Extracellular vesicles of the probiotic E. coli O83 activate innate immunity and prevent allergy in mice

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Abstract

Background: *E. coli* O83 (Colinfant Newborn) is a Gram-negative (G-) probiotic bacterium used in the clinic. When administered orally, it reduces allergic sensitisation but not allergic asthma. Intranasal administration may be more effective as it reaches the lungs directly. G- bacteria release outer membrane vesicles (OMVs) to communicate with the environment. Here we investigate whether intranasally administered *E. coli* O83 OMVs (EcO83-OMVs) can reduce allergy in mice. **Methods:** EcO83-OMVs were isolated by ultracentrifugation and characterised with respect to their number, morphology (shape and size), composition (proteins and lipopolysaccharide; LPS), recognition by innate receptors (using transfected HEK293 cells) and immunomodulatory potential (in naïve splenocytes and bone marrow-derived dendritic cells; BMDCs). Their allergy-preventive effect was investigated in a mouse model of allergic airway inflammation. **Results:** EcO83-OMVs are spherical nanoparticles with a size of about 110 nm. They contain LPS and protein cargo. We identified a total of 1120 proteins, 136 of which were enriched in OMVs compared to parent bacteria. Proteins from the flagellum dominated. OMVs activated the pattern recognition receptors TLR2/4/5 as well as NOD1 and NOD2. EcO83-OMVs were internalised by epithelial cells and induced the production of pro- and anti-inflammatory cytokines in splenocytes and BMDCs. Intranasal administration of EcO83-OMVs inhibited airway hyperresponsiveness, decreased airway eosinophilia, Th2 cytokine production and mucus secretion. **Conclusion:** We demonstrate for the first time that intranasally administered OMVs from probiotic G- bacteria have an anti-allergic effect. Our study highlights the advantages of OMVs as a safe platform for prophylactic treatment of allergy.

INTRODUCTION

Allergies are one of the most common chronic inflammatory diseases, the prevalence of which has increased in industrialised countries in recent decades, resulting in a significant socioeconomic health burden (1). To date, the only disease-modifying treatment available is allergen-specific immunotherapy (AIT). Although highly effective, AIT has several disadvantages such as regular administration over a long period of time and the occurrence of adverse reactions (2).

The exact mechanisms responsible for the development of allergies are largely unknown, but both genetic and environmental factors play a role (3). The increasing prevalence of allergic diseases has been linked to changes in the microbial load in the environment due to profound changes in lifestyle and hygiene (4,5). Therefore, restoring a balanced microbiota at mucosal surfaces, such as the lung and gut, with probiotics is a promising strategy to prevent or treat allergies. Several clinical trials have tested the effect of oral administration of live probiotic bacterial strains in the treatment of allergic diseases (6). Although some beneficial effects of probiotic bacteria in reducing allergic sensitisation have been reported (7,8), the efficacy in preventing or treating allergic asthma appears inconsistent (9–11).

Studies in mice have shown that intranasal administration of*Lacticaseibacillus paracasei* NCC246 during aerosol challenge reduced the recruitment of inflammatory cells and the concentrations of IL-5 in the lungs of ovalbumin (OVA)-sensitised and challenged mice (12). Along these lines, intranasal administration of*Lacticaseibacillus rhamnosus* GG, but not the *L. rhamnosus* strain GR-1, prevented allergic asthma in a mouse model (13). We have shown that *Bifidobacterium longum* NCC 3001, but not*L. paracasei* , administered intranasally prior to sensitisation and challenge, suppressed allergen-specific immune responses in mice (14).

The Gram-negative (G-) probiotic bacterial strain *E. coli* A0 34/86 (*E. coli* O83; serotype O83:K24:H31) is a commercially available live oral vaccine (Colinfant Newborn). Although oral administration of *E. coli* O83 to high-risk infants reduced allergic sensitisation (skin disease), this bacterium did not provide protection against the development of respiratory allergies (15,16). We recently demonstrated that the route of delivery of probiotics might be important since *E. coli* O83 administered intranasally reduced allergic lung inflammation in mice. Mechanistically, the TLR4 signalling pathway was required for this beneficial effect (17).

Probiotic bacteria are generally well tolerated and considered safe. However, probiotic therapy in clinics may be associated with some risks in vulnerable target groups such as pregnant women, young children, and immunocompromised individuals (18). Although rare, intentional supplementation of probiotics was linked with side effects such as systemic infections, toxic metabolic activities, or gene transfer (19). Therefore, the discovery of non-living bacterial products that modulate immune function and can be conveniently used in daily life will open up new possibilities for the prevention or treatment of allergies.

G- bacteria produce extracellular vesicles (EVs) called outer membrane vesicles (OMVs). They are produced during normal bacterial growth by detachment of the outer membrane and consist largely of outer membrane components such as lipopolysaccharide (LPS), lipids and proteins, but may also contain material from the periplasmic space or from within the bacterial cell, such as nucleic acids (20). Several studies have investigated OMVs of pathogenic (21–24) or non-pathogenic (25–27)*E. coli* strains, but OMVs of *E. coli* O83 have not yet been investigated. OMVs contain microbe-associated molecular patterns (MAMPs) that give them immunostimulatory properties. They can interact with different pattern recognition receptors (PRRs) in and on host cells to stimulate innate and adaptive immune responses (28). As natural and non-replicative particles, OMVs are ideal candidates for use as safe vaccine platforms due to their nanosize (20-200 nm), low toxicity, cargo protection, ability to cross biological barriers, and the simplicity of their manufacturing and purification processes (29–31). Although some OMVs-based vaccines against infectious diseases are already on the market (32), the potential of OMVs to treat non-communicable diseases, such as allergies, has not yet been satisfactorily investigated.

