

Modulation of outer membrane vesicle-based immune responses by cathelicidins



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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 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 *B. bronchiseptica* 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.

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1. 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 *Neis-*

seria 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

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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 1 β (IL-1 β) 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 potentially 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.

2. Materials and methods

2.1. 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.

2.2. 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 [31] (pH 7.4) containing 0.02 % (w/v) KCl, 0.05 % (w/v) KH₂PO₄, 0.01 % (w/v) MgCl₂•6 H₂O (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 25,922 was grown in lysogeny broth (LB) containing 1 % yeast extract, 0.5 % tryptone (both Becton, Dickinson and Company) and 1 % NaCl (Merck).

2.3. OMV isolation

OMVs were isolated as described before [32]. In short, bacteria were grown overnight to an OD₅₉₀ 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 4700g. The supernatant was passed through a 0.45 μ m 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.

2.4. 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 μ L of sample, supplemented with 2 % SDS (Invitrogen, Carlsbad, California, USA), were incubated with 200 μ L 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.

2.5. 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 % CO₂. For stimulation, 5x10⁴ 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 μ g/mL OMVs, 10 ng/mL *B. bronchiseptica* LPS (unless stated otherwise), 1x10⁶ 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),

Table 1
Sequence, organism of origin, number of amino acids (No. aa) and charge of studied peptides [33–37].

Peptide	Sequence	Origin	No. aa	Charge
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES	Human	37	6+
CATH-2	RFGRLRKIRRFKPKVTITIQGSARF-NH ₂	Chicken	26	8+
PMAP-36	Ac-GRFRRLRKKTRKRLKKIGKVLKWIPIVIGSIPLGCC	Porcine	36	13+
PMAP-23	RIIDLWVRVRPQPKFVTWVR	Porcine	23	6+
PR-39	RRRPRPPYLPRPPPPFPRLPPRIPPGFPPRFPPRFPP	Porcine	39	10+
K9CATH	RLKELITGGQKIGEKIRIRIGQRIKDFKLNQLPREEKS	Canine	38	5+
IDR-1018	VRLLVAVRIWRR-NH ₂	Synthetic	12	5+
IDR-2005	VRLLVVRVRIWRR-NH ₂	Synthetic	12	6+

Nystatin (Merck) and ethylisopropyl amiloride (EIPA, Sigma-Aldrich) were used as uptake inhibitors.

2.6. 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 μ L 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 μ L block buffer at room temperature (RT) (PBS with 1 % BSA (Sigma Aldrich)). Then 100 μ L of either standard or sample was added and the plate was incubated for 2 h at RT. The plate was incubated with 100 μ L detection antibody (diluted as described in the kits' protocol) in reagent buffer (PBS / 1 % BSA (Sigma Aldrich)) for 2 h at RT. Next, 100 μ L mAb Streptavidin-HRP (R&D Systems) was added to the plate and incubated for 20 min at RT. Subsequently, 100 μ L 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 μ L 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).

2.7. Griess assay

The Griess assay was performed to measure NO production. Either 50 μ L sodium nitrite (Sigma Aldrich) diluted in DMEM as standard or 50 μ L sample was added in a 96-wells plate. Samples were incubated for 5 min with 50 μ L 1 % sulfanilamide and 5.1 % *ortho*-phosphoric acid (both from Merck) at RT in the dark. Afterwards 50 μ L 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).

2.8. 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 μ L 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.

2.9. 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 μ L of OMV solution. PMAP-36 was prepared in an identical buffer to a concentration of 200 μ M. PMAP-36 was titrated into the chamber, with 1.96 μ L per titration with a 300 s interval. Experiments were performed at 37 °C and analyzed using the NanoAnalyze software (TA instruments, Asse, Belgium).

2.10. 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 to 45 °C were measured. Samples were equilibrated for 2 min and each temperature measurement was 5 min.

