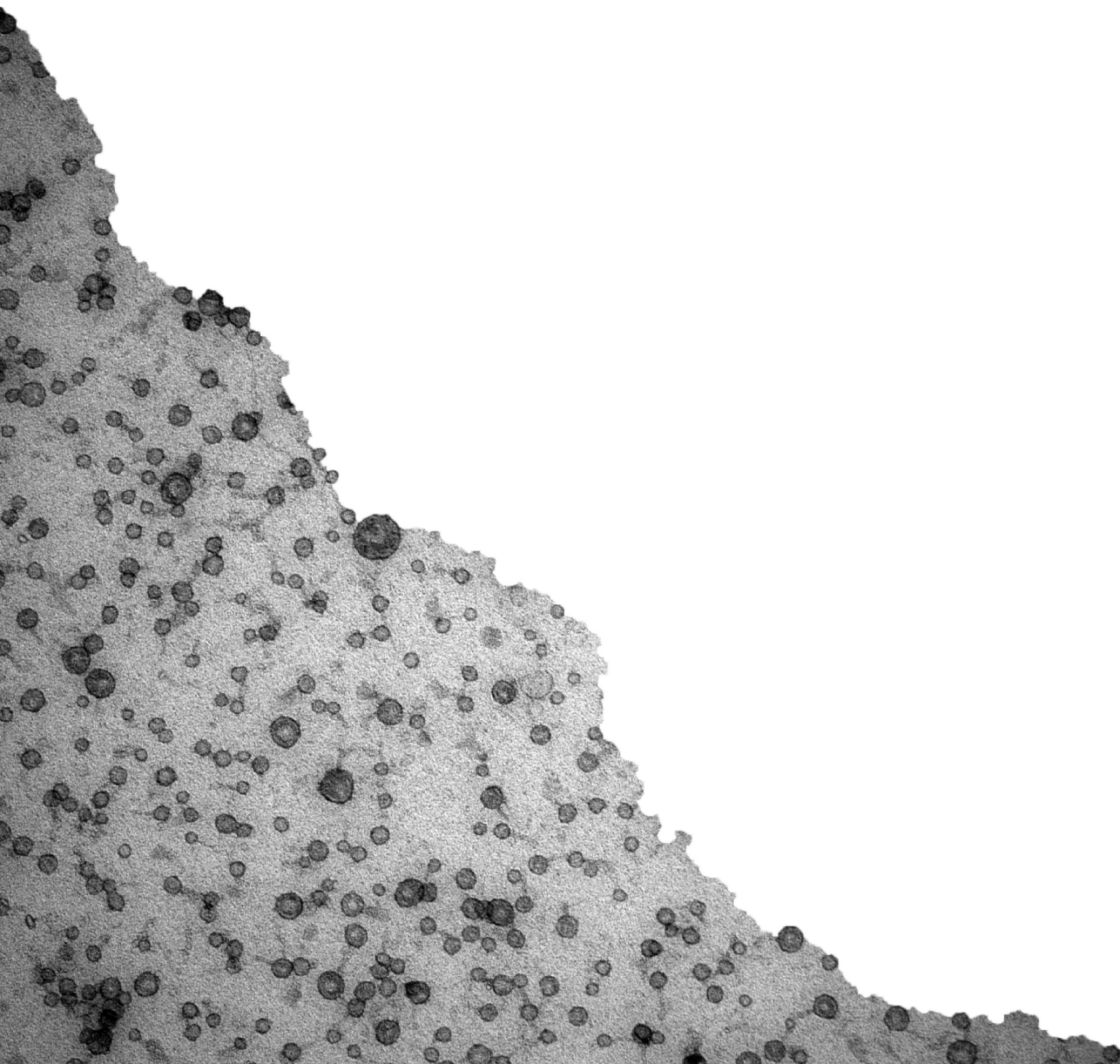
Supplementary Figure 3: HDPs are not cytotoxic and do not activate RAW264.7 cells. *Cells were stimulated with only a dose of HDP. (a) Metabolic activity of RAW cells was determined using the WST-1 assay. Cell stimulation with only medium was set to 100%, represented by the dotted line. (b) NO production was measured by Griess assay, only for 4 µM of HDP (n=2-4). M = cell medium, B = Tris-buffer.*



Supplementary Figure 4: Stimulation of HEK-Blue (a) Null1 and (b) hTLR3 cell lines with OMVs. *Cells were stimulated with 0.025 µg/mL sOMVs (s) or hOMVs (h), DMEM-medium (M) or 10 µg/mL Poly I:C (P).*



Supplementary Figure 5: Cytotoxicity and activation of macrophages stimulated with OMVs, different uptake inhibitors or heat-killed E. coli combined with different uptake inhibitors. *RAW264.7 cells were stimulated with 0.025 µg/mL sOMVs, hOMVs, Fillipin (3 µg/mL), Chlorpromazine (CPZ, 10 µM), methyl-β-cyclodextrin (MbCD, 10 mM), Nystatin (50 µM), Ethylisopropyl amiloride (EIPA, 15 µM) or heat-killed E. coli ($1*10^6$ CFU/mL, HK bact) alone or combined with an uptake inhibitor. WST-1 reagent was used to determine metabolic activity (n=3-4), Griess assay was used to determine macrophage activation (n=2-3).*



Chapter 7

General discussion

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Summary

The scope of this thesis is the interplay between Host Defense Peptides (HDPs) and Outer Membrane Vesicles (OMVs), as summarized in Figure 1. HDPs have both antimicrobial and immunomodulatory properties. OMVs contain multiple antigens in their native environment and are therefore promising for vaccine development. It was also thought that OMVs could protect bacteria against killing by HDPs, which was described before [1,2], but additionally it was investigated whether OMV release could be a response of bacteria upon HDPs in the environment. This increase in OMV yield could also be used for vaccine production, if OMV properties are not significantly altered. HDPs are known to have membrane-active properties [3], but the role of lipopolysaccharide (LPS)-binding in killing of Gram-negative bacteria is not yet evident. Therefore, LPS-binding was correlated with HDP antibacterial effectivity, to distinguish between LPS acting as anchor and facilitating HDP functions or LPS acting as a sink and inhibiting HDP functions. Furthermore, immunomodulatory capabilities of HDPs were investigated, since HDPs are known to neutralize LPS-induced toll-like receptor (TLR) 4 activation [4] but this neutralization has not yet been described for OMV-induced activation. If HDPs could balance OMV-induced immune responses, the combination could be promising for vaccine development.

In chapter 3, it was shown that OMVs can indeed act as a bacterial defense against HDPs. Not only were OMV quantities increased after stimulation with sub-lethal concentrations of HDPs, but also could addition of isolated OMVs to bacterial cultures protect against HDP killing. However, this was not true for all HDPs tested. The differences in the mechanisms of action of HDPs were further elucidated in chapter 4. CATH-2 and PMAP-36 were shown to be membrane active, and permeabilize membranes within 20 minutes, but their effectivity did not correlate with LPS-binding. PMAP-23 showed an interaction with LPS and probably acts via a carpet model, but antibacterial effectivity was not correlated with LPS affinity. PR-39 was shown to be purely intracellularly active and to not affect bacterial membranes at all but did show binding to LPS. Stronger LPS-binding even correlated with enhanced bacterial killing for PR-39. Not only HDPs were shown to affect bacterial membranes, but also heat was shown to affect bacteria and enhance OMV release. In chapter 5, PMAP-36 was studied in more detail with respect to OMV induction and also to the immunomodulation of OMVs. Induction with PMAP-36 resulted in the presence of the peptide in isolated OMVs but this presence did not affect immunomodulation. When PMAP-36 was added to spontaneously formed OMVs (sOMVs) after isolation, it did show a neutralizing effect on the immune response evoked in porcine bone marrow-derived M1 macrophages (pBMDM1). Therefore, a large array of HDPs was investigated for their immunomodulatory capabilities in

combination with OMVs (chapter 6). Four out of the eight HDPs tested showed immunomodulatory effects, being LL-37, CATH-2, PMAP-36 and K9CATH. They were further investigated for their specific TLR neutralizing capabilities. This revealed that OMVs were not only able to stimulate TLR4 and TLR2, which was expected since their ligands are LPS and lipoproteins respectively, but that TLR5 and TLR9 activation also occurred, suggesting that flagellae and DNA in OMV preparations may contribute to immune activation. TLR neutralization was HDP specific, but TLR5 was consistently not neutralized by any peptide tested. Concluding, OMVs indeed play a role in defense against HDPs and HDPs are able to modulation OMV-induced immune responses. However, LPS-binding did not seem to correlate with HDP antibacterial activity.