In this study, we hypothesised that non-proliferating *E. coli*O83-derived vesicles have immunomodulatory potential and can recapitulate the allergy-preventive effect of their parent strain in a mouse model of experimental asthma, providing a mucosal vaccine candidate with fewer risks associated with their use in humans.

MATERIALS AND METHODS

Bacteria

Escherichia coli strain A0 34/86 (*E. coli* O83; serotype O83:K24:H31) was cultured as described before (17).

For experiments in cell culture, the bacteria were inactivated. Details about bacteria source, culture, growing curve, fixation, and preparation of lysate are given in the Appendix.

Isolation and characterization of *E. coli* O83-derived outer membrane vesicles (EcO83-OMVs)

Vesicles were isolated according to published protocol (33). Details about the visualization and characterization of EcO83-OMVs are described in the Appendix.

Liquid chromatography/mass spectrometry (LC-MS) analyses of proteins

The label-free proteomics analyses were performed as previously described (34). The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (35) with the dataset identifier PXD040963. For a detailed description of the methodology, please refer to the Appendix.

Culture and stimulation of A549 and human embryonic kidney (HEK) 293 cells

Recognition of EcO83-OMVs by innate pattern recognition receptors (PRRs) was tested on HEK 293 cells transfected with human receptors hNOD1, hNOD2, hTLR2, hTLR5 and hTLR4/CD14/MD2. The human lung epithelial cell line A549 was used for the uptake assay. Cells were cultured according to the published protocols (36–38) using the appropriate culture media (Table S1). Culture conditions, stimulation conditions, and methods for analysis, as well as the methodology for the internalisation assay are given in the Appendix.

Mice

Wild type BALB/c mice (female, aged 6-8 weeks) were purchased from Charles River (Sulzfeld, Germany). Animals were kept in conventional housing at the Institute for Pathophysiology and Allergy Research of the Medical University of Vienna with free access to food and water. Experiments were approved by the Animal Experimentation Committee of the Medical University of Vienna and the Austrian Federal Ministry of Education, Science and Culture (BMBWF-66.009/0277-V/3b/2019). **Isolation and stimulation of splenocytes and bone marrow-derived dendritic cells (BMDCs) derived from naïve mice**

Spleens were isolated, cultured, stimulated and supernatants were analysed as previously described (39). The isolation and differentiation of the bone marrow cells into BMDCs was performed as described previously (40). Detailed description of the culture, stimulation and analysis is given in the Appendix.

Allergic sensitisation, allergic challenge, and intranasal treatment with EcO83-OMVs

Airway inflammation was induced according to published protocol (39). Briefly, BALB/c mice were sensitised intraperitoneally (i.p.) with either OVA/aluminiumhydroxide (alum) or phosphate-buffered saline (PBS)/alum on days 0 and 14 and challenged intranasally (i.n.) with either OVA or PBS on days 21-24. Mice received i.n 0.1 µg or 1 µg EcO83-OMVs or 0.9% NaCl prior to each OVA administration. Airway hyperresponsiveness (AHR) was measured by whole-body plethysmography (39). For more detail on the methodology, please refer to the Appendix.

Characterisation of cell populations in the bronchoalveolar lavage (BAL)

Lungs were lavaged with PBS and cells were collected and stained as described before (39). For details on the methodology, please refer to the Appendix.

Lung histology and isolation and restimulation of lung cells *ex vivo*

Histological evaluation of lungs and isolation, stimulation, and analysis of pulmonary single cell suspensions were performed according to published protocol (39). Detailed methodology is given in the Appendix.

Statistics

Statistical analysis was performed in GraphPad Prism Software 9 (GraphPad Software Inc.) using One-Way ANOVA or Two-Way ANOVA followed by post-hoc Tukey's multiple comparison test. Data obtained from internalisation assay were analysed by comparing the area under the curve (AUC) by unpaired t-test. Data are shown as mean ±SD. Significant differences are marked as *p<0.5; **p<0.05; ***p<0.01, ****p<0.001.

RESULTS

Probiotic *E. coli* O83 produces outer membrane vesicles

EcO83-OMVs were isolated from cell-free culture supernatants by ultracentrifugation (Figure 1A). Transmission electron microscopy (TEM) visualization showed that EcO83-OMVs are spherical structures with a diameter of approximately 100 to 200 nm (Figure 1B and 1C). Figure 1B shows a TEM image of the budding of vesicles on the surface of the parent bacteria and their release into the surrounding medium. Dynamic light scattering (DLS) measurement revealed high concentration of vesicles with a mean size around 110 nm (Figure 1D). In total, we isolated 7 x 10^{12} particles from a two litre culture of *E. coli* O83 (O.D. = 2) (Table S2). To compare the protein profile of the vesicles with that of the parental bacteria, we performed automated electrophoresis with the Bioanalyzer. As shown in Figure 1E, the lysate of the whole bacteria and the OMVs had different protein profiles. Several prominent bands of about 19, 34, 49 and 64 kDa, can be identified in the OMV sample (Figure 1E; Table S3). The most dominant protein had a size of 64 kDa and accounted for 27.5% of the total protein content (Table S3). In addition, we performed a LC-MS-based label-free protein quantification to compare the protein expression profile in *E. coli* O83 bacteria and EcO83-OMVs in more detail. While most of the identified proteins (545) are shared by the OMVs and the parent bacteria, there is a group of 136 proteins (Figure 1F), including flagellar proteins FlgL 1 and 3, FlgE, FlgG, and FliC, detected exclusively in the vesicles (Figure 1G and Table S4). The molecular mass of flagellin ranges from 37 to 67 kDa depending on the*E. coli* strain (41). Our preliminary sequencing data confirm that the band of 64 kDa is *E. coli* O83 flagellin (data not shown). LPS is the major MAMP of the outer membrane of Gram- bacteria and we have shown that LPS content in OMVs is approximately 5 times higher than the protein content. (Table S2).