2.11. QuantiBlue assay

HEK-Blue cell line NULL1, TLR2, TLR3, TLR4, TLR5 and TLR9 (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 μ g/ml Zeocin, 100 μ g/ml Normocin and selective antibiotics specific for each cell line: TLR2 and TLR4 (30 μ g/mL Blastidicin, 200 μ g/mL Hygromycin), TLR3 and TLR5 (30 μ g/mL Blastidicin) and TLR9 (10 μ g/mL Blastidicin). 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 5×10^4 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 μ L supernatant was mixed with 180 μ L of Quanti-Blue (InvivoGen) in a clear flat-bottom immune non-sterile 96-well plate (Thermo Fisher). After 1 h the absorbance was determined at 230 nm with the FLUOstar Omega microplate reader.

2.12. Fluorescence activated cell sorting (FACS) analysis

RAW264.7 cells were seeded as described above and stimulated for 2 h with 1 mg/mL FITC-dextran in the presence or absence of uptake inhibitors. Afterwards, cells were incubated with 50 μ L 0.5 mM ethylenediaminetetraacetic acid (EDTA) in PBS for 5 min at 37 °C. Cells were resuspended with an additional 150 μ L PBS and transferred to a V-bottom 96-wells plate (Corning Incorporated). Plates were centrifuged at 1800 rpm for 2 min at 4 °C and supernatant was discarded. Cells were resuspended in 100 μ L PBS containing 0.5 % BSA and measured using the FACS CANTO (Becton, Dickinson and Company). Data analysis was performed using Flowjo version 10.

3. Results

3.1. 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 μ g/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, TNF α 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 TNF α release, as well as in the anti-inflammatory IL-10 release.

3.2. Immune modulation of HDPs is minimal

HDPs from different species were investigated for their modulation of NO production and TNF α 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 TNF α and IL-10 release, only for hOMVs. PMAP-36 showed a decrease of NO production at high (4 μ M) concentrations, but an increase in TNF α at low (0.5 μ M) 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 TNF α , 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.

To control for any responses caused by HDPs alone, metabolic activity and NO production of RAW cells was assessed after stimulation with 4 μ M 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 μ M or 4 μ M). 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 TNF α release was consequently decreased and, although not significant, IL-10 release showed a decreasing trend by all four cathelicidins as well.

3.3. 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 μ M PMAP-36 in the case of hOMVs. 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.

3.4. 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 (M β CD) 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 in this experimental setup. No cytotoxic effects were observed for the other inhibitors, only some metabolic activation of macrophages was observed (Fig. S5a). Additionally, macrophages were stimulated with FITC-dextran and the highest concentration of inhibitor, to control for activity of the inhibitors. This showed that all inhibitors were able to decrease uptake of FITC-dextran; however, this decrease was only significant for CPZ and M β CD (Fig. S5b).

3.5. 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

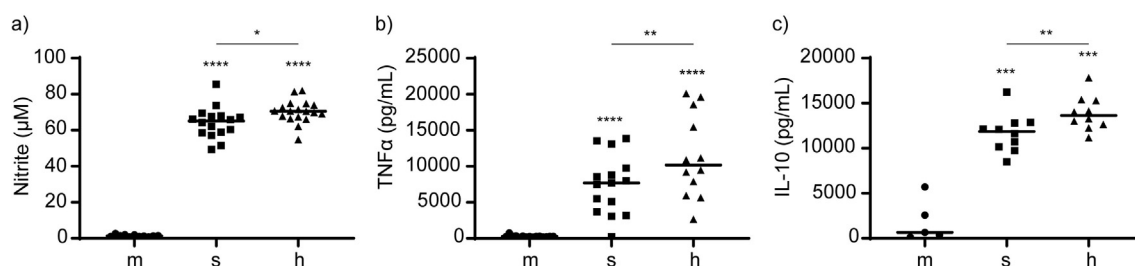


Fig. 1. Activation of RAW264.7 cells upon stimulation with spontaneous and heat-induced OMVs. RAW cells were stimulated with OMVs. (a) NO production was measured by Griess assay, (b) TNF α and (c) IL-10 using ELISA. m = medium, s = sOMVs, h = hOMVs. Results were analyzed using a paired mixed-model analysis with Geisser-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.

