Video Article

Legionella pneumophila Outer Membrane Vesicles: Isolation and Analysis of Their Pro-inflammatory Potential on Macrophages

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Abstract

Bacteria are able to secrete a variety of molecules via various secretory systems. Besides the secretion of molecules into the extracellular space or directly into another cell, Gram-negative bacteria can also form outer membrane vesicles (OMVs). These membrane vesicles can deliver their cargo over long distances, and the cargo is protected from degradation by proteases and nucleases.

Legionella pneumophila (*L. pneumophila*) is an intracellular, Gram-negative pathogen that causes a severe form of pneumonia. In humans, it infects alveolar macrophages, where it blocks lysosomal degradation and forms a specialized replication vacuole. Moreover, *L. pneumophila* produces OMVs under various growth conditions. To understand the role of OMVs in the infection process of human macrophages, we set up a protocol to purify bacterial membrane vesicles from liquid culture. The method is based on differential ultracentrifugation. The enriched OMVs were subsequently analyzed with regard to their protein and lipopolysaccharide (LPS) amount and were then used for the treatment of a human monocytic cell line or murine bone marrow-derived macrophages. The pro-inflammatory responses of those cells were analyzed by enzyme-linked immunosorbent assay. Furthermore, alterations in a subsequent infection were analyzed. To this end, the bacterial replication of *L. pneumophila* in macrophages was studied by colony-forming unit assays.

Here, we describe a detailed protocol for the purification of *L. pneumophila* OMVs from liquid culture by ultracentrifugation and for the downstream analysis of their pro-inflammatory potential on macrophages.

Video Link

The video component of this article can be found at https://www.jove.com/video/55146/

Introduction

Bacteria can secrete virulence factors via different mechanisms[1]. Besides the well-known secretory systems, Gram-negative bacteria can exchange information and deliver virulence factors via outer membrane vesicles (OMVs), which are small, spheroid vesicles 10-300 nm in diameter and with a bilayered membrane structure. They are secreted in a variety of growth environments (liquid culture, solid culture, and biofilms) and in all growth phases[2,3]. OMVs are an important means of transportation (*e.g.,* for proteins, adhesins, toxins, and enzymes, as well as for LPS, which is found on the OMV surface)[4]. The intraluminal cargo is protected from proteolytic degradation, so it is able to act over long distances, and the vesicles can be found in body fluids and distant organs[5,6,7,8]. They can not only be recognized and taken up by eukaryotic cells[9,10], but furthermore, they are able to facilitate the binding of bacteria and their invasion into host cells[4]. *Legionella pneumophila* (*L. pneumophila*) is a Gram-negative bacterium that can release OMVs. In the human lung, it primarily infects alveolar macrophages, even though its natural host are freshwater amoebae[11]. An *L. pneumophila* infection can cause Legionnaires' disease, a severe form of pneumonia[12]. It blocks phagosome-lysosome fusion in the host cell. It also recruits host organelles, whereby a replication niche, the *Legionella*-containing vacuole (LCV), is formed[13,14]. Lysosomal degradation is inhibited not only by effector protein translocation via the type IV secretion system, but also by the release of OMVs[15].

The purification of OMVs from bacterial cultures is required to analyze their effect on recipient cells. Earlier studies focused on the protein content of *L. pneumophila* OMVs and on the influence of the vesicles on alveolar epithelial cells[16], but later studies with human lung tissue transplants demonstrated that *L. pneumophila* OMVs are taken up by alveolar macrophages[17].

As OMVs present pathogen-associated molecular patterns (PAMPs) and other bacterial antigens, they might have an impact on the infection of eukaryotic cells and modulate the host immune response[18]. *L. pneumophila* OMVs rapidly fuse with host cell membranes and, moreover, they

activate the membranous TLR2[19]. As it is known that *L. pneumophila* OMVs stimulate macrophages and epithelial cells in a pro-inflammatory manner[16,17], we analyzed the impact of OMVs on the infection process in human and murine macrophages.

Here, we describe a protocol for the cultivation of *L. pneumophila* in liquid culture to isolate the secreted OMVs by differential ultracentrifugation and to assess the impact of the vesicles on eukaryotic host cells, either directly or following an infection.