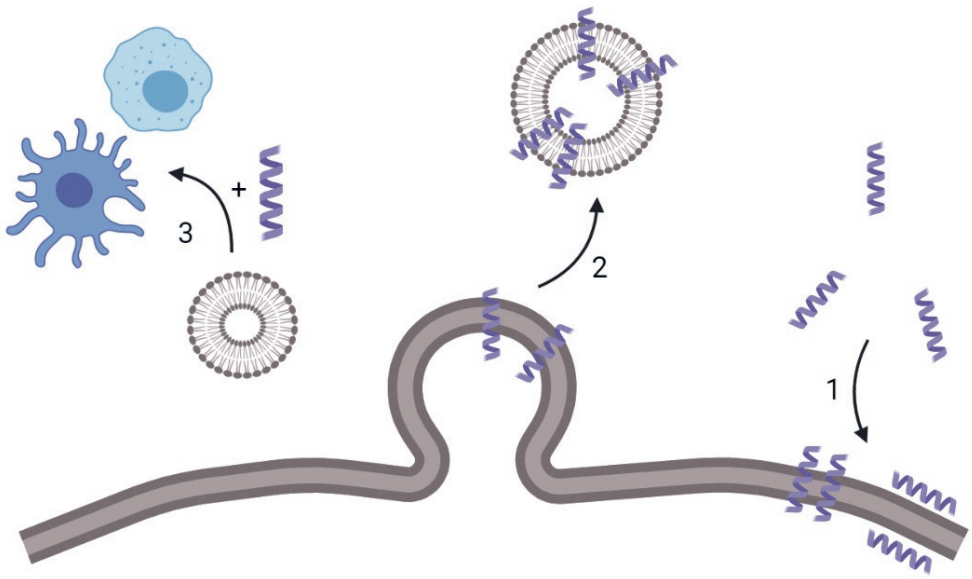


Figure 1: Scope of the thesis summarized. *1. Mechanisms of HDP action were described in chapter 4, as well as interactions with LPS, an abundant molecule in the outer membrane of Gram-negative bacteria. 2. OMVs as defense against HDPs was described in chapter 3, as well as properties of HDP-induced OMVs in chapters 3 and 5. 3. HDPs were combined with OMVs to investigate their OMV-modulating potential in chapters 5 and 6. Created with BioRender.com*

How OMVs affect HDPs function

HDPs are part of the innate immune system and are small molecules (<100 amino acids) with often cationic and amphipathic properties [3]. They have both direct antimicrobial activities as well as immunomodulatory functions [5]. Since they have various bacterial targets, both intracellular and membrane components, resistance against HDPs is not frequently observed [6]. However, some mechanisms have developed in bacteria, such as degradation of HDPs by proteases [7,8]. Another common mechanism used by bacteria is repulsion of HDPs by altering the charge of their membranes [9,10]. Efflux pumps, known to confer resistance against antibiotics, have also been implicated in bacterial defense against HDPs, such as the *mtr* efflux system in *Neisseria gonorrhoeae* [11]. Additionally, proteins can be secreted by bacteria that interfere with HDP killing by binding HDPs or altering HDP properties, such as citrullination of HDPs to reduce their charge and function [12,13] . Two earlier studies also showed that OMVs can protect *Helicobacter pylori* and *Escherichia coli* against killing by LL-37 [1,2]. All currently known defense mechanisms are summarized in **Figure 2**. In **chapter 3** it was confirmed that OMVs can protect not only *E. coli* but also *Bordetella bronchiseptica* and *Pseudomonas aeruginosa* against HDPs, against LL-37 but also against CATH-2 and PMAP-36. This protection could be established via two different mechanisms. Firstly, the OMVs could be produced as a decoy, in response to the bacterium having sensed the presence of HDPs in the environment. The other mechanism could be that the bacterium senses the HDPs when they are in the bacterial membrane and disposes of affected membrane via secretion in the form of an OMV. The latter is more likely, since for the first mentioned mechanism the bacterium needs to retrieve sensors from the environment which relay information about the presence of HDPs, and then in response needs to start enhancing OMV production. In that same timeframe, HDPs will have reached the bacterial membrane, and probably OMVs will be too late to act as a decoy. Sensing HDPs in the bacterial membrane is quicker and more likely, suggesting OMVs are produced as a response to the presence of HDP in the membrane, to dispose of affected membrane.

Different sensor molecules are known to detect HDPs in the environment and turn on a cascade of reactions in the bacterial cell. In the Gram-positive *Staphylococcus aureus*, the sensor protein ApsS not only alters the bacterial membrane charge upon detection of HDPs, but also activates a putative HDP transporter system [14]. In the Gram-negative *Salmonella typhimurium*, the sensor kinase PhoQ was shown to be activated by LL-37 [15]. However, most HDPs do not target proteins but interact with membrane lipids. Insertion of HDPs will affect membrane fluidity, which in turn could also be sensed by the bacterium [16].

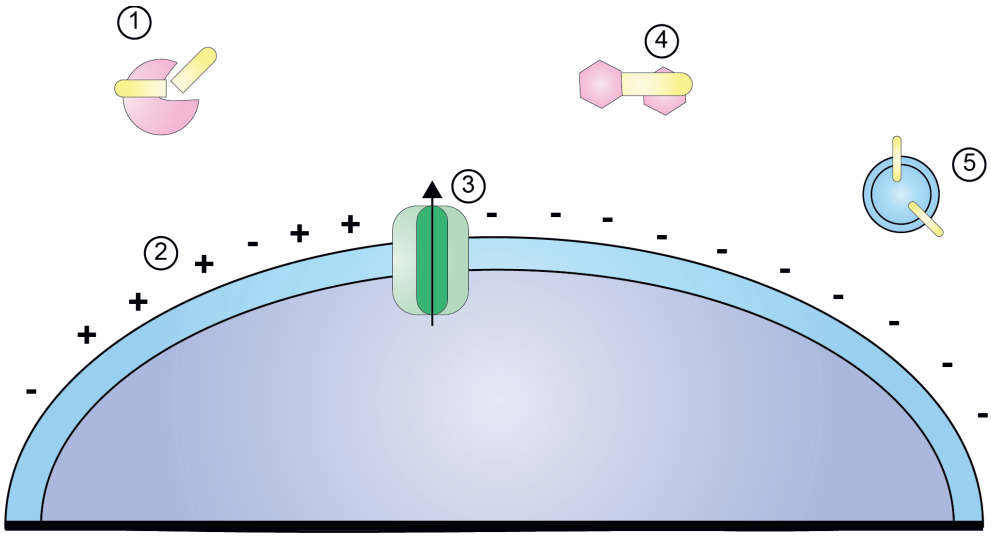


Figure 2: Currently known bacterial resistance mechanisms against HDPs. *1. Proteases may be secreted to cleave HDPs. 2. Membrane charge of the bacterium may be altered. 3. HDPs may be exported via efflux pumps. 4. HDPs may be inhibited by binding to secreted bacterial proteins. 5. OMVs may bind and thereby hinder HDPs.*

To properly distinguish between the two mechanisms, one would have to follow labelled HDPs added to a bacterium with labelled membrane. Nevertheless, OMV production will result in loss of bacterial membrane staining, so it would be ideal to ensure newly formed lipids and therefore membrane will also stay labelled. A baseline of OMV production will need to be established after addition of buffer and be compared to OMV production after addition of sub-lethal concentrations of HDPs. FITC-labelled CATH-2 was previously shown to localize to the bacterial membrane and the label did not interfere with CATH-2 function, showing the possibilities of HDP labelling [17,18]. Also labelling of LL-37 did not seem to interfere with its antimicrobial mechanisms [19].

In **chapter 3**, three different bacteria were tested and these bacteria responded differently to the three HDPs tested. PMAP-36 could induce OMV release in all three bacterial species. CATH-2 was able to induce OMV release in *E. coli* and *B. bronchiseptica*, although it could not induce OMV release in *P. aeruginosa*. The minimal bactericidal concentration (MBC) of CATH-2 for *P. aeruginosa* was four-fold higher than the MBC for *E. coli*, perhaps explaining why no OMV induction was observed at the concentrations used. CATH-2 is a membrane-active HDP and might be less effective against *P. aeruginosa* due to differences in membrane components, such as LPS, which is mostly penta-acylated for *P. aeruginosa* and hexa-acylated for *E. coli*. It was shown that the penta-acylated *P. aeruginosa* LPS was less efficient in activating human TLR4 [20] which could suggest it is also less efficiently recognized by other immune components such as HDPs. Additionally, *P. aeruginosa* could

have other defense mechanisms against HDPs, such as using its elastase which was shown to degrade LL-37 [21].

On the other hand, of the three HDPs tested, LL-37 was not able to induce OMVs in any of the bacterial species tested. Concentrations up to 10 μM LL-37 were tested which did not result in increased OMV release for *E. coli* or *P. aeruginosa*. This could indicate that LL-37 acts intracellularly and few HDPs are sensed in the bacterial membrane and therefore, no release of OMVs is necessary to dispose of affected membrane. This showed that HDPs employ different antibacterial mechanisms and bacteria act accordingly.

The different mechanisms HDPs employ for their bactericidal activities were further explored in **chapter 4**. It is still unsure whether LPS-binding aids HDPs in their function or whether LPS-binding acts as a sink and prevents HDPs from reaching their actual targets [22]. No conclusive evidence was found for either hypothesis, only for PR-39 the data suggests that stronger LPS-binding aids this HDP in its function. However, CATH-2 and PMAP-23 were shown to bind all LPS species tested in **chapter 4**, independent of the LPS structure, whether it was rough or smooth LPS or whether it was penta- or hexa-acylated. Furthermore, MBCs were not influenced by the strength of the binding. Therefore, it is most likely that LPS-binding is balanced by HDPs and does not strongly enhance or inhibit HDP bactericidal activity. Furthermore, HDPs could interact with other components in the bacterial membrane, such as anionic phospholipids, to exhibit their membrane-active mechanism.