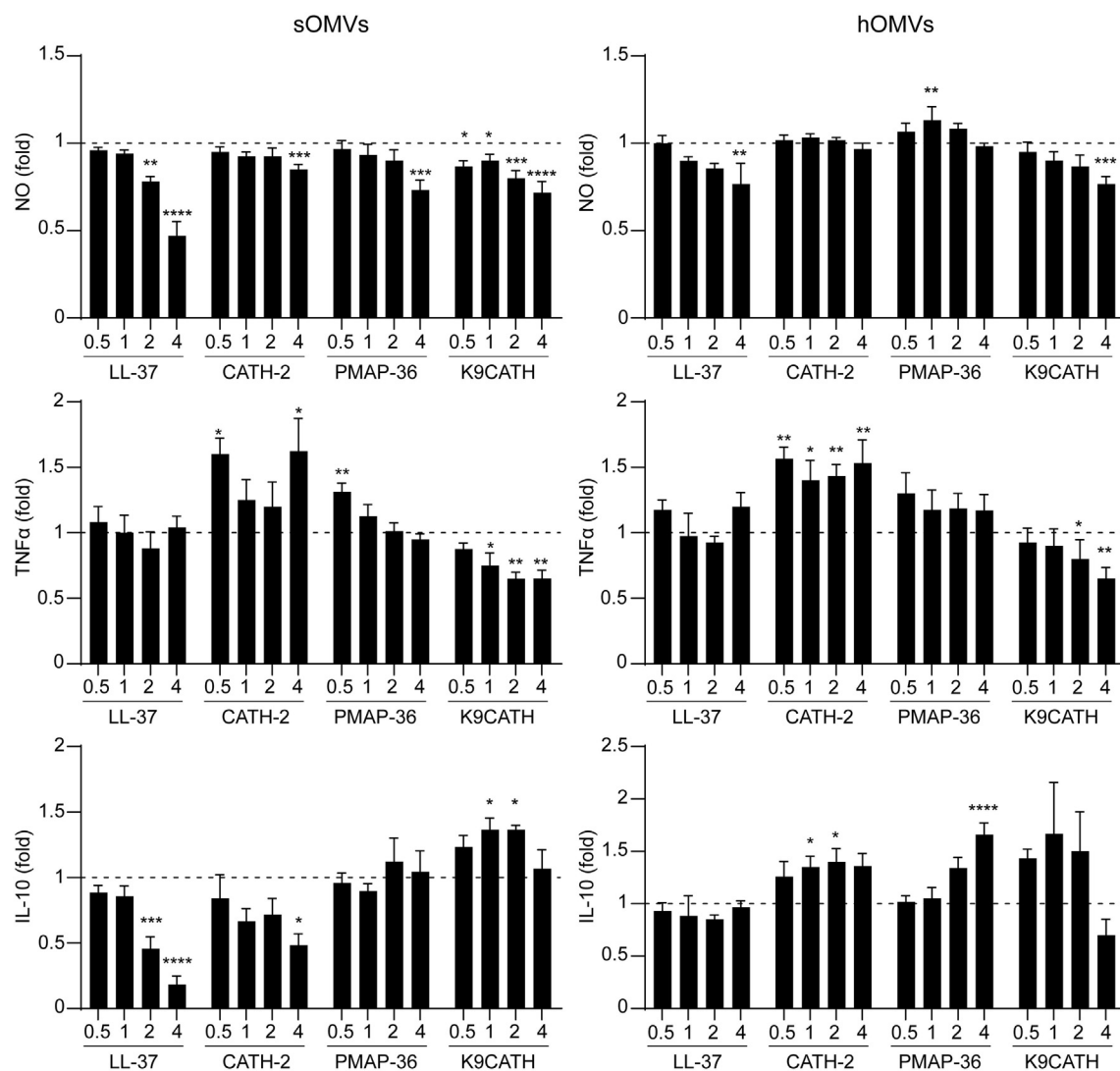


Fig. 2. Modulation of RAW264.7 cell activation by cathelicidins upon stimulation with spontaneous and heat-induced OMVs. RAW cells were stimulated with 0.025 μg/mL OMVs and a dose of LL-37, CATH-2, PMAP-36 or K9CATH (0.5, 1, 2 or 4 μM). Results are normalized to signals obtained by only OMV stimulation (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 Fig. 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	-	-	