EcO83-OMVs activate the surface receptors TLR4, TLR2, and TLR5 and the intracellular receptors NOD1 and NOD2

OMVs derived from Gram- bacteria have previously been shown to interact with the host via their cargoes such as LPS, lipoproteins and flagellin (23,42,43) leading to modulation of the innate immune response. We have previously shown that *E. coli* O83 prevents the development of allergies in mice and that this effect depends on its interaction with TLR4 (17). Here we show that EcO83-OMVs, similar to the parent bacteria, target TLR4, confirming the presence of the TLR4 ligands on the bacteria and also on their vesicles (Figure 2A). Similarly, we observed the involvement of TLR2 (lipoprotein receptor) and TLR5 (flagellin receptor) in the interaction with EcO83-OMVs (Figure 2A). TLR2, but not TLR5, was activated by the parent bacteria, confirming our observation from proteomic analysis showing that flagellum-associated proteins are more abundant in OMVs than in the parent bacteria.

Unlike TLR2/4/5, which recognise microbial ligands on the cell surface, NOD1 and NOD2 sense bacterial products such as peptidoglycan in the cytosol (44,45) and bacterial OMVs have been shown to deliver peptidoglycan into the cytoplasm (45). NOD1 and NOD2 recognised EcO83-OMVs and whole bacteria lysate, and EcO83-OMVs interact with these receptors in a dose-dependent manner (Figure 2B).

To further confirm that EcO83-OMVs are successfully internalised into eukaryotic cells, we performed an uptake assay using the human lung epithelial cell line A549. EcO83-OMVs were labelled with octadecyl rhodamine B chloride and added to the cells. The fluorescence of this probe is quenched at high concentration and dequenched when the probe is diluted after fusion of the vesicles with the membrane of cells. Increased fluorescence indicates uptake of EcO83-OMVs by epithelial cells (Figure 3A and 3B). Cells incubated with medium only were used as a negative control. Internalisation of the vesicles by the cells appears to be an active process, as fluorescence did not increase when the same experiment was performed at 4°C (Figure 3C).

EcO83-OMVs have an immunomodulatory effect *in vitro*

EcO83-OMVs induced high levels of IL-10, IL-17 and IFN-γ in naïve splenocytes in a dose-dependent manner (Figure 4A). The pro-inflammatory cytokine TNF-α was induced to a much lesser extent and IL-6 was not

induced by OMVs at any of the concentration used (Figure 4A). EcO83-OMVs are potent inducers of IL-10, a cytokine with regulatory potential, as 1 ng of the vesicles induced levels comparable to the response elicited by 10^7 CFU/ml fixed *E. coli* O83 bacteria (Figure 4A). Dendritic cells (DCs) are the most potent antigen-presenting cells and are directly associated with the instruction and regulation of the adaptive immune response (46). BMDCs stimulated by vesicles produced significant amounts of IL-6, IL-23 and IL-1β (Figure 4B). The levels of IL-10 and IL-12p70 were not significantly different from those triggered by the medium alone (Figure 4B).

Preventive effect of intranasally administered EcO83-OMVs on experimental asthma

In our previous study, intranasal administration of live *E. coli*O83 reduced OVA-induced allergic asthma in a mouse model (17). Here we tested whether EcO83-OMVs can recapitulate the beneficial effects of live bacteria (Figure 5A). In this model, repeated exposure of mice to OVA leads to AHR, the main feature of asthma (Figure 5B). Intranasal pretreatment with 1 μg EcO83-OMVs significantly decreased PenH levels after exposure to methacholine compared to sham-treated OVA-sensitised mice, demonstrating the protective effect of vesicles (Figure 5B).

Intranasal treatment of mice with EcO83-OMVs reduced the total number of cells in the lung (Figure 5C) and the number of eosinophils in the BAL compared to the sham-treated OVA group (Figure 5D). The number of macrophages, neutrophils and lymphocytes in the BAL did not change in the EcO83-OMVs group compared to the allergic control group (Figure 5D). Decreased levels of allergen-specific IgA and IgE in BAL fluid were observed in the OMV-treated mice compared to controls (Figure S1).

Histological analysis of lung sections confirmed reduced eosinophil infiltration (Figure 5E) and a decrease in mucus-producing cells (Figure 5F) in mice treated with 1 μg EcO83-OMVs. The lower dose reduced the disease score based on evaluation of H&E-stained samples (Figure 5G), but did not decrease the disease score based on mucus production (Figure 5H). Single-cell lung suspensions were stimulated with OVA and the levels of IL-4, IL-5, IL-13, IL-10 and IFN-γ were measured in the supernatant. The group treated with 1 μg EcO83-OMVs exhibited significantly lower levels of IL-4, IL-5, IL-13 and IL-10 compared to sham-treated controls (Figure 6). Treatment with 0.1 μg EcO83-OMVs reduced the levels of IL-5, IL-13 but not IL-4 and IL-10 (Figure 6) compared to allergic controls. IFN-γ levels were increased in both treatment groups compared to the allergic control, but the effect was not significant (Figure 6).