Protocol

1. Prepare Medium and Agar Plates

1. Prepare 1 L of broth medium (YEB). Dissolve 10 g of ACES and 10 g of yeast extract in 900 mL of distilled water. Adjust the pH to 6.9 with KOH (5 N). Add 10 mL of L-cysteine (0.4 g in 10 mL of distilled water) and 10 mL of $Fe(NO_3)_3x9H_2O$ (0.25 g in 10 mL of distilled water). Fill up to 1 L with distilled water and filter sterilize the solution (pore size: 0.22 µm). Store at 4 °C.
2. Prepare buffered charcoal yeast extract (BCYE) agar plates. Dissolve 10 g of ACES and 10 g of yeast extract in 900 mL of distilled water. Adjust the pH to 6.9 with KOH (5 N). Add 15 g of agar and 2.5 g of activated charcoal. Fill up to 1 L with distilled water and autoclave.
    1. Add 10 mL of L-cysteine (0.4 g in 10 mL of distilled water) and 10 mL of $Fe(NO_3)_3x9H_2O$ (0.25 g in 10 mL of distilled water, both sterilized by filtration through 0.22-µm pores) to cooled BCYE (approximately 50 °C). Pour plates and store at 4 °C.

2. Cultivate *L. pneumophila*

1. Spread *L. pneumophila* strain Corby (wild type, WT) on BCYE agar plates and incubate them at 37 °C for 3 days. Inoculate 10 mL of YEB at an OD_{600} of 0.3 with *L. pneumophila* from the preculture plate; incubate the bacteria at 37 °C on a rotating shaker (150 rpm) for 6 h.
2. Verify the purity of the liquid culture by spreading 100 µL of the suspension on a blood agar plate. Incubate overnight at 37 °C.
3. Add the remaining liquid culture to 90 mL of fresh YEB medium and incubate on a rotating shaker (37 °C and 150 rpm) to reach an OD_{600} of 3.0-3.5, which takes approximately 16-20 h.

3. Prepare and Quantify *L. pneumophila* OMVs

NOTE: Carry out all of the following centrifugation steps under sterile conditions and at 4 °C.

1. Centrifuge the liquid culture at 4,000 x g for 20 min to pellet the bacteria. Transfer the supernatant to fresh centrifuge tubes, discard the bacterial pellet, and repeat the centrifugation (4,000 x g for 20 min). Repeat this step once.
2. Sterile-filter the remaining supernatant twice (pore size: 0.22 µm). Transfer the bacteria-free supernatant to ultracentrifuge tubes and ultracentrifuge at 100,000 x g for 3 h.
3. Decant the supernatant and discard it. Resuspend the OMV pellet in sterile phosphate-buffered saline (PBS) and ultracentrifuge (100,000 x g for 3 h) to remove contaminating proteins and LPS.
4. Discard the supernatant and resuspend the OMV pellet in 500 µL of sterile PBS. Streak 20 µL on a blood agar plate and on a BCYE agar plate to exclude bacterial contamination of the prepared vesicles. Incubate the blood agar plate overnight and the BCYE agar plate for 3 days (both at 37 °C).
5. Quantify the protein amount obtained from the OMV preparation using a bicinchoninic acid assay according to the manufacturer's instructions.
   NOTE: The concentration of 100 mL of *L. pneumophila* culture is usually 1 µg/µL. Store the prepared and quantified OMVs at -20 °C.

4. Pre-treat Macrophages

1. Prepare THP-1 cells.
   NOTE: THP-1 is a monocytic cell line derived from a leukemia patient.
    1. Add $2x10^5$ THP-1 cells per 24 wells and differentiate them by adding 20 nM phorbol 12-myristate 13-acetate (PMA) into macrophage-like cells. Incubate for 24 h at 37 °C.
    2. Replace the medium with 500 µL of fresh medium and incubate for another 24 h; the optimal medium for THP-1 cells is composed of RPMI 1640 high glucose supplemented with 10% fetal calf serum.
2. Isolate the murine bone marrow-derived macrophages (mBMDM), as described in Reference 20.
3. Treat THP-1-derived macrophages or mBMDM with OMVs.
    1. Thaw the OMVs prepared in step 3 and add them according to their protein amount (0.1, 1, and 10 µg/mL) to the human or murine macrophages. Incubate the macrophages with OMVs at 37 °C for at least 20 h. Use the supernatant for ELISA or move on with step 5.

5. Infect the Macrophages and Assess Bacterial Replication with a Colony-Forming Unit (CFU) Assay

1. Use *L. pneumophila* from step 2.1, pre-treated THP-1 cells or mBMDM from step 4.3, and not pre-treated macrophages as controls ($2x10^5$/24 wells). Do not exchange the medium.