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 were involved in an endothermic reaction with PMAP-36, but the highest interac-

tion peak located 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,

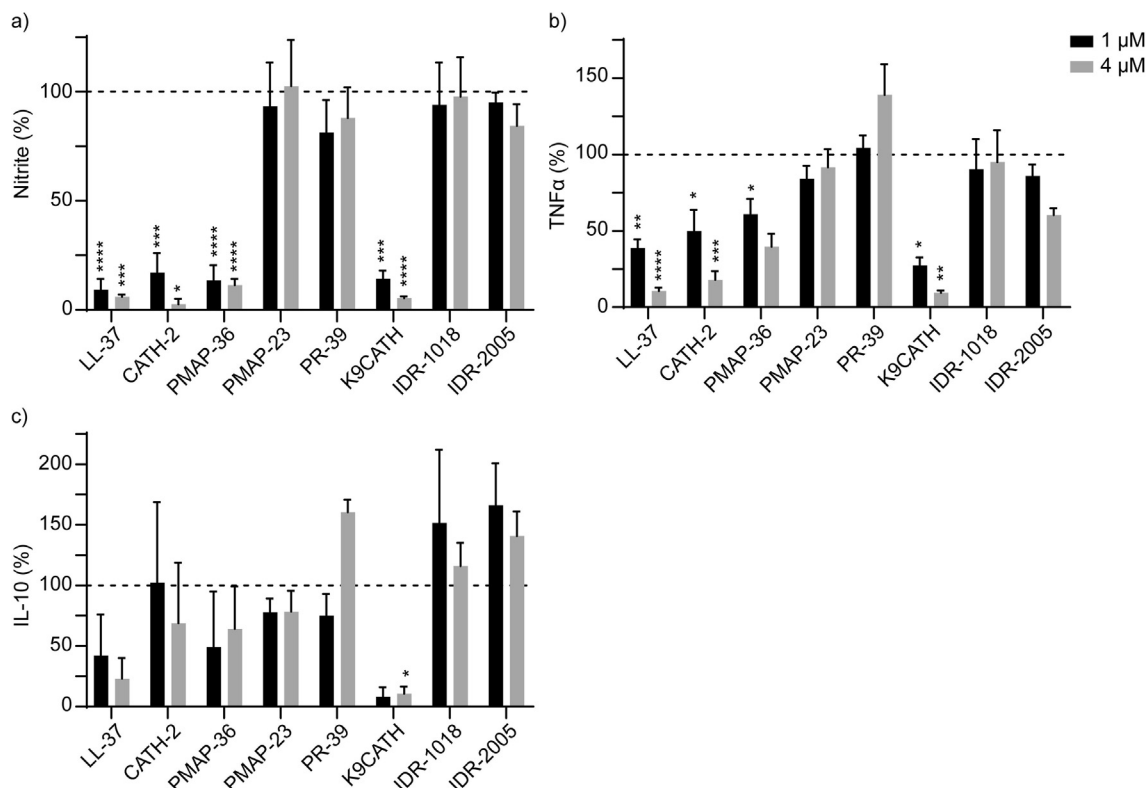


Fig. 3. LPS neutralizing capability of HDPs. RAW264.7 cells were stimulated with *B. bronchiseptica* LPS and a dose of HDP, stimulation with only LPS was set to 100 %. (a) NO production was measured by Griess assay, (b) TNFα and (c) IL-10 using ELISA. Results were analyzed with a two-way ANOVA and Dunnett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

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.

4. 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. LPS detoxification by genetic modifications or removal of LPS by detergents are known methods to decrease LPS-mediated side effects, but these methods can alter antigen composition, and therefore immune responses, due to loss of lipoproteins [29]. HDPs are known to efficiently neutralize LPS-evoked TLR4 activation [19] and do not alter antigenic properties of the OMVs. However, 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 [42]. 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 [32], 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 and mice [43].