The systemic response was assessed by measuring the level of specific antibodies in serum and by the production of cytokines in OVA-restimulated spleen cell cultures. Treatment with EcO83-OMVs had no effect on the levels of OVA-specific serum antibodies (Figure S2A) and OVA-specific IFN-γ in stimulated splenocytes (Figure S2B). We observed a decrease in the levels of IL-5 in OVA-stimulated splenocytes (Figure S2B) after treatment with 1 μg EcO83-OMVs and IL-4, IL-5 and IL-10 after treatment with 0.1 μg EcO83-OMVs compared to sham-treated OVA-sensitised controls.

DISCUSSION

This is the first study demonstrating a protective effect of OMVs from a probiotic G- bacterium in a mouse model of allergic asthma. Here we describe the isolation, identification, biophysical characterisation, interaction with innate receptors and anti-allergic properties of*E. coli* O83 OMVs.

Although numerous epidemiological studies propose that continuous high exposure to environmental bacteria and endotoxins has a protective effect against allergic sensitisation and asthma, the exact mechanisms are not yet clear (47,48). Preclinical studies confirmed the beneficial effect of intranasal administration of farm dust (48,49) or farm dust microbes (50–52) and suggested that LPS is at least largely responsible for the beneficial effect (48,49,53). We have shown in our previous study that intranasally administered *E. coli* O83 reduced allergy in a TLR4-dependent manner, suggesting the role of LPS (17). Here we show that EcO83-OMVs, like their parent bacteria contain endotoxin and are recognised by TLR4. The effects of intranasal administration of LPS on pulmonary allergy are complex and to some extent controversial, with some studies showing a worsening, while others show a reduction in allergy (54,55). It is now understood that variables

such as timing, dosage, chemical structure, and the resulting biological activity of the used LPS can affect the type of immune response that occurs (56).

In terms of timing, EcO83-OMVs were administered concomitantly with the allergen, at the time of allergic sensitization and challenge, using the same experimental protocol as we used for the application of live bacteria in our previous study (17). In this sense, Tulic *et al* . reported that *Salmonella typhimurium* LPS reduced pulmonary allergy when administered prior or up to 4 days after OVA sensitisation, but worsened it when administered more than 6 days after sensitisation (57). Furthermore, Bickert *et al* . have shown that LPS from*E. coli* 026:B6 reduced eosinophilia only when applied at the time of OVA challenge but not when administered before or after sensitisation (58). Whether prophylactic administration of vesicles prior to sensitisation and challenge has the potential to reduce allergy or whether the beneficial effect is long-lasting remains to be investigated.

It has been demonstrated that the dose of LPS delivered intranasally also determines the direction in which the immune system reacts. A high dose (100 μg) of LPS from a bacterial strain other than *E. coli*O83 reduced allergy to inhaled allergens in mice, but a low dose (0.1 μg) had the reverse effect (59). Here we show that the higher dose (1 μg) of EcO83-OMVs reduced allergy, whereas a lower dose (0.1 μg) did not worsen the disease severity but improved allergic inflammation in several parameters. Although LPS is a dominant OMV antigen, our results cannot be directly compared with those of Eisenbarth *et al.* also because they used LPS from other bacteria and it is known that LPS can vary greatly between different bacterial strains.

Flagella synthesis has been shown to play a key role in the budding of OMVs from *E. coli* (60). Here we show that OMVs from *E. coli* O83 contain flagellin (FliC), a major structural protein of bacterial flagellum, and proteins required for anchoring the flagella in the membrane (flagellar hook-associated proteins FlgK, FlgL; hook protein FlgE; basal body rod proteins FlgB, FlgC, FlgE, FlgF; and L-ring protein FlgH). These proteins showed a significant fold enrichment in vesicles compared to the whole bacteria.. The cost of constructing flagella is high in bacteria such as *E. coli* , and the reason behind the release of these energy intensive molecules into the environment has yet to be investigated.

Antigen-presenting cells such as DCs recognise flagellin on their apical surface through TLR5, which activates NF-kB and MAPK, leading to the production of pro-inflammatory cytokines (61). In our studies, EcO83-OMVs, containing high levels of flagellin, induced the expression of pro-inflammatory cytokines such as IL-6, IL-12 and TNF-α in BMDCs, and the involvement of TLR5 in their recognition was confirmed in TLR5-transfected HEK293 cells. Vesicle-stimulated BMDCs also produce caspase-1-dependent IL-1β, suggesting activation of the NLR neuronal apoptosis inhibitory protein 5 or 6 (NAIP5/6), the sensor for intracellular flagellin, followed by assembly of the NLRC4 inflammasome (62). However, vesicle-induced IL-1β levels were several hundred-fold lower compared to levels induced by whole bacteria, suggesting that vesicles can be viewed as potent immunomodulators that lack some of the negative properties of the parent bacteria. On the other hand, vesicles but not whole bacteria were recognized by TLR5, implying that this receptor is important for vesicles recognition but less important for whole bacteria recognition.

In vivo, we have shown that intranasal treatment with vesicles reduces the number of eosinophils in the lungs compared to allergic controls. A similar effect was observed with intranasal application of recombinant flagellin (63,64). A recent study suggests that the binding of flagellin to surface receptors on eosinophils such as TLR5 may prevent eosinophil sensitisation (65). According to Luo *et al* ., flagellin can reduce oxidative stress in eosinophils (64). We are currently conducting studies to elucidate the effect of EcO83-OMVs on oxidative stress in immune cells.