2. Infect THP-1 cells with *L. pneumophila* Corby WT and mBMDM with a flagellin-lacking mutant of *L. pneumophila* Corby (both with a multiplicity of infection (MOI) of 0.5; 1x10^5 *L. pneumophila*/24 wells) and incubate for 24 and 48 h, respectively. Prepare both *L. pneumophila* Corby (WT or flagellin-lacking mutant) as described in step 2.1.
3. Lyse the cells in their medium by the addition of saponin (final concentration: 0.1%) and incubate at 37 °C for 5 min.
4. Resuspend the bacteria by pipetting and transfer the suspension to a reaction vessel. Prepare serial dilutions of the *L. pneumophila*-containing media in sterile PBS.
5. Streak 50 µL of the required dilutions on BCYE agar plates and incubate for 3 days at 37 °C.
6. Visually count the formed colonies. Calculate the CFU $(\frac{CFU}{mL} = counted\ colonies \cdot dilution\ factor)$. Normalize the CFU count result to not pre-treated but infected macrophages, which are set to 100%.

Representative Results

The experimental setup to prepare *L. pneumophila* OMVs and to analyze their influence on the pro-inflammatory response of macrophages following infection is depicted in **Figure 1**. The pro-inflammatory potential of the prepared OMVs can be analyzed on PMA-differentiated THP-1 cells, which is shown in **Figure 2**. THP-1 cells respond with a time- and dose-dependent increase of IL-8 and IL-6 secretion. Additionally, the influence of different TLRs on *L. pneumophila* OMV recognition can be analyzed by using mBMDM from different genetic backgrounds, as presented by the CXCL1 ELISA in **Figure 3**. mBMDM from WT mice secreted CXCL1 after OMV stimulation, while mBMDM TLR2/4$^{-/-}$ secreted significantly less. To study the impact of *L. pneumophila* OMVs on bacterial replication in THP-1 macrophages, cells were pre-incubated with OMVs and then additionally infected with *L. pneumophila* (**Figure 4 A**). The pre-stimulation of THP-1-derived macrophages first reduces the bacterial replication after 24 h of infection, but it leads to a doubling in CFU count at the later time point (48 h p.i.). The impact of Toll-like receptor (TLR) signaling on OMV recognition following the infection of the macrophages can be assessed by mBMDM, as presented in **Figure 4 B**. Bacterial replication increases by tenfold in mBMDM from WT animals after OMV pre-incubation, while TLR2$^{-/-}$ and TRIF/MyD88$^{-/-}$ cells do not show this increase in *L. pneumophila* replication.

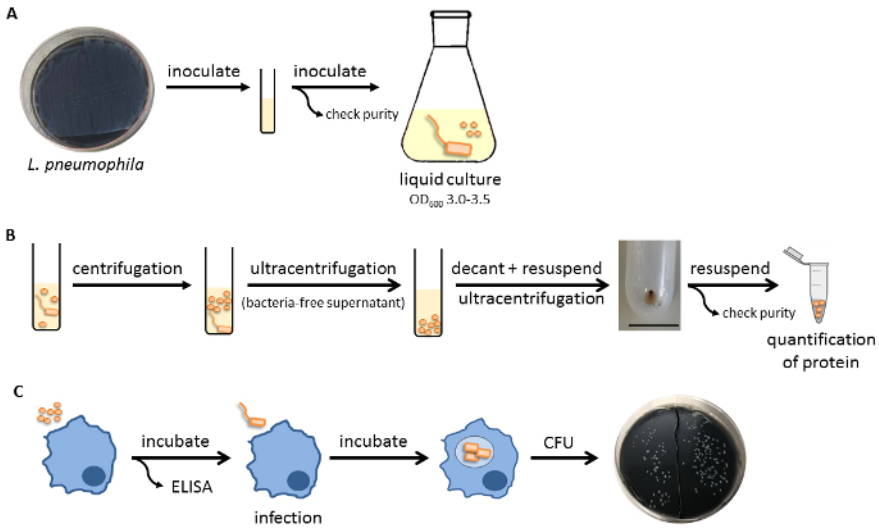

Figure 1: Experimental procedure. (A) *L. pneumophila* Corby WT from 10-cm BCYE agar plates are used to inoculate a small liquid culture (10 mL), which is transferred into 90 mL of fresh YEB medium after 6 h. A small volume is also plated on a blood agar plate to check for purity. Bacteria are incubated at 37 °C until the early stationary phase (OD_{600} = 3.0-3.5). **(B)** The liquid culture is centrifuged and sterile-filtered to remove the bacteria. The *Legionella*-free supernatant is then ultracentrifuged to obtain an OMV pellet, which is resuspended in PBS and ultracentrifuged again. The isolated vesicles are resuspended, checked for purity, and quantified for the protein amount. The scale bar represents 2.5 cm. **(C)** Human or murine macrophages are stimulated with the quantified OMVs. The cell culture supernatant can be used for ELISA, or macrophages can be infected with *L. pneumophila* to determine bacterial replication by CFU assay on 10-cm BCYE agar plates. Please click here to view a larger version of this figure.