Despite the lack of conclusive evidence found regarding the role of LPS-binding in HDP mechanisms, different mechanisms of the HDPs studied were described in detail in **chapter 4**. *E. coli* expressing mCherry in the periplasm and GFP in the cytoplasm enabled detailed studies into inner and outer membrane damage by the HDPs. This revealed that CATH-2 and PMAP-36 are strongly membranolytic HDPs, as has been suggested before [18,23]. PMAP-23 showed formation of smaller pores, which required higher concentrations of peptide and longer periods of time to form, confirming PMAP-23 acts via the carpet model described earlier [24,25]. PR-39 is only intracellularly active, which was suspected [26,27], but the assay showed clearly that PR-39 did not even form small pores in the bacterial membrane. This suggests PR-39 either enters the bacterial cell without inducing membrane damage or it enters the bacterial cell via a different route, such as via receptors.

Nevertheless, HDPs are commonly shown to interact with LPS and this needs to be understood better. Assessing MBCs and binding affinities of LPS mutants towards HDPs, where only one part of the LPS is mutated, might provide information about the components involved in LPS-binding and how stronger or weaker binding correlates with

the MBC. However, the loss of one acyl chain in the LPS structure might result in small differences of HDP function and may not lead to relevant or significant changes. Additionally, bacterial membranes consist of many more components than only LPS and investigating affinities in more complex systems could provide more relevant information, for instance by using liposomes with mixed lipid composition potentially even with one membrane protein integrated.

How HDPs can affect OMV properties

Not only can OMVs influence the functions of HDPs, HDPs can also influence the properties of OMVs. More specifically the immunological properties of OMVs. OMVs have been shown to induce good immune responses and have great potential for bacterial vaccine development due to the presence of multiple important antigens in their native environment [28,29]. Different TLRs are stimulated by OMV preparations, as described in **chapter 6**, where human TLR2, TLR4, TLR5 and TLR9 are activated by OMVs. Further purification is recommended, since TLR5 activation by flagellae, co-purified during OMV isolation, was observed, and this TLR5 activation could not be decreased by HDPs. LPS however, despite being a requirement for proper stimulation of immune cells, could also cause over-stimulation of immune cells. Therefore, HDPs could be employed to neutralize and balance OMV-evoked immune responses, which could limit possible side-effects in vaccine usage.

HDPs possess immunomodulatory activities and are known to efficiently neutralize LPS-induced TLR4 activation [4,5,30]. However, this was always studied with pure LPS, while LPS is mostly present in intact bacterial membranes. When PMAP-36 was combined with *B. bronchiseptica* OMVs in **chapter 5**, it was shown to decrease release of cytokines produced by porcine bone marrow-derived M1 macrophages (pBMDM1) [31]. When an array of HDPs was investigated in **chapter 6** however, PMAP-36 was not so efficient in decreasing macrophage activation by OMVs. This shows that the choice of test system can influence results, since in **chapter 6** a mouse macrophage cell line (RAW264.7) and a human embryonic kidney cell line (HEK293) expressing a single human TLR (hTLR) and a reporter gene were used instead of the above-mentioned porcine macrophages. Additionally, LL-37 was most efficient in decreasing hTLR activation, further demonstrating species-specific effects. Most relevant data for human use will probably be obtained by using human peripheral blood mononuclear cells (PBMCs).

Although cell lines might not be the most relevant test systems, they do provide useful information. The HEK-Blue cell line expressing a single human TLR receptor and a reporter gene provided information about specific TLRs that could be activated by *B. bronchiseptica* OMVs. Uncertainty remains about location of expression of intracellular TLRs, like TLR9,

but this could be investigated by using a fluorescently labelled TLR9 agonist and observing staining with fluorescence microscopy. The HEK-Blue hTLR system also provided information about neutralization of OMV-induced TLR activation by HDPs. This did not always correlate with neutralization observed in RAW264.7 cells. And remarkably, TLR4 neutralization, for which the HDPs are well known, was less pronounced than described before [4]. This could be due to LPS not being freely available but present in OMVs. Additionally, a host-specific effect might explain why porcine and chicken peptides were less effective in neutralizing hTLR4 activation.

This does raise the question about the mechanism of TLR neutralization by HDPs. It is commonly thought that HDPs bind TLR agonists and thereby prevent them from binding to TLRs and activating immune cells. However, LL-37 was still able to decrease LPS-induced TLR4 activation when used to only pre-treat THP-1 cells [32]. Similarly, CATH-2 and PMAP-36 were shown to bind LPS with higher affinity compared to LL-37, yet LL-37 was more efficient in neutralizing LPS-induced TLR4 activation [23]. The neutralization effect of HDPs could be compared to a TLR4-neutralizing antibody to distinguish between pure TLR4 blocking effects and possible downstream effects.

Furthermore, HDPs were previously described to enhance DNA-induced TLR9 activation in plasmacytoid dendritic cells and B cells, for which DNA-binding by HDPs is necessary [30]. In **chapter 6** however, it was observed that HDPs slightly decreased TLR9 responses in macrophages, perhaps due to decreasing OMV uptake. This emphasizes the importance of the test system and the influence it can have on the results.

HDP immunomodulatory functions in more complex systems need to be investigated further, but especially LL-37 and CATH-2 have shown potential in human and mouse systems, respectively. Other obstacles may need to be overcome though, such as high production costs. Currently, HDPs are most often chemically synthesized, which is costly, but recombinant expression of HDPs is also commonly used [33,34]. One drawback is toxicity of the HDP for the bacterial expression system but expressing HDPs as fusion proteins or using eukaryotic expression systems can circumvent this. Furthermore, HDPs often display poor stability *in vivo.* HDPs are sensitive to degradation, both by pathogen proteases as well as host proteases [35]. Additionally, HDP activities are often impaired by fluctuating pH and ion concentrations [36], especially presenting problems in the gastrointestinal tract. Several approaches exist to circumvent these issues, such as the use of D-amino acids instead of the naturally occurring L-amino acids or cyclization of HDPs [37–39].

Perspectives of HDPs and OMVs

HDPs possess antimicrobial activities and often target multiple sites in a pathogen [3]. They show potential as alternatives to antibiotics, which are necessary due to increase of antibiotic resistance [40]. Numerous HDPs have been described so far, with amphibians possessing the largest array of peptides [41]. Therefore, both broad-spectrum HDPs, targeting both Gram-negative and Gram-positive bacteria for instance, and narrow-spectrum HDPs can be found. PMAP-36 and CATH-2 were both shown to be effective against both Gram-positive and Gram-negative bacterial species [42,43]. Nisin however is very potent against Gram-positive bacteria, but less effective against Gram-negative bacteria [44]. Furthermore, cathelicidin-related (CR-) peptides were designed to specifically target *P. aeruginosa* and *S. aureus*, common pathogens in cystic fibrosis lungs [45]. Broad-spectrum HDPs could be deployed when bacteria cannot be identified, due to time constraints or other factors. Narrow-spectrum HDPs are advantageous to limit systemic effects of the peptide and leave commensal bacteria unharmed. Furthermore, it limits the possibility of resistance development in possible other pathogens present. Precise delivery of HDPs could also limit the systemic effects described above, such as topical application in gel-based formulations or inhalation of HDP powder-based formulation for pneumonic administration. Two HDP-derived peptides were shown to maintain activity in hydrogels and eradicate wound infections when applied topically [46,47]. However, modification of HDPs might be necessary, since many HDPs lose activity in high salt concentrations or physiological media [4]. Even for a rationally designed peptide, P7, minimal bactericidal concentrations were shown to increase 8- to 30-fold in higher salt concentrations [48]. However, modulation of CR-peptide 156 resulted in a peptide, CR-172, that retained activity in low pH and high salt conditions [45]. More detailed information on the antibacterial mechanisms of HDPs is necessary to wisely modify HDPs.

Structural studies could provide detailed information about the mode of binding in a membranous environment. It is known that HDPs often are unstructured in solution and only adopt a folded conformation upon interaction with membranes. Therefore, most NMR experiments were performed in a membrane-mimicking environment, using micelles. LL-37 is extensively studied and NMR aided in the elucidation of the different functional domains of LL-37 [49–51]. NMR studies in dodecyl phosphocholine micelles revealed a kink in the helical conformation of LL-37 at residue K12 [52] and it was shown in another study that only the C-terminal region is necessary for antimicrobial activity and interactions with dioctanoyl phosphatidylglycerol micelles [53]. Several other HDPs studied in an membrane-mimicking environment also revealed to have a kink, separating the peptide into two domains [54–57], indicating this kink is important for HDP function. Nevertheless, the most relevant information will be obtained if HDPs are studied in a

proper membranous environment, for instance with the use of solid-state NMR (ssNMR) [58]. ssNMR studies on the peptide antibiotic nisin revealed details about the pore formation and essential residues that interact with Lipid II in the bacterial membrane [59]. Another peptide antibiotic, teixobactin, was shown to form large clusters on the bacterial membrane, which highly depended on the membrane charge [60]. This shows that there is no consensus among antibacterial membrane-active peptides, with each peptide possibly interacting differently with the bacterial membrane.