Not only immune cells but also airway structural cells can regulate innate immunity to allergens (66,67). We have shown that EcO83-OMVs are actively internalised by airway epithelial cells. Several pathways for internalisation of OMVs are known, such as macropinocytosis, endocytosis, membrane fusion or lipid raft formation (68). In a study by Canas *et al.* , OMVs from probiotic and commensal *E. coli*strains entered epithelial cells via clathrin-mediated endocytosis and activated the intracellular receptor NOD1 (26). The

biomolecules of the bacterial cell wall, such as LPS, can influence the pathway of vesicular uptake and consequently the entry kinetics and efficiency (69). The specific pathway mediating the internalisation of EcO83-OMVs remains to be explored.

Recent studies have demonstrated the feasibility of using OMVs to deliver heterogeneous antigens. Eastwood *et al* . used an innovative expression system in *E. coli* based on a simple peptide tag that results in a high yield of functional proteins packaged into the vesicles (70). Another study used the "plug-and-play" approach to decorate *S. typhimurium* OMVs with the spike receptor-binding domain (71). This technology makes it possible to decorate the surface of OMVs with a variety of antigens or even multiple antigens. This could be of interest for the production of engineered OMVs decorated with allergens/peptides for specific immunotherapy. In our previous study, we constructed recombinant *E. coli* Nissle 1917 expressing the chimera with major birch and grass pollen allergenic protein/peptides and showed that its intranasal application prevented poly-sensitization in mice (72). Therefore, future studies in our laboratory will focus on developing a recombinant *E. coli* O83 to produce OMVs decorated with fluorescent markers or mono- and poly-allergens for specific immunotherapy.

In summary, we show that the probiotic *E. coli* O83 produces OMVs that exhibit potent immunomodulatory and anti-allergic properties. The ease of manufacture and the availability of technologies for genetically engineered vesicles, decorated with heterologous antigens in the lumen or on the surface, provide an extraordinarily versatile platform with great potential for therapeutic applications of bacterial vesicles in allergy research and clinic.

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FIGURE LEGENDS

Figure 1 : **Isolation and characterisation of EcO83-OMVs. (A)** OMVs were isolated from culture supernatant of*E. coli* O83 by ultracentrifugation. **(B)** Visualisation of the budding process of EcO83-OMVs and **(C)** of the isolated EcO83-OMVs by TEM. Scale bar = 200 nm. **(D)** The size of the isolated EcO83-OMVs were determined by Dynamic Light Scattering.**(E)** Protein profiles of *E. coli* O83 lysate and EcO83-OMVs analysed by Bioanalyzer. **(F)** Intersection of proteins associated with *E. coli* O83 lysate and EcO83-OMVs depicted by Venn diagram **(G)** Volcano plot visualization of enriched proteins in EcO83-OMVs compared to *E. coli* O83 lysate with annotated flagella proteins.

Figure 2: Receptor activation by EcO83-OMVs. HEK-293 cells expressing **(A)** hTLR2, hTLR4 and hTLR5, **(B)** hNOD1 and hNOD2 were treated with the respective positive control (LPS 1 µg/ml, MDP 5 µg/ml, Pam3CSK 1 µg/ml or recFLA-ST 100 ng/ml), fixed *E. coli* O83 (10^7 CFU/ml), EcO83-OMVs (OMVs; 1 ng/ml, 10 ng/ml, and 100 ng/ml for TLR4; and 10 ng/ml, 100 ng/ml and 1000 ng/ml for TLR2, TLR5, NOD1 and NOD2) or medium only. Receptor activation was detected as an increase in IL-8 production. Data were analysed using a One-Way ANOVA followed by post-hoc Tukey's multiple comparison test. *p<0.5; **p<0.05; ***p<0.01, ****p<0.001; + = significant difference between positive and negative

control. Data are representative of three independent experiments. Average ± SD is shown. Significant differences between EcO83-OMVs and the negative controls are indicated n.d. = not detected.

Figure 3: Uptake of EcO83-OMVs by human lung epithelial cells. Human A549 lung epithelial cells were incubated with octadecyl rhodamine B chloride-labelled EcO83-OMVs (OMVs) for 24 h at 37 °C and fluorescence was measured hourly for 8 h. Unstimulated cells served as controls (Medium). **(A)** Shown is the increase in RFU and**(B)** the area under the curve. **(C)** A549 cells were incubated with octadecyl rhodamine B chloride-labelled EcO83-OMVs (OMVs) for 8 h at 4 °C. **(A)** Data were analysed using a Two-Way ANOVA followed by post-hoc Tukey's multiple comparison test **(B and C)**or unpaired t-test. **p<0.05; ****p<0.001. Data are representative of three independent experiments. Average ± SD is shown. RFU = relative fluorescence units; AUC = area under the curve.

Figure 4: Stimulation of naïve mouse splenocytes and BMDCs with EcO83-OMVs. (A) Mouse splenocytes and **(B)** BMDCs were stimulated with LPS (1 µg/ml), fixed *E. coli* O83 (10^7 CFU/ml), EcO83-OMVs (OMVs; 1 ng/ml, 10 ng/ml, and 100 ng/ml) for 72 and 48 h, respectively. Unstimulated cells served as controls (Medium). Cytokine levels were measured in the cell culture supernatants by ELISA. Data was analysed using a One-Way ANOVA followed by post-hoc Tukey's multiple comparison test. *p<0.5; **p<0.05; ***p<0.01. **(A)** Data are representative of four and **(B)** two independent experiments, respectively. Average ± SD is shown. Significant differences between EcO83-OMVs and the negative controls are indicated. BMDC = bone marrow-derived dendritic cell; n.d. = not detected.