HDPs are thought to modulate TLR responses by interactions with either the TLR itself, the TLR agonist or both [41]. Interactions with TLRs can decrease agonist binding, while interactions with TLR agonists can increase uptake of intracellular TLR agonists especially. However, currently no structure-activity relationship has been found, nor does the immunomodulatory activity corresponds to antibacterial activity of HDPs. Therefore, 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

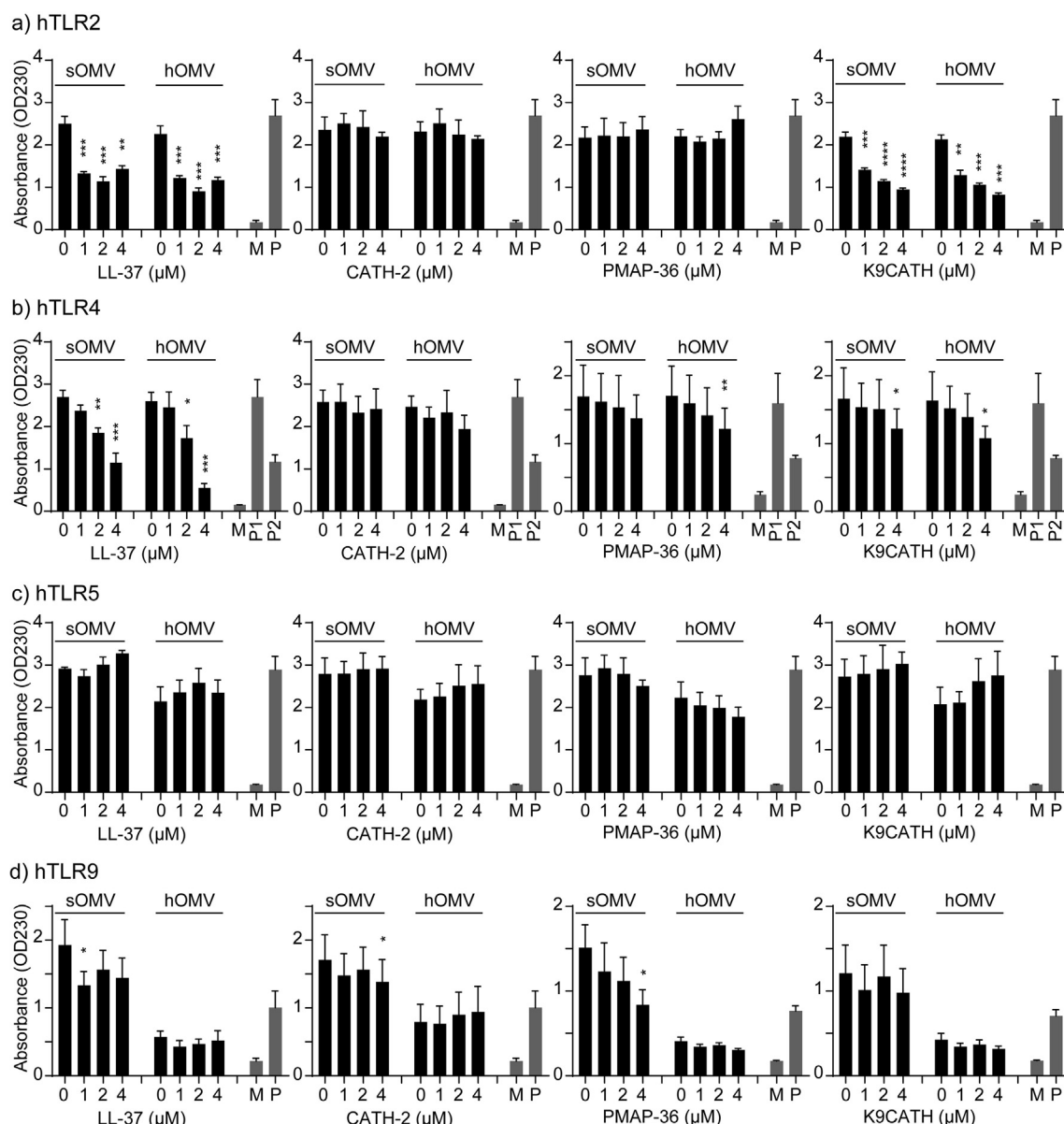


Fig. 4. Screen of TLR activation in HEK-cells and modulation by cathelicidins. HEK-Blue (a) hTLR2, (b) hTLR4, (c) hTLR5 or (d) hTLR9 were stimulated with 0.025 μg/mL sOMVs or hOMVs and a dose of peptide. (M) is DMEM-medium and (P) is a positive control, which differed per cell-line: TLR2: 100 ng/ml Pam3CSK4, 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.