Previously it was thought that resistance against HDPs could not be developed by microbes, but several defense mechanisms have already risen in bacteria [3,6,61–64]. OMVs were shown in this thesis to also play a role in bacterial defense against HDPs [65]. Further proteomic studies and RNA sequencing could provide information on other proteins and genes involved in bacterial defense against HDPs, which could aid to circumvent bacterial adaptation. Proteomic analysis of *Streptococcus pneumonia* demonstrated multiple adaptation mechanisms were induced after treatment with LL-37 [10]. Selecting HDPs less reliant on electrostatic interaction with the bacterial membrane for their antibacterial activity would circumvent bacterial adaptation of membrane charge. Additionally, using cyclic or D-enantiomers of HDPs would inhibit proteolytic degradation by bacteria.

For HDPs to be used as alternatives to antibiotics, combination therapies might be most promising [66]. LL-37 and derivatives showed synergism with vancomycin to combat vancomycin resistant *Staphylococcus aureus* [67]. LL-37 displayed also synergy with several other antibiotics against *Salmonella enterica* [68]. Synergism is mostly based on combinations of antimicrobials with different bacterial targets, e.g. a membrane-active HDP with an intracellularly active antibiotic [69]. To overcome OMVs as bacterial defense, anti-vesiculation drugs could be promising new antimicrobials to be developed and used in combination therapy with HDPs.

A different approach to combat bacterial infections is by the use of vaccines. OMVs are promising components to be used in bacterial vaccines, which can be either homologous or heterologous vaccines by expression of non-native antigens on or in OMVs [70,71]. Low yields can be overcome by several different methods, such as genetic modifications or heat induction [72–74], and purification can simply be achieved by size-based methods [75]. Balancing the evoked immune responses could be achieved by addition of HDPs, although minimal modulations were found in the currently used test systems. Studies in PBMCs could provide more information to develop HDPs as balancing vaccine adjuvants for OMV-based vaccines.

Synthetic innate defense regulator peptides are a class of peptides showing great potential as simulatory adjuvants in vaccine formulations. IDR peptides are derived from the bovine HDP BMAP-27 and are specifically developed for their immunomodulatory abilities [76,77]. Immunization with microparticles against *Bordetella pertussis* in mice, containing the IDR-1002 peptide, resulted in higher production of cytokines, IgG and IgA antibodies [78]. Addition of IDR-HH_2 to a hepatitis B vaccine formulation also resulted in increased cytokine production after immunization and higher antibody titers against hepatitis B [79]. The combination of IDRs with polyphosphazene and oligodeoxynucleotides resulted in a adjuvant formulation capable of boosting vaccine-induced immunizations in multiple systems tested [80], showing the potential of HDPs as vaccine adjuvants.

References

[1] A.J. Manning, M.J. Kuehn, Contribution of bacterial outer membrane vesicles to innate bacterial defense, BMC Microbiol. 11 (2011) 1–14. doi:10.1186/1471-2180-11-258.

[2] B.O. Murray, R.A. Dawson, L.M. Alsharaf, J. Anne Winter, Protective effects of Helicobacter pylori membrane vesicles against stress and antimicrobial agents, Microbiology. 166 (2020) 751–758. doi:10.1099/mic.0.000934.

[3] N. Mookherjee, M.A. Anderson, H.P. Haagsman, D.J. Davidson, Antimicrobial host defence peptides: functions and clinical potential, Nat. Rev. Drug Discov. 19 (2020) 311–332. doi:10.1038/s41573-019-0058-8.

[4] M. Coorens, M.R. Scheenstra, E.J.A. Veldhuizen, H.P. Haagsman, Interspecies cathelicidin comparison reveals divergence in antimicrobial activity, TLR modulation, chemokine induction and regulation of phagocytosis, Sci. Rep. 7 (2017) 1–11. doi:10.1038/srep40874.

[5] R. van Harten, E. van Woudenbergh, A. van Dijk, H. Haagsman, Cathelicidins: Immunomodulatory Antimicrobials, Vaccines. 6 (2018) 63. doi:10.3390/vaccines6030063.

[6] A. Peschel, H.G. Sahl, The co-evolution of host cationic antimicrobial peptides and microbial resistance, Nat. Rev. Microbiol. 4 (2006) 529–536. doi:10.1038/nrmicro1441.

[7] S. Stumpe, R. Schmid, D.L. Stephens, G. Georgiou, E.P. Bakker, Identification of OmpT as the Protease That Hydrolyzes the Antimicrobial Peptide Protamine before It Enters Growing Cells of Escherichia coli, J. Bacteriol. 180 (1998) 4002–4006. doi:10.1128/JB.180.15.4002-4006.1998.

[8] T. Guina, E.C. Yi, H. Wang, M. Hackett, S.I. Miller, A PhoP-regulated outer membrane protease of Salmonella enterica serovar typhimurium promotes resistance to alpha-helical antimicrobial peptides., J. Bacteriol. 182 (2000) 4077–86. doi:10.1128/jb.182.14.4077-4086.2000.

[9] C.M. Ernst, P. Staubitz, N.N. Mishra, S.-J. Yang, G. Hornig, H. Kalbacher, A.S. Bayer, D. Kraus, A. Peschel, The Bacterial Defensin Resistance Protein MprF Consists of Separable Domains for Lipid Lysinylation and Antimicrobial Peptide Repulsion, PLoS Pathog. 5 (2009) e1000660. doi:10.1371/journal.ppat.1000660.

[10] P.-A. Mücke, S. Maaß, T.P. Kohler, S. Hammerschmidt, D. Becher, Proteomic Adaptation of Streptococcus pneumoniae to the Human Antimicrobial Peptide LL-37, Microorganisms. 8 (2020) 413. doi:10.3390/microorganisms8030413.

[11] W.M. Shafer, X.D. Qu, A.J. Waring, R.I. Lehrer, Modulation of Neisseria

gonorrhoeae susceptibility to vertebrate antibacterial peptides due to a member of the resistance/nodulation/division efflux pump family, Proc. Natl. Acad. Sci. 95 (1998) 1829–1833. doi:10.1073/pnas.95.4.1829.

[12] X. Lauth, M. von Köckritz-Blickwede, C.W. McNamara, S. Myskowski, A.S. Zinkernagel, B. Beall, P. Ghosh, R.L. Gallo, V. Nizet, M1 Protein Allows Group A Streptococcal Survival in Phagocyte Extracellular Traps through Cathelicidin Inhibition, J. Innate Immun. 1 (2009) 202–214. doi:10.1159/000203645.

[13] T. Stobernack, M. du T. Espina, L.M. Mulder, L.M.P. Medina, D.R. Piebenga, G. Gabarrini, X. Zhao, K.M.J. Janssen, J. Hulzebos, E. Brouwer, T. Sura, D. Becher, A.J. van Winkelhoff, F. Götz, A. Otto, J. Westra, J.M. van Dijl, A secreted bacterial peptidylarginine deiminase can neutralize human innate immune defenses, MBio. 9 (2018) 1–15. doi:10.1128/mbio.01704-18.

[14] M. Li, D.J. Cha, Y. Lai, A.E. Villaruz, D.E. Sturdevant, M. Otto, The antimicrobial peptide-sensing system aps of Staphylococcus aureus, Mol. Microbiol. 66 (2007) 1136–1147. doi:10.1111/j.1365-2958.2007.05986.x.

[15] M.W. Bader, S. Sanowar, M.E. Daley, A.R. Schneider, U. Cho, W. Xu, R.E. Klevit, H. Le Moual, S.I. Miller, Recognition of antimicrobial peptides by a bacterial sensor kinase, Cell. 122 (2005) 461–472. doi:10.1016/j.cell.2005.05.030.

[16] H. Strahl, J. Errington, Bacterial Membranes: Structure, Domains, and Function, Annu. Rev. Microbiol. 71 (2017) 519–538. doi:10.1146/annurev-micro-102215-095630.

[17] V.A.F. Schneider, M. Coorens, J.L.M. Tjeerdsma-van Bokhoven, G. Posthuma, A. van Dijk, E.J.A. Veldhuizen, H.P. Haagsman, Imaging the Antistaphylococcal Activity of CATH-2: Mechanism of Attack and Regulation of Inflammatory Response, MSphere. 2 (2017) e00370-17. doi:10.1128/mSphere.00370-17.

[18] V.A.F. Schneider, M. Coorens, S.R. Ordonez, J.L.M. Tjeerdsma-Van Bokhoven, G. Posthuma, A. Van Dijk, H.P. Haagsman, E.J.A. Veldhuizen, Imaging the antimicrobial mechanism(s) of cathelicidin-2, Sci. Rep. 6 (2016) 1–11. doi:10.1038/srep32948.