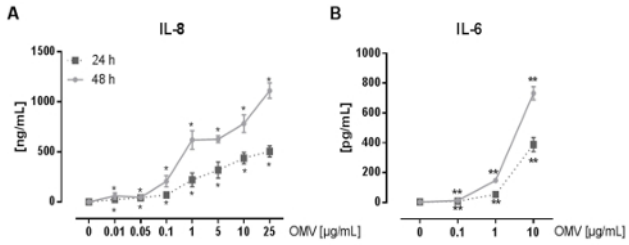

Figure 2: Pro-inflammatory activation of THP-1 cells by *L. pneumophila* OMVs. (A) Here, the monocytic THP-1 cell line is used as a model for alveolar macrophages. PMA-differentiated THP-1 cells were treated with increasing doses of *L. pneumophila* OMVs (0.01-25 µg/mL) for 24 and 48 h, respectively. The cell-free supernatant was used for IL-8 ELISA. The mean values of three independent experiments ± SEM are shown. THP-1 cells responded to as little as 0.01 µg/mL *L. pneumophila* OMVs with significant IL-8 secretion, which was time- and dose-dependent. **(B)** *L. pneumophila* OMVs (0.1-10 µg/mL) were used to stimulate PMA-differentiated THP-1 cells. The supernatant was collected after 24 and 48 h of incubation, and the released IL-6 was measured in the supernatant via ELISA. The mean values of three independent experiments ± SEM are shown. THP-1 cells secreted significant amounts of IL-6, even with the lowest dose of OMVs (0.1 µg/mL). The secretion of IL-6 increased with increasing OMV doses and with prolonged incubation times. Statistics: Mann-Whitney test; *p < 0.05 and **p < 0.01 compared to the corresponding 0 µg/mL OMV. Reprinted with permission from Reference 20. Please click here to view a larger version of this figure.

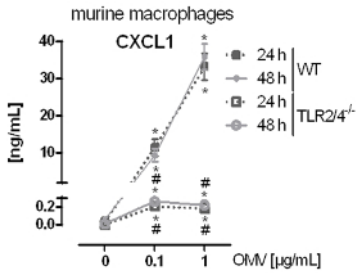

Figure 3: The pro-inflammatory activation of macrophages depends on TLR2/4. mBMDM from WT and TLR2/4$^{-/-}$ mice were incubated with *L. pneumophila* OMVs (0.1 or 1 µg/mL). CXCL1 secretion was analyzed by ELISA after 24 and 48 h, respectively. The mean values ± SEM of three independent experiments are shown. mBMDM from WT mice responded with a dose-dependent CXCL1 secretion after *L. pneumophila* OMV incubation. TLR2/4$^{-/-}$ mBMDM secreted significantly less CXCL1 compared to WT mBMDM, and this secretion did not increase dose-dependently. Statistics: Mann-Whitney test; *p < 0.05 compared to the corresponding 0 µg/mL OMV; #p < 0.05 compared to an equally treated WT sample. Reprinted with permission from Reference 20. Please click here to view a larger version of this figure.