expression of TNF α 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 TNF α 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 Fig. 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 fourfold (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 [41]. 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 [41,44,45]. 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.

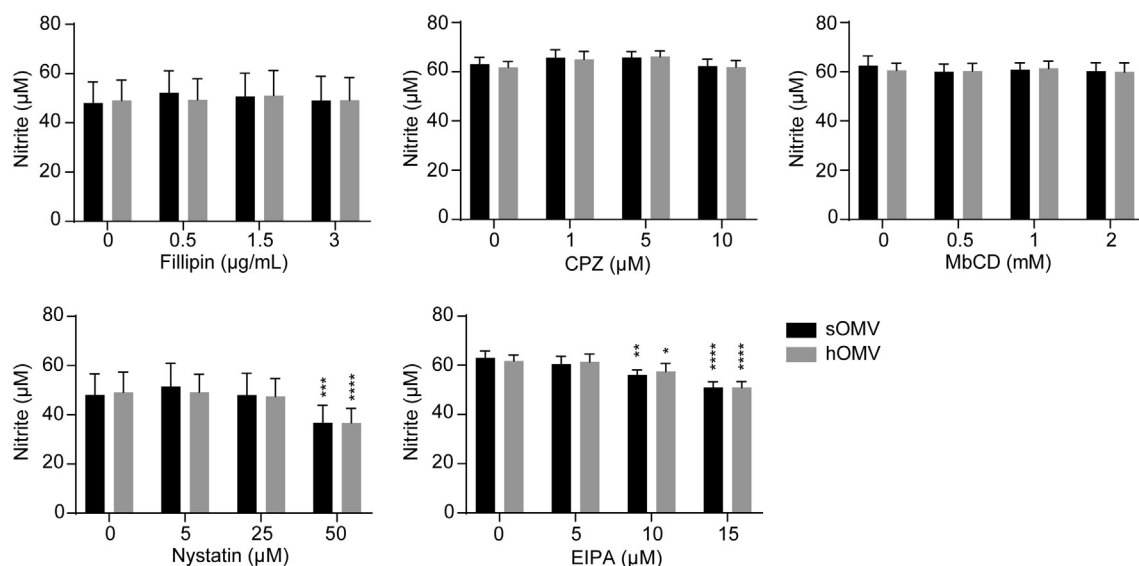


Fig. 5. Effect of uptake inhibitors on macrophage activation by OMVs. RAW264.7 cells were stimulated with 0.025 $\mu\text{g/mL}$ sOMVs or hOMVs, together with a dose of Fillipin, Chlorpromazine (CPZ), methyl- β -cyclodextrin (MbCD), Nystatin or Ethylisopropyl amiloride (EIPA). Results were analyzed using 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.