[19] S.R. Ordonez, I.H. Amarullah, R.W. Wubbolts, E.J.A. Veldhuizen, H.P. Haagsman, Fungicidal Mechanisms of Cathelicidins LL-37 and CATH-2 Revealed by Live-Cell Imaging, Antimicrob. Agents Chemother. 58 (2014) 2240–2248. doi:10.1128/AAC.01670-13.

[20] S.I. Miller, R.K. Ernst, M.W. Bader, LPS, TLR4 and infectious disease diversity, Nat. Rev. Microbiol. 3 (2005) 36–46. doi:10.1038/nrmicro1068.

[21] A. Schmidtchen, I.M. Frick, E. Andersson, H. Tapper, L. Björck, Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide

LL-37, Mol. Microbiol. 46 (2002) 157–168. doi:10.1046/j.1365-2958.2002.03146.x.

[22] N. Papo, Y. Shai, A molecular mechanism for lipopolysaccharide protection of gram-negative bacteria from antimicrobial peptides, J. Biol. Chem. 280 (2005) 10378–10387. doi:10.1074/jbc.M412865200.

[23] M.R. Scheenstra, M. van den Belt, J.L.M. Tjeerdsma-van Bokhoven, V.A.F. Schneider, S.R. Ordonez, A. van Dijk, E.J.A. Veldhuizen, H.P. Haagsman, Cathelicidins PMAP-36, LL-37 and CATH-2 are similar peptides with different modes of action, Sci. Rep. 9 (2019) 4780. doi:10.1038/s41598-019-41246-6.

[24] B. Orioni, G. Bocchinfuso, J.Y. Kim, A. Palleschi, G. Grande, S. Bobone, Y. Park, J. Il Kim, K. soo Hahm, L. Stella, Membrane perturbation by the antimicrobial peptide PMAP-23: A fluorescence and molecular dynamics study, Biochim. Biophys. Acta - Biomembr. 1788 (2009) 1523–1533. doi:10.1016/j.bbamem.2009.04.013.

[25] D. Roversi, V. Luca, S. Aureli, Y. Park, M.L. Mangoni, L. Stella, How many antimicrobial peptide molecules kill a bacterium? The case of PMAP-23, ACS Chem. Biol. 9 (2014) 2003–2007. doi:10.1021/cb500426r.

[26] H.G. Boman, B. Agerberth, A. Boman, Mechanisms of action on Escherichia coli of cecropin P1 and PR-39, two antibacterial peptides from pig intestine, Infect. Immun. 61 (1993) 2978–2984. doi:10.1128/iai.61.7.2978-2984.1993.

[27] B. Agerberth, H. Gunne, J. Odeberg, P. Kogner, H.G. Boman, G.H. Gudmundsson, PR-39, a proline-rich peptide antibiotic from pig, and FALL-39, a tentative human counterpart, in: Vet. Immunol. Immunopathol., Elsevier, 1996: pp. 127–131. doi:10.1016/S0165-2427(96)05676-0.

[28] L. van der Pol, M. Stork, P. van der Ley, Outer membrane vesicles as platform vaccine technology, Biotechnol. J. 10 (2015) 1689–1706. doi:10.1002/biot.201400395.

[29] M.D. Balhuizen, E.J.A. Veldhuizen, H.P. Haagsman, Outer Membrane Vesicle Induction and Isolation for Vaccine Development, Front. Microbiol. 12 (2021) 629090. doi:10.3389/fmicb.2021.629090.

[30] M.R. Scheenstra, R.M. van Harten, E.J.A. Veldhuizen, H.P. Haagsman, M. Coorens, Cathelicidins Modulate TLR-Activation and Inflammation, Front. Immunol. 11 (2020) 1137. doi:10.3389/fimmu.2020.01137.

[31] M.D. Balhuizen, C.M. Versluis, R.M. van Harten, E.F. de Jonge, J.F. Brouwers, C.H.A. van de Lest, E.J.A. Veldhuizen, J. Tommassen, H.P. Haagsman, PMAP-36 reduces the innate immune response induced by Bordetella bronchiseptica-derived outer membrane vesicles, Curr. Res. Microb. Sci. 2 (2021) 100010. doi:10.1016/j.crmicr.2020.100010.

[32] N. Mookherjee, K.L. Brown, D.M.E. Bowdish, S. Doria, R. Falsafi, K. Hokamp, F.M. Roche, R. Mu, G.H. Doho, J. Pistolic, J.-P. Powers, J. Bryan, F.S.L. Brinkman, R.E.W. Hancock, Modulation of the TLR-Mediated Inflammatory Response by the Endogenous Human Host Defense Peptide LL-37, J. Immunol. 176 (2006) 2455–2464. doi:10.4049/jimmunol.176.4.2455.

[33] M.E. Pachón-Ibáñez, Y. Smani, J. Pachón, J. Sánchez-Céspedes, Perspectives for clinical use of engineered human host defense antimicrobial peptides, FEMS Microbiol. Rev. 41 (2017) 323–342. doi:10.1093/femsre/fux012.

[34] R. Gaglione, K. Pane, E. Dell'Olmo, V. Cafaro, E. Pizzo, G. Olivieri, E. Notomista, A. Arciello, Cost-effective production of recombinant peptides in Escherichia coli, N. Biotechnol. 51 (2019) 39–48. doi:10.1016/J.NBT.2019.02.004.

[35] P. Mallia, J. Footitt, R. Sotero, A. Jepson, M. Contoli, M.B. Trujillo-Torralbo, T. Kebadze, J. Aniscenko, G. Oleszkiewicz, K. Gray, S.D. Message, K. Ito, P.J. Barnes, I.M. Adcock, A. Papi, L.A. Stanciu, S.L. Elkin, O.M. Kon, M. Johnson, S.L. Johnston, Rhinovirus infection induces degradation of antimicrobial peptides and secondary bacterial infection in chronic obstructive pulmonary disease, Am. J. Respir. Crit. Care Med. 186 (2012) 1117–1124. doi:10.1164/rccm.201205-0806OC.

[36] A.A. Pezzulo, X.X. Tang, M.J. Hoegger, M.H. Abou Alaiwa, S. Ramachandran, T.O. Moninger, P.H. Karp, C.L. Wohlford-Lenane, H.P. Haagsman, M. Van Eijk, B. Bánfi, A.R. Horswill, D.A. Stoltz, P.B. Mc Cray, M.J. Welsh, J. Zabner, Reduced airway surface pH impairs bacterial killing in the porcine cystic fibrosis lung, Nature. 487 (2012) 109–113. doi:10.1038/nature11130.

[37] A. Zaet, P. Dartevelle, F. Daouad, C. Ehlinger, F. Quilès, G. Francius, C. Boehler, C. Bergthold, B. Frisch, G. Prévost, P. Lavalle, F. Schneider, Y. Haïkel, M.-H. Metz-Boutigue, C. Marban, D-Cateslytin, a new antimicrobial peptide with therapeutic potential, Sci. Rep. 7 (2017) 15199. doi:10.1038/s41598-017-15436-z.

[38] L. Zhang, R.L. Gallo, Antimicrobial Peptides, Springer Singapore, Singapore, 2019. doi:10.1007/978-981-13-3588-4.

[39] E.M. Molhoek, A. Van Dijk, E.J.A. Veldhuizen, H.P. Haagsman, F.J. Bikker, Improved proteolytic stability of chicken cathelicidin-2 derived peptides by d-amino acid substitutions and cyclization, Peptides. 32 (2011) 875–880. doi:10.1016/j.peptides.2011.02.017.

[40] D.R. Dodds, Antibiotic resistance: A current epilogue, Biochem. Pharmacol. 134 (2017) 139–146. doi:10.1016/J.BCP.2016.12.005.

[41] J.M. Ageitos, A. Sánchez-Pérez, P. Calo-Mata, T.G. Villa, Antimicrobial peptides (AMPs): Ancient compounds that represent novel weapons in the fight against bacteria, Biochem. Pharmacol. 133 (2017) 117–138. doi:10.1016/j.bcp.2016.09.018.

[42] Y. Lv, J. Wang, H. Gao, Z. Wang, N. Dong, Q. Ma, A. Shan, Antimicrobial Properties and Membrane-Active Mechanism of a Potential α-Helical Antimicrobial Derived from Cathelicidin PMAP-36, PLoS One. 9 (2014) e86364. doi:10.1371/journal.pone.0086364.

[43] A. van Dijk, E.M. Molhoek, E.J.A. Veldhuizen, J.L.M.T. van Bokhoven, E. Wagendorp, F. Bikker, H.P. Haagsman, Identification of chicken cathelicidin-2 core elements involved in antibacterial and immunomodulatory activities, Mol. Immunol. 46 (2009) 2465–2473. doi:10.1016/j.molimm.2009.05.019.

[44] Q. Li, M. Montalban-Lopez, O.P. Kuipers, Increasing the Antimicrobial Activity of Nisin-Based Lantibiotics against Gram-Negative Pathogens, Appl. Environ. Microbiol. 84 (2018) e00052-18. doi:10.1128/AEM.00052-18.