Figure 5: Effect of intranasal application of Eco83-OMVs on the development of allergic airway inflammation. (A) Experimental setup for intranasal application of EcO83-OMVs and induction of experimental allergic airway inflammation to OVA. BALB/c mice were sensitised (i.p.) with either OVA/alum (groups Sham/OVA, OMVs 0.1 µg/OVA and OMVs 1 µg/OVA) or PBS/alum (group Sham/PBS) on days 0 and 14 and challenged (i.n.) with either OVA or PBS on days 21-24 with. Mice were treated i.n. with 30 µl of 0.1 µg EcO83-OMVs (group OMVs 0.1 µg/OVA), 1 µg EcO83-OMVs (group OMVs 1 µg/OVA) or 0.9% NaCl (groups Sham/PBS and Sham/OVA).**(B)** AHR was measured in response to increasing doses of metacholine by whole body plethysmography. Penh = enhanced pause.**(C-D)** Differential cell counts in bronchoalveolar lavage (BAL):**(C)** Total cell count and **(D)** total numbers of macrophages, eosinophils, neutrophils and lymphocytes. **(E-F)**Representative H&E and PAS-stained lung tissue sections showing recruited inflammatory cells and mucus-producing goblet cells, respectively. **(G-H)** Quantification of histological experiments (Disease score 0-3 for PAS and 1-4 for H&E). Scale bar = 50 µm.**(B, C, E and F)** Data were analysed using a One-Way ANOVA or**(D)** Two-Way ANOVA followed by post-hoc Tukey's multiple comparison test. * p<0.5; **p<0.05; ***p<0.01; ****p<0.001. n = 5/group. Data are representative of three independent experiments. Average ± SD is shown. Significant differences between Sham/OVA and EcO83-OMVs treatment groups (OMVs 0.1 µg/OVA or OMVs 1 µg/OVA) are indicated. alum = aluminiumhydroxide; i.n. = intranasal, i.p. = intraperitoneal; OVA = ovalbumin; OMVs = EcO83-OMVs, H&E = haematoxylin and eosin; PAS = periodic acid-Schiff.

Figure 6: Effect of intranasal application of Eco83-OMVs on the cellular recall response in the lung . Mice were treated as shown in the Figure 5A. Single cell lung suspensions were stimulated with OVA for 72 h. Cytokines were measured in the supernatant by ELISA. Data were analysed using a One-Way ANOVA followed by post-hoc Tukey's multiple comparison test**.** * p<0.5; ***p<0.01, ***p<0.001, n = 5/group. Data are representative of three independent experiments. Significant differences between sham-treated allergic mice (group Sham/OVA) and EcO83-OMVs treatment groups (OMVs 0.1 µg/OVA or OMVs 1 µg/OVA) are indicated. OVA = ovalbumin; OMVs = EcO83-OMVs.