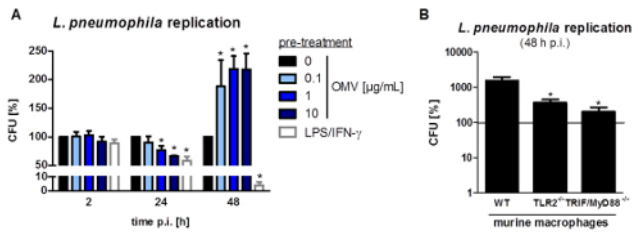

Figure 4: *L. pneumophila* OMV pre-incubation increases bacterial replication in macrophages. (A) Differentiated THP-1 cells were pre-incubated with OMVs (0.1, 1, or 10 µg/mL) or LPS/IFN-γ (200 ng/mL each) or were left untreated for control. After pre-incubation (20 h), THP-1 cells were infected with *L. pneumophila* Corby WT (MOI 0.5) for 2, 24, and 48 h, respectively. THP-1 cells were lysed by the addition of saponin, and the bacteria were plated on BCYE agar plates. CFUs were calculated relative to 0 µg/mL OMV after every time point. The bars represent the mean values ± SEM of three independent experiments, each performed in technical duplicates. There were no differences in bacterial uptake (2 h post-infection (p.i.)) in comparison to not pre-treated cells. Alterations in bacterial replication were determined after 24 and 48 h, respectively. LPS/IFN-γ pre-treated THP-1 cells showed a reduction in bacterial load 24 h p.i. This was also observed dose-dependently for *L. pneumophila* OMV pre-treated cells. At the later time point (48 h p.i.), OMV pre-treated THP-1 cells showed a doubling in *L. pneumophila* replication, whereas LPS/IFN-γ pre-treated macrophages showed a further reduction of bacterial load. Statistics: Mann-Whitney test; *p < 0.05 and **p < 0.01 compared to the corresponding 0 µg/mL OMV. **(B)** mBMDM from mice with different genetic backgrounds (WT, TLR2$^{-/-}$, and TRIF/MyD88$^{-/-}$) were pre-incubated with 0.1 µg/mL *L. pneumophila* OMVs for 20 h and were then infected with a flagellin-deficient mutant of *L. pneumophila* Corby (MOI 0.5) for 48 h. mBMDM were lysed by the addition of saponin, and the *Legionella* were plated on BCYE agar plates. The CFU were calculated relative to 0 µg/mL OMV, indicated by the solid line. The bars represent the mean values ±± SEM of three independent experiments, each performed in duplicates. mBMDM from WT mice showed an increase in *L. pneumophila* replication after OMV pre-treatment. TLR2$^{-/-}$ macrophages showed significantly reduced *Legionella* replication, which was comparable to TRIF/MyD88$^{-/-}$ mBMDM. Statistics: Mann-Whitney test; p < 0.05 compared to the WT sample. Reprinted with permission from Reference 20. Please click here to view a larger version of this figure.

Discussion

The OMVs of bacterial pathogens and the impact of these membrane vesicles on their target cells are currently being intensively studied. For example, *Clostridium perfringens*-derived OMVs induce cytokine secretion in macrophages, B lymphocytes can be activated by OMVs from *Borrelia burgdorferi*, and *Helicobacter pylori*-released membrane vesicles can act on gastric epithelial cells[21,22,23]. *L. pneumophila*, an intracellular pathogen that can induce a severe form of atypical pneumonia, also releases OMVs that are able to activate lung epithelial cells and macrophages[16,19]. Here, we present a detailed protocol for the small-scale isolation of *L. pneumophila* OMVs from liquid culture to study the potential role of OMVs in pneumonia. It is critical to work under sterile conditions and to rule out contamination from other bacteria in order to obtain a pure *L. pneumophila*-derived OMV preparation. The isolation of OMVs includes a filtration step through 0.22-µm pores in order to prevent the contamination of the obtained OMV pellet with *L. pneumophila*, even though this reduces the OMV yield, since the largest OMVs are lost by this filtration step.

Furthermore, we tested the response of human and murine macrophages to those isolated vesicles and infected cells with *L. pneumophila* to more closely approximate the situation in *Legionella* pneumonia, where OMVs are released inside the LCV by extracellular bacteria[15]. The employed OMV doses have been estimated according to the free OMV amount in an *in vitro* infection of human macrophages after 24 h of incubation (described in Reference 20). For the stimulation of other recipient cells or *in vivo* experiments, other OMV doses might be necessary and must be established. The analysis of the effect of *L. pneumophila* OMVs represents an advancement to the protocol described by Jager and Steinert[24].

Here, PMA-differentiated THP-1 cells serve as a model for alveolar macrophages due to the limited availability of primary human material. The addition of PMA differentiates the monocytic THP-1 cells into macrophage-like cells[25]. Furthermore, they are a well-known model cell line for *L. pneumophila* studies[26]. Besides this human monocytic cell line, mBMDM cells are used. mBMDM are widely accepted for the study of the effects of *L. pneumophila*[27,28,29]. The possibility of using genetic knockouts for different TLRs or other proteins make them a valuable tool for studying OMV effects. In order to lower the amount of mice per experiment, mBMDM are used instead of alveolar macrophages due to the limitations of the macrophages. Key experiments might require alveolar macrophages for validation.

Besides the herein-described