[45] M. van Eijk, A. van Dijk, C.K. van der Ent, H.G.M. Arets, E. Breukink, N. van Os, R. Adrichem, S. van der Water, R. Lino Gómez, M. Kristensen, M. Hessing, S. Jekhmane, M. Weingarth, R.A.W. Veldhuizen, E.J.A. Veldhuizen, H.P. Haagsman, PepBiotics, novel cathelicidin-inspired antimicrobials to fight pulmonary bacterial infections, Biochim. Biophys. Acta - Gen. Subj. 1865 (2021) 129951. doi:10.1016/J.BBAGEN.2021.129951.

[46] K.W. Woodburn, J.M. Jaynes, L.E. Clemens, Evaluation of the Antimicrobial Peptide, RP557, for the Broad-Spectrum Treatment of Wound Pathogens and Biofilm, Front. Microbiol. 10 (2019) 1688. doi:10.3389/fmicb.2019.01688.

[47] J. Håkansson, J.P. Cavanagh, W. Stensen, B. Mortensen, J.-S. Svendsen, J. Svenson, In vitro and in vivo antibacterial properties of peptide AMC-109 impregnated wound dressings and gels, J. Antibiot. (Tokyo). 74 (2021) 337–345. doi:10.1038/s41429-021-00406-5.

[48] N. Klubthawee, R. Aunpad, A Thermostable, Modified Cathelicidin-Derived Peptide With Enhanced Membrane-Active Activity Against Salmonella enterica serovar Typhimurium, Front. Microbiol. 11 (2021) 1–14. doi:10.3389/fmicb.2020.592220.

[49] G. Wang, B. Mishra, R.F. Epand, R.M. Epand, High-quality 3D structures shine light on antibacterial, anti-biofilm and antiviral activities of human cathelicidin LL-37 and its fragments, Biochim. Biophys. Acta - Biomembr. 1838 (2014) 2160–2172. doi:10.1016/j.bbamem.2014.01.016.

[50] J. Johansson, G.H. Gudmundsson, M.E. Rottenberg, K.D. Berndt, B. Agerberth, Conformation-dependent Antibacterial Activity of the Naturally Occurring Human Peptide LL-37, J. Biol. Chem. 273 (1998) 3718–3724. doi:10.1074/JBC.273.6.3718.

[51] G. Wang, Structures of human host defense cathelicidin LL-37 and its smallest

antimicrobial peptide KR-12 in lipid micelles, J. Biol. Chem. 283 (2008) 32637–32643. doi:10.1074/jbc.M805533200.

[52] F. Porcelli, R. Verardi, L. Shi, K.A. Henzler-Wildman, A. Ramamoorthy, G. Veglia, NMR structure of the cathelicidin-derived human antimicrobial peptide LL-37 in dodecylphosphocholine micelles, Biochemistry. 47 (2008) 5565–5572. doi:10.1021/bi702036s.

[53] X. Li, Y. Li, H. Han, D.W. Miller, G. Wang, Solution structures of human LL-37 fragments and NMR-based identification of a minimal membrane-targeting antimicrobial and anticancer region, J. Am. Chem. Soc. 128 (2006) 5776–5785. doi:10.1021/ja0584875.

[54] Y. Xiao, A.I. Herrera, Y.R. Bommineni, J.L. Soulages, O. Prakash, G. Zhang, The central kink region of fowlicidin-2, an α-helical host defense peptide, is critically involved in bacterial killing and endotoxin neutralization, J. Innate Immun. 1 (2009) 268–280. doi:10.1159/000174822.

[55] O. Toke, Z. Bánóczi, P. Király, R. Heinzmann, J. Bürck, A.S. Ulrich, F. Hudecz, A kinked antimicrobial peptide from Bombina maxima. I. Three-dimensional structure determined by NMR in membrane-mimicking environments, Eur. Biophys. J. 40 (2011) 447–462. doi:10.1007/s00249-010-0657-0.

[56] P.B. Timmons, D. O'Flynn, J.M. Conlon, C.M. Hewage, Structural and positional studies of the antimicrobial peptide brevinin-1BYa in membrane-mimetic environments, J. Pept. Sci. 25 (2019) e3208. doi:10.1002/psc.3208.

[57] S. Yang, C.W. Lee, H.J. Kim, H.-H. Jung, J. Il Kim, S.Y. Shin, S.-H. Shin, Structural analysis and mode of action of BMAP-27, a cathelicidin-derived antimicrobial peptide, Peptides. 118 (2019) 170106. doi:10.1016/j.peptides.2019.170106.

[58] M.A. Sani, F. Separovic, Antimicrobial Peptide Structures: From Model Membranes to Live Cells, Chem. - A Eur. J. 24 (2018) 286–291. doi:10.1002/chem.201704362.

[59] J. Medeiros-Silva, S. Jekhmane, A.L. Paioni, K. Gawarecka, M. Baldus, E. Swiezewska, E. Breukink, M. Weingarth, High-resolution NMR studies of antibiotics in cellular membranes, Nat. Commun. 9 (2018) 3963. doi:10.1038/s41467-018-06314-x.

[60] R. Shukla, J. Medeiros-Silva, A. Parmar, B.J.A. Vermeulen, S. Das, A.L. Paioni, S. Jekhmane, J. Lorent, A.M.J.J. Bonvin, M. Baldus, M. Lelli, E.J.A. Veldhuizen, E. Breukink, I. Singh, M. Weingarth, Mode of action of teixobactins in cellular membranes, Nat. Commun. 11 (2020) 2848. doi:10.1038/s41467-020-16600-2.

[61] D.I. Andersson, D. Hughes, J.Z. Kubicek-Sutherland, Mechanisms and consequences of bacterial resistance to antimicrobial peptides, Drug Resist.

Updat. 26 (2016) 43–57. doi:10.1016/j.drup.2016.04.002.

[62] A. Peschel, How do bacteria resist human antimicrobial peptides?, Trends Microbiol. 10 (2002) 179–186. doi:10.1016/S0966-842X(02)02333-8.

[63] B. Bechinger, S.U. Gorr, Antimicrobial Peptides: Mechanisms of Action and Resistance, J. Dent. Res. 96 (2017) 254–260. doi:10.1177/0022034516679973.

[64] H.-S. Joo, C.-I. Fu, M. Otto, Bacterial strategies of resistance to antimicrobial peptides, Philos. Trans. R. Soc. B Biol. Sci. 371 (2016) 20150292. doi:10.1098/rstb.2015.0292.

[65] M.D. Balhuizen, A. van Dijk, J.W.A. Jansen, C.H.A. van de Lest, E.J.A. Veldhuizen, H.P. Haagsman, Outer Membrane Vesicles Protect Gram-Negative Bacteria against Host Defense Peptides, MSphere. 6 (2021) e00523-21. doi:10.1128/mSphere.00523-21.

[66] J.M. Sierra, E. Fusté, F. Rabanal, T. Vinuesa, M. Viñas, An overview of antimicrobial peptides and the latest advances in their development, Expert Opin. Biol. Ther. 17 (2017) 663–676. doi:10.1080/14712598.2017.1315402.

[67] J.F. Shurko, R.S. Galega, C. Li, G.C. Lee, Evaluation of LL-37 antimicrobial peptide derivatives alone and in combination with vancomycin against S. aureus, J. Antibiot. (Tokyo). 71 (2018) 971–974. doi:10.1038/s41429-018-0090-7.

[68] G. Sakoulas, M. Kumaraswamy, A. Kousha, V. Nizet, Interaction of Antibiotics with Innate Host Defense Factors against Salmonella enterica Serotype Newport, MSphere. 2 (2017) e00410-17. doi:10.1128/mSphere.00410-17.

[69] M.S. Zharkova, D.S. Orlov, O.Y. Golubeva, O.B. Chakchir, I.E. Eliseev, T.M. Grinchuk, O. V. Shamova, Application of Antimicrobial Peptides of the Innate Immune System in Combination With Conventional Antibiotics—A Novel Way to Combat Antibiotic Resistance?, Front. Cell. Infect. Microbiol. 9 (2019) 1–23. doi:10.3389/fcimb.2019.00128.

[70] M.H. Daleke-Schermerhorn, T. Felix, Z. Soprova, C.M. ten Hagen-Jongman, D. Vikström, L. Majlessi, J. Beskers, F. Follmann, K. de Punder, N.N. van der Wel, T. Baumgarten, T. V. Pham, S.R. Piersma, C.R. Jiménez, P. van Ulsen, J.W. de Gier, C. Leclerc, W.S.P. Jong, J. Luirinka, Decoration of outer membrane vesicles with multiple antigens by using an autotransporter approach, Appl. Environ. Microbiol. 80 (2014) 5854–5865. doi:10.1128/AEM.01941-14.

[71] W.S. Jong, M.H. Daleke-Schermerhorn, D. Vikström, C.M. ten Hagen-Jongman, K. de Punder, N.N. van der Wel, C.E. van de Sandt, G.F. Rimmelzwaan, F. Follmann, E.M. Agger, P. Andersen, J.-W. de Gier, J. Luirink, An autotransporter display platform for the development of multivalent recombinant bacterial vector vaccines, Microb. Cell Fact. 13 (2014) 162. doi:10.1186/s12934-014-0162-8.