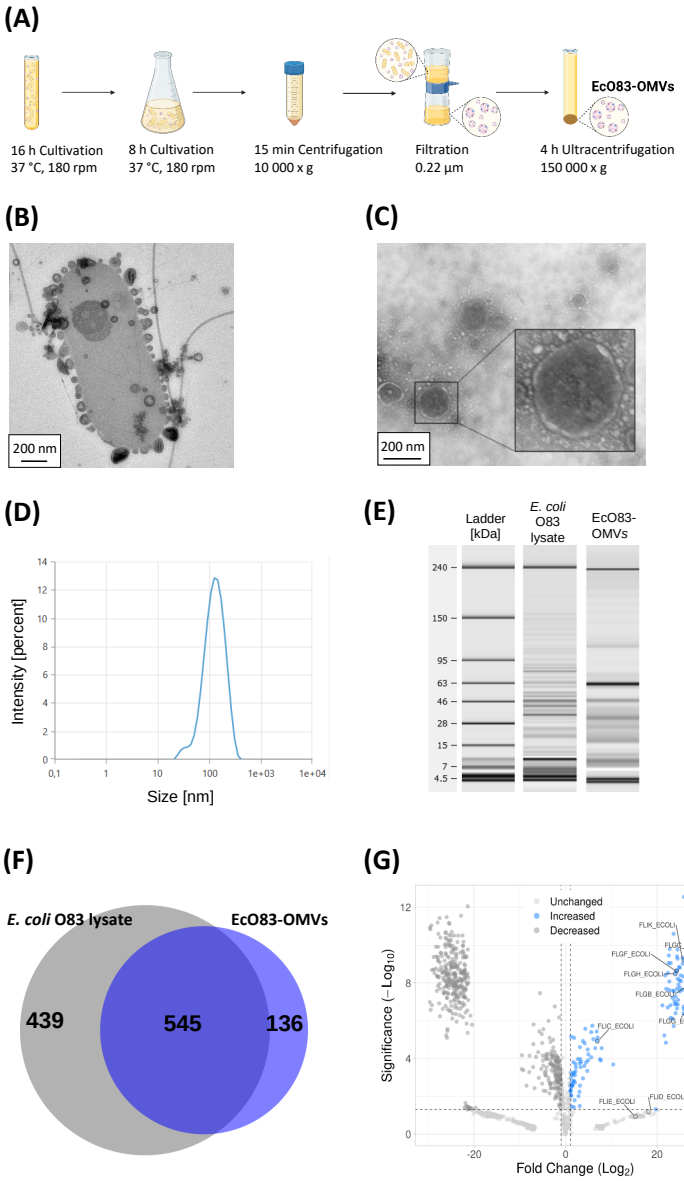


Figure 1_Schmid *et al.*

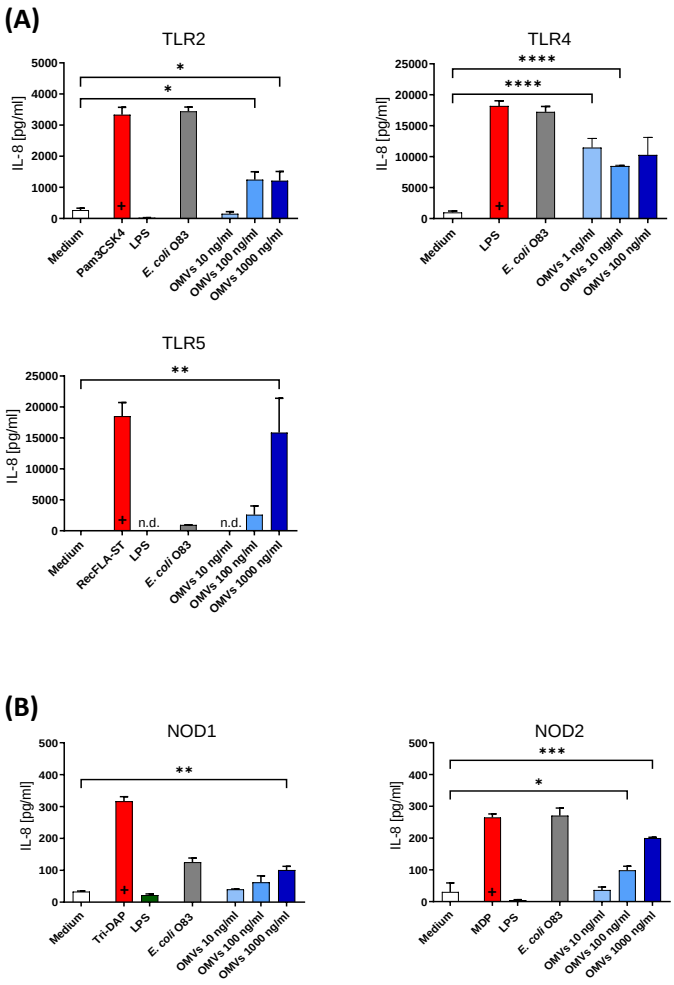
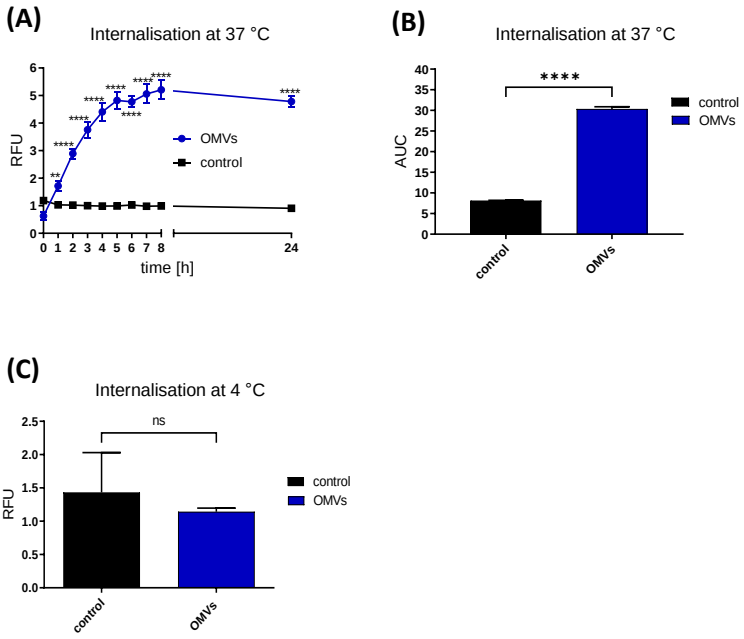


Figure 2_Schmid *et al.*



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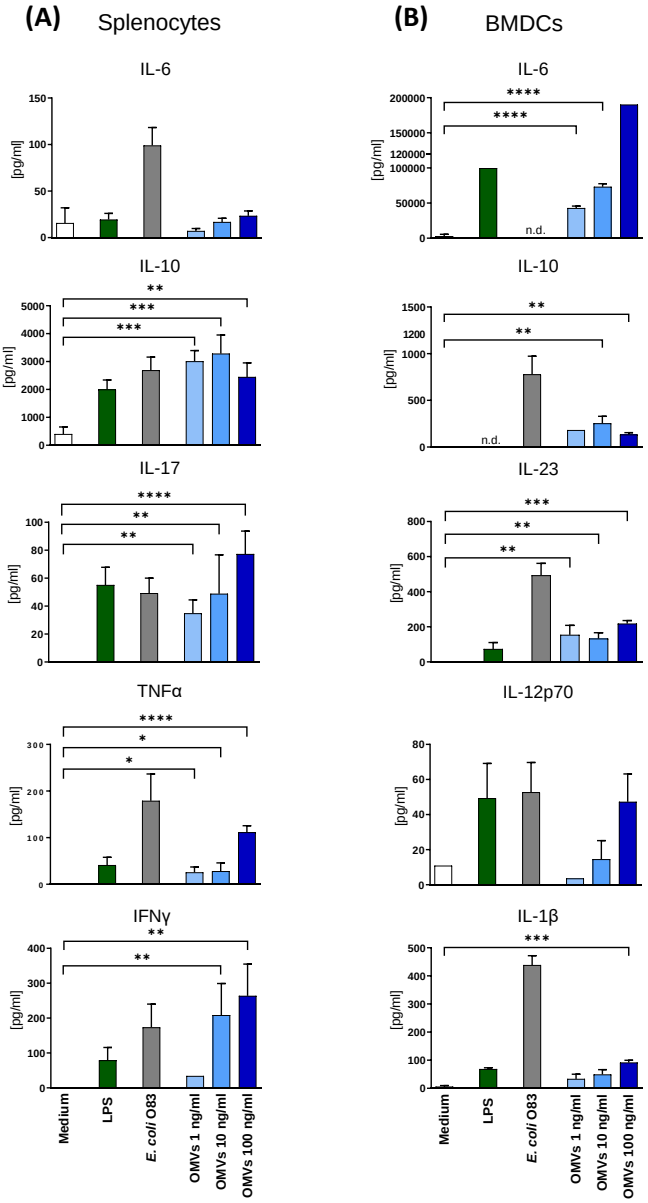