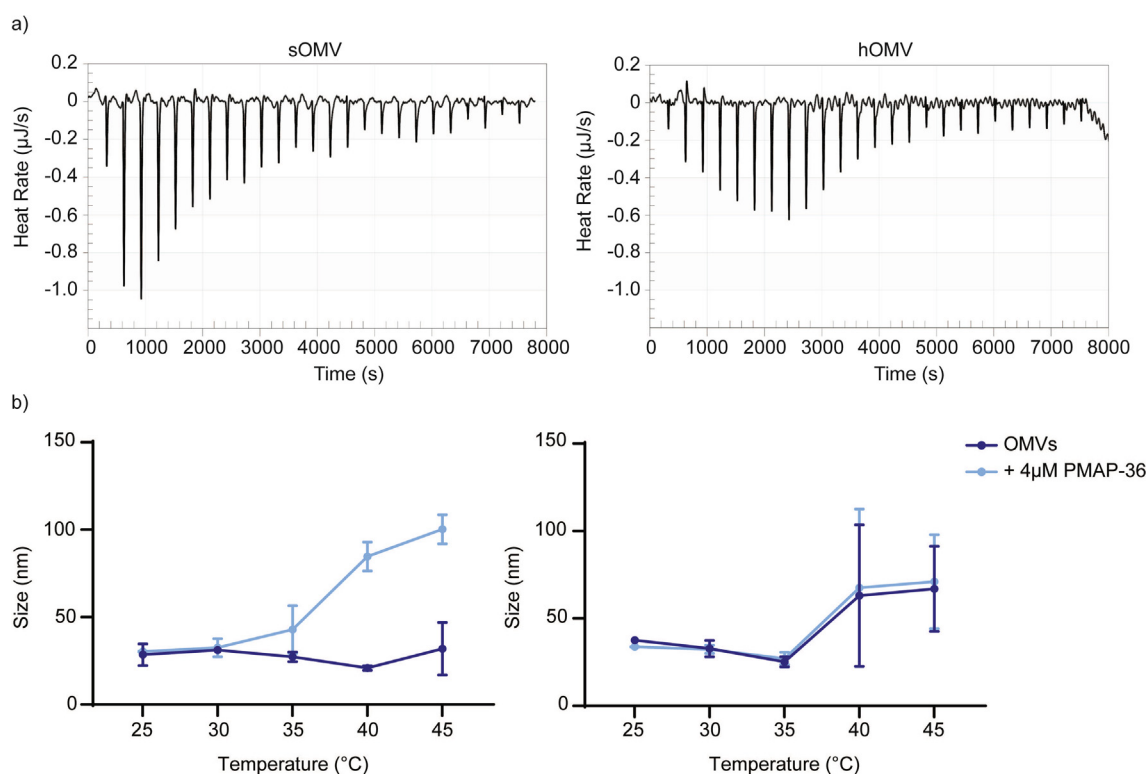


Fig. 6. Interaction between PMAP-36 and spontaneous or heat-induced OMVs. (a) ITC was used to investigate binding between 200 μM PMAP-36 and spontaneous or heat-induced OMVs (30 $\mu\text{g/mL}$ protein). Spectra obtained indicate minor differences in affinity and binding mode. (b) DLS was used to observe 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.

The human LL-37 seems more efficient in modulating immune responses in the human TLR HEK-Blue system, while 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 that HDPs not only block immune responses by interacting with OMVs, but also with the immune cell, as described for LL-37. In those studies, $\text{TNF}\alpha$ 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 in this experimental setup. However, uptake of OMVs can differ between cell types. Not all uptake inhibitors are equally efficient in every cell type, so RAW264.7 macrophages might not respond well to the inhibitors used [48], especially since uptake of FITC-dextran was only slightly decreased by the uptake inhibitors (Fig. S5b). Alternatively, OMVs can be labelled and followed, a methodology which showed that *E. coli* OMVs are endocytosed by RAW264.7 cells [42]. 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]. 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. 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 [32]. 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 [32]. 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 [32]. 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). Additionally, only OMVs from *B. bronchiseptica* were tested in this study. Since structures of TLR agonists may differ between bacterial species, this may alter immunomodulation of HDPs as well. Many factors can influence OMV interactions with immune cells and HDPs and this needs to be taken into account when contemplating vaccine potential of HDPs in combination with 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.

5. 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 lines. 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.

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CRediT authorship contribution statement

Melanie D. Balhuizen: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Chantal M. Versluis:** Investigation. **Monica O. van Grondelle:** Investigation. **Edwin J.A. Veldhuizen:** Conceptualization, Supervision, Writing – review & editing. **Henk P. Haagsman:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.vaccine.2022.03.015..>

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