[72] E.F. de Jonge, M.D. Balhuizen, R. van Boxtel, J. Wu, H.P. Haagsman, J. Tommassen, Heat shock enhances outer-membrane vesicle release in Bordetella spp., Curr. Res. Microb. Sci. 2 (2021) 100009. doi:10.1016/j.crmicr.2020.100009.

[73] T. Henry, S. Pommier, L. Journet, A. Bernadac, J.P. Gorvel, R. Lloubès, Improved methods for producing outer membrane vesicles in Gram-negative bacteria, Res. Microbiol. 155 (2004) 437–446. doi:10.1016/j.resmic.2004.04.007.

[74] M.J.H. Gerritzen, D.E. Martens, R.H. Wijffels, L. van der Pol, M. Stork, Bioengineering bacterial outer membrane vesicles as vaccine platform, Biotechnol. Adv. 35 (2017) 565–574. doi:10.1016/j.biotechadv.2017.05.003.

[75] J. Klimentová, J. Stulík, Methods of isolation and purification of outer membrane vesicles from gram-negative bacteria, Microbiol. Res. 170 (2015) 1–9. doi:10.1016/j.micres.2014.09.006.

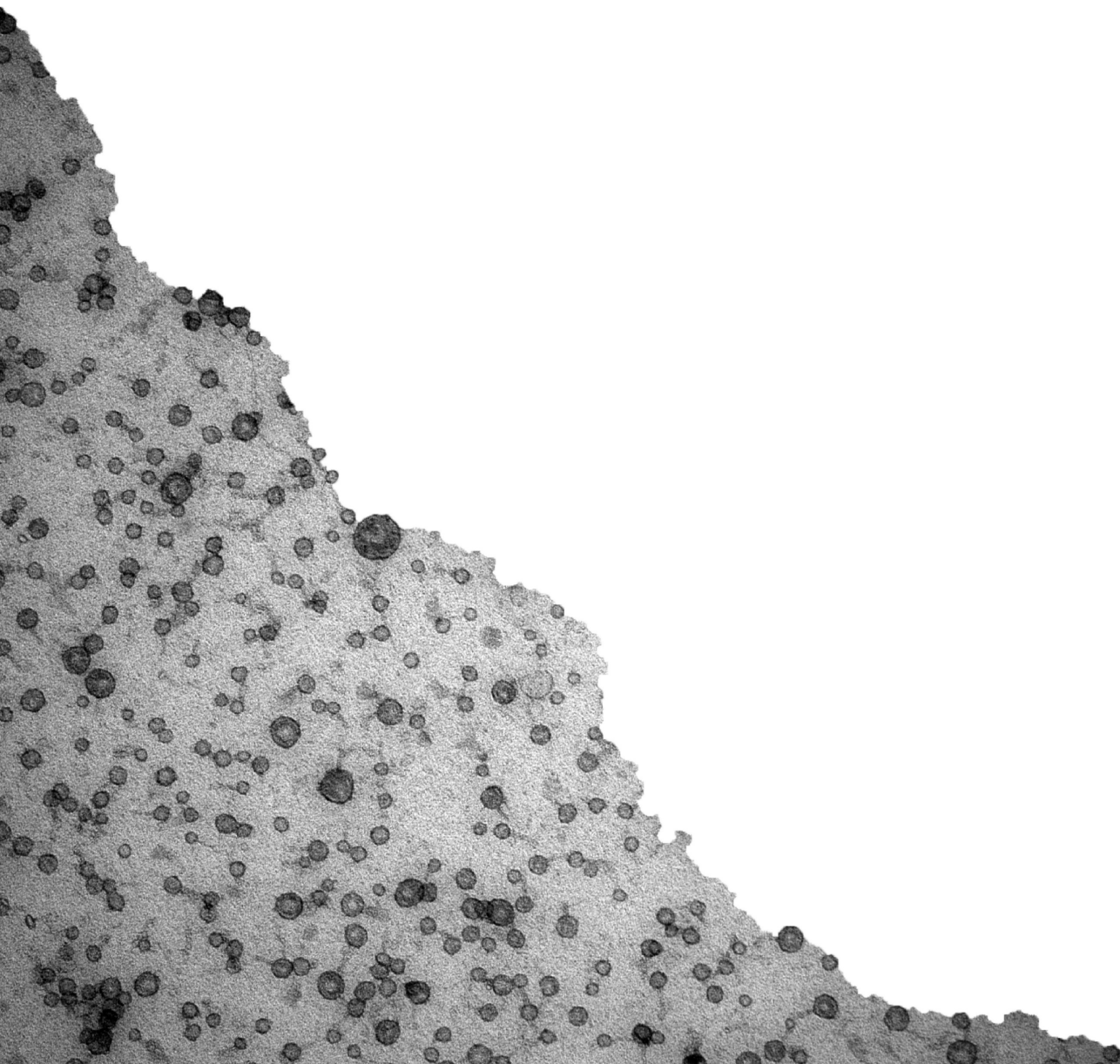
[76] K.C. Wuerth, R. Falsafi, R.E.W. Hancock, Synthetic host defense peptide IDR-1002 reduces inflammation in Pseudomonas aeruginosa lung infection, PLoS One. 12 (2017) 1–18. doi:10.1371/journal.pone.0187565.

[77] R.E.W. Hancock, E.F. Haney, E.E. Gill, The immunology of host defence peptides: Beyond antimicrobial activity, Nat. Rev. Immunol. 16 (2016) 321–334. doi:10.1038/nri.2016.29.

[78] S. Garlapati, N.F. Eng, T.G. Kiros, J. Kindrachuk, G.K. Mutwiri, R.E.W. Hancock, S.A. Halperin, A.A. Potter, L.A. Babiuk, V. Gerdts, Immunization with PCEP microparticles containing pertussis toxoid, CpG ODN and a synthetic innate defense regulator peptide induces protective immunity against pertussis, Vaccine. 29 (2011) 6540–6548. doi:10.1016/j.vaccine.2011.07.009.

[79] C.H. Yu, Z.C. Luo, M. Li, L. Lu, Z. Li, X.Z. Wu, Y.Z. Fan, H.L. Zhang, B.L. Zhou, Y. Wan, K. Men, Y.M. Tian, S. Chen, F.J. Yuan, R. Xiang, L. Yang, Synthetic innate defense regulator peptide combination using CpG ODN as a novel adjuvant induces long-lasting and balanced immune responses, Mol. Med. Rep. 13 (2016) 915–924. doi:10.3892/mmr.2015.4581.

[80] R. Garg, L. Babiuk, S. van Drunen Littel-van den Hurk, V. Gerdts, A novel combination adjuvant platform for human and animal vaccines, Vaccine. 35 (2017) 4486–4489. doi:10.1016/j.vaccine.2017.05.067.



Appendices

A

Nederlandse samenvatting

Inleiding

Het immuunsysteem bestaat uit een aangeboren en aangeleerd deel en in dit proefschrift gaat het met name over het aangeboren deel. Dit is weer verder onder te verdelen in een cellulair deel, waarbij onder andere witte bloedcellen zorgen voor bescherming tegen pathogenen, en een moleculair deel, waarbij eiwitten worden uitgescheiden die onder andere zelfstandig pathogenen kunnen uitroeien. Een groep van die eiwitten vormt de klasse van gastheerverdedigingspeptiden. Deze peptiden, kleine eiwitten, kunnen bacteriën en virussen doden en immuuncellen stimuleren. Zo ondersteunen zij op twee verschillende manieren het immuunsysteem.

De pathogenen waar dit proefschrift vooral over gaat zijn bacteriën. Bacteriën zijn onder te verdelen in Gram-positieve en Gram-negatieve bacteriën; dit proefschrift beschrijft Gram-negatieve bacteriën. Deze bacteriën hebben twee membranen met daartussen een suikerlaag om zichzelf te beschermen tegen de buitenwereld. Wanneer de gastheerverdedigingspeptiden deze bacteriën aanvallen komen ze eerst de membraan tegen. Sommige peptiden zullen proberen deze membraan lek te prikken en zo de bacterie te doden. Andere peptiden zullen alleen een klein gaatje maken, zodat ze binnenin de bacterie essentiële processen kunnen stilleggen en zo de bacterie kunnen doden.

Samenvatting van het proefschrift

Bacteriën kunnen dus aangevallen worden door deze gastheerverdedigingspeptiden, maar kunnen zichzelf ook verdedigen. In **hoofdstuk 3** wordt beschreven wat er gebeurt als we een niet-dodelijke dosis peptide aan drie verschillende Gram-negatieve bacteriën toevoegen. Specifiek wordt gekeken naar de secretie van buitenmembraanblaasjes. Van de twee membranen die deze bacterie heeft, kan namelijk de buitenste worden uitgescheiden als een soort blaasje. De bacterie doet dit van nature, om afvalproducten op te ruimen of te communiceren met andere bacteriën. Maar het blijkt dus dat als Gram-negatieve bacteriën worden blootgesteld aan gastheerverdedigingspeptiden deze blaasjes ook worden uitgescheiden. We denken dat dit een soort verdedigingsmechanisme is van de bacterie. Wanneer deze blaasjes namelijk worden onderzocht, blijkt dat de peptiden in de blaasjes aanwezig zijn. Dit suggereert dat de bacterie probeert om het stuk membraan waar peptide zit uit te scheiden in de vorm van een buitenmembraanblaasje, en zo zichzelf te beschermen.