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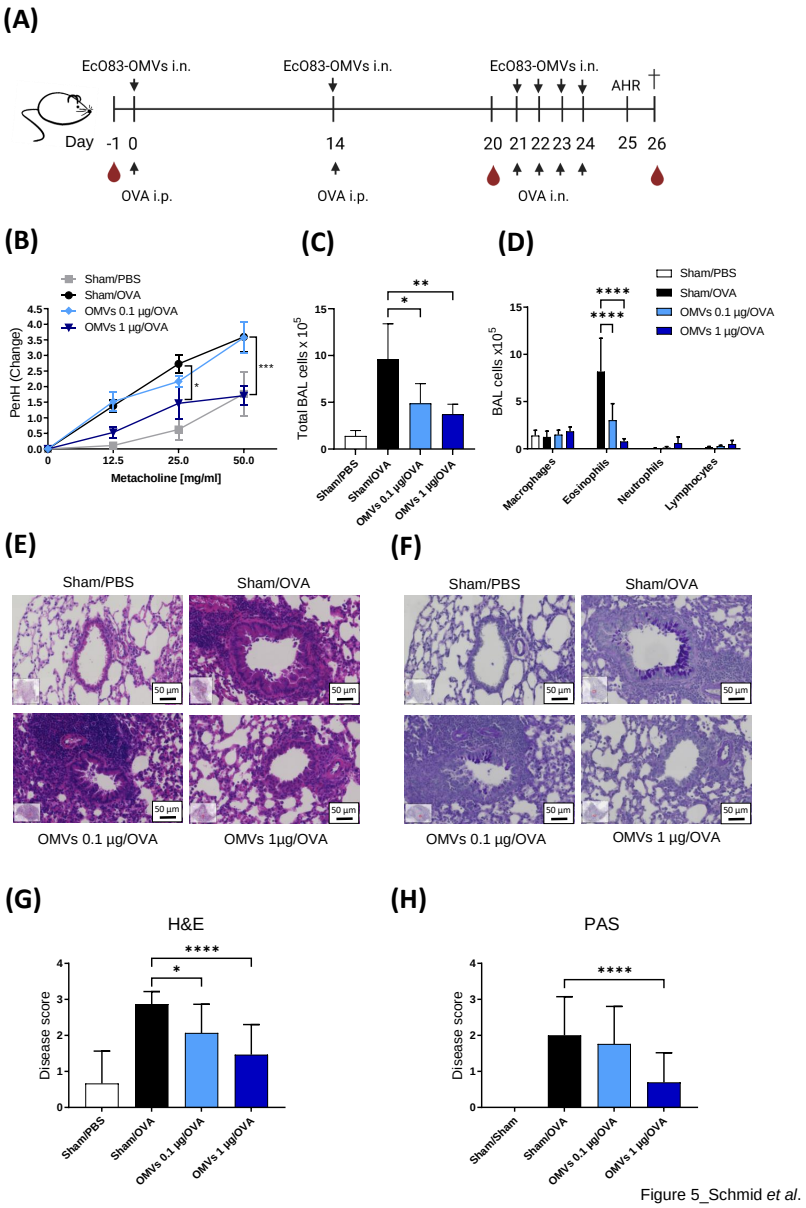

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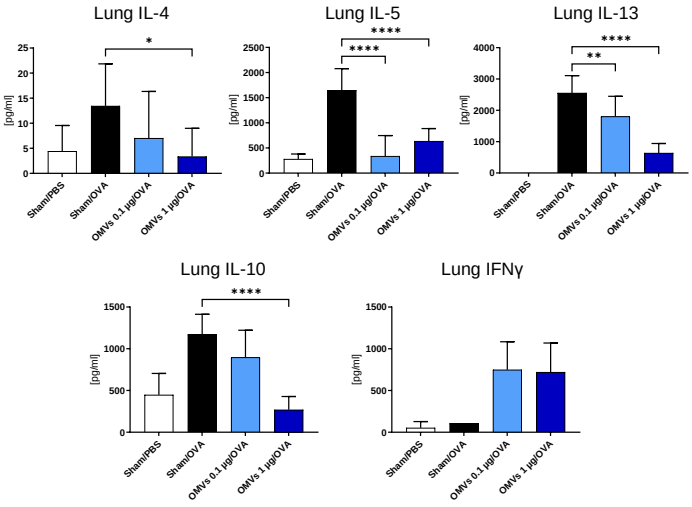

Figure 6_Schmid *et al*