Een belangrijk molecuul in de buitenmembraan van Gram-negatieve bacteriën is lipopolysacharide (LPS). Dit molecuul gaat een binding aan met de gastheerverdedigings-peptiden wanneer de bacterie wordt aangevallen. Lange tijd was het onduidelijk of deze binding nodig is voor peptiden om de bacterie te kunnen doden of dat het juist een verdediging was van de bacterie zodat het peptide niet de cel kan binnendringen. In **hoofdstuk 4** geven de experimenten hier meer duidelijkheid over. Er is gekeken naar de binding tussen verschillende LPS-structuren en peptiden en of die binding gecorreleerd is met de effectiviteit van peptiden. Hieruit bleek dat een sterkere binding met LPS voordelig was voor een peptide, PR-39, maar dat de activiteit van drie andere peptiden, CATH-2, PMAP-36 en PMAP-23, niet significant beïnvloed werd door binding aan LPS. Ook is er gekeken naar het mechanisme waarmee de peptiden bacteriën doden. Hieruit bleek dat CATH-2 en PMAP-36 duidelijk membraanactief zijn en grote poriën maken in de bacterie. PMAP-23 maakte alleen kleine poriën en PR-39 liet de membraan volledig intact. Dit laat dus zien dat verschillende peptiden duidelijk andere mechanismen gebruiken en dat binding aan LPS in de bacteriemembraan alleen voor het intracellulair actieve peptide PR-39 bijdraagt aan de antimicrobiële activiteit.

In **hoofdstuk 5** worden immuun-modulerende functies van de peptiden beschreven. De buitenmembraanblaasjes zijn namelijk niet alleen interessant voor de bacterie. Deze blaasjes lijken op de buitenkant van de bacterie maar kunnen zich niet vermenigvuldigen. Dit betekent dat er zeer veel antigenen aanwezig zijn op de blaasjes maar dat ze niet gevaarlijk zijn; ideaal om een vaccin van te maken dus. Het enige nadeel is de aanwezigheid van het bovengenoemde LPS-molecuul wat kan functioneren als een endotoxine en een te extreme reactie van het immuunsysteem kan opwekken. Daarom moet dit LPS gedeeltelijk geneutraliseerd worden en hiervoor zijn de gastheerpeptiden een goede optie. Het is namelijk al eerder beschreven dat deze peptiden LPS-activatie van macrofagen kunnen neutraliseren. In **hoofdstuk 5** wordt dieper ingegaan op één varkensbacterie en één varkenspeptide en beschreven hoe dit peptide de productie van ontstekingsmoleculen kan remmen.

De resultaten waren veelbelovend en in **hoofdstuk 6** worden experimenten beschreven die de immuun-modulerende activiteiten van acht verschillende peptiden onderzoeken. Hieruit bleek dat vier peptiden de activatie van macrofagen door buitenmembraanblaasjes konden onderdrukken maar dat het ontstekingsremmend effect minder sterk was dan wanneer deze macrofagen werden gestimuleerd met zuiver LPS. Buitenmembraan-blaasjes bevatten namelijk niet alleen LPS, maar ook eiwitten, DNA en suikers die macrofagen ook kunnen activeren. Met specifieke cellijnen is onderzocht op welke manier activatie zou kunnen verlopen om zo meer inzicht te krijgen in de algemene immuunrespons en de bruikbaarheid van de buitenmembraanblaasjes als bacterieel vaccin. Hieruit bleek dat de blaasjes de receptoren voor lipoproteïne, zweepstaartjes en

DNA kunnen activeren. Dit zou voordelig kunnen zijn, omdat dit een gevarieerde immuunrespons opwekt en zo het risico minimaliseert dat de bacterie eiwitten kan veranderen om de immuunrespons te ontwijken. Wat wel bleek is dat de gastheerverdedigingspeptiden in meer of mindere mate de activatie door lipoproteïne en DNA konden neutraliseren, maar de activatie door zweepstaartjes niet. Verdere zuivering om deze zweepstaartjes te verwijderen kan zorgen voor een beter gebalanceerde immuun-activatie.

Conclusie

In dit proefschrift zijn de interacties tussen gastheerverdedigingspeptiden en bacteriële buitenmembraanblaasjes beschreven. Allereerst bleek het dat deze buitenmembraan-blaasjes gebruikt worden door bacteriën om zichzelf te beschermen tegen gastheerverdedigingspeptiden. Daarentegen werd er geen duidelijke correlatie gevonden tussen binding van gastheerverdedigingspeptiden met LPS, wat aanwezig is in de buitenmembraanblaasjes, en de effectiviteit van deze peptiden. De buitenmembraan-blaasjes bleken wel geschikt om een immuunrespons op te wekken en zo de gastheer te beschermen tegen de bacterie. Deze immuunrespons werd veroorzaakt door verschillende componenten van de buitenmembraanblaasjes, welke in verschillende mate geneutraliseerd konden worden door de gastheerverdedigingspeptiden. Dit laat zien dat de gastheerverdedigingspeptiden potentieel hebben om de immuunrespons van bacteriële vaccins te balanceren.

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Dankwoord

Hier ligt hij dan, mijn proefschrift. Het resultaat van vier jaar hard werken. En stiekem ben ik best wel trots op het resultaat. Trots op dat ik dit heb kunnen en mogen doen, want we weten allemaal dat een PhD doen geen kleinigheidje is. En ondanks dat een PhD een individueel project is, had ik dit nooit alleen kunnen doen. Dus ik wil graag een paar mensen bedanken.

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Curriculum Vitae

Melanie Balhuizen was born on September 29th, 1994 in Amersfoort, The Netherlands. She finished her high school education in 2012 at Het Baarnsch Lyceum, Baarn and started her Chemistry bachelor at Utrecht University with support of a grant from the TopSectorChemie program. She finished her bachelor and honors program in 2015 and continued her studies at Utrecht University with the master Molecular and Cellular Life Sciences. During her master's program she performed a nine-month internship in the lab of Madelon Maurice, under the supervision of Eline van Kappel, at the department of Cell Biology at the University Medical Center Utrecht. Additionally, she performed a six-month industry internship at Bioceros (now known as Polpharma) under the supervision of Stan de Kleijn. Melanie obtained her master's degree in August 2017 and started her PhD in September in the group of Henk Haagsman at the department of Molecular Host Defence at the Veterinary Faculty of Utrecht University. This PhD project was part of a larger consortium with several industry partners. The research of her PhD has been described in this thesis and with this work she received a second prize for best oral communication at the IMAP-2019. Additionally, several chapters have been published in peer-reviewed scientific journals.

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List of publications

Al-Adwani, S., Wallin, C., **Balhuizen, M.D.**, Veldhuizen, E.J.A., Coorens, M., Landreh, M., Végvári, Á., Smith, M.E., Qvarfordt, I., Lindén, A., et al. (2020). Studies on citrullinated LL-37: detection in human airways, antibacterial effects and biophysical properties. Sci. Rep. 10, 1–14.

Peng, L., Du, W., **Balhuizen, M.D.**, Haagsman, H.P., de Haan, C.A.M., and Veldhuizen, E.J.A. (2020). Antiviral Activity of Chicken Cathelicidin B1 Against Influenza A Virus. Front. Microbiol. 11, 426.

de Jonge, E.F., **Balhuizen, M.D.**, van Boxtel, R., Wu, J., Haagsman, H.P., and Tommassen, J. (2021). Heat shock enhances outer-membrane vesicle release in Bordetella spp. Curr. Res. Microb. Sci. 2, 100009.

Balhuizen, M.D., Versluis, C.M., van Harten, R.M., de Jonge, E.F., Brouwers, J.F., van de Lest, C.H.A., Veldhuizen, E.J.A., Tommassen, J., and Haagsman, H.P. (2021). PMAP-36 reduces the innate immune response induced by *Bordetella bronchiseptica*-derived outer membrane vesicles. Curr. Res. Microb. Sci. 2, 100010.

Balhuizen, M.D., Veldhuizen, E.J.A., and Haagsman, H.P. (2021). Outer Membrane Vesicle Induction and Isolation for Vaccine Development. Front. Microbiol. 12, 629090.

van Harten, R.M., Tjeerdsma-van Bokhoven, J.L.M., de Greeff, A., **Balhuizen, M.D.**, van Dijk, A., Veldhuizen, E.J.A., Haagsman, H.P., and Scheenstra, M.R. (2021). D-enantiomers of CATH-2 enhance the response of macrophages against *Streptococcus suis* serotype 2. J. Adv. Res. https://doi.org/10.1016/j.jare.2021.05.009

Balhuizen, M.D., van Dijk, A., Jansen, J.W.A., van de Lest, C.H.A., Veldhuizen, E.J.A., and Haagsman, H.P. (2021). Outer Membrane Vesicles Protect Gram-negative Bacteria against Host Defense Peptides. mSphere. 6, 4: e00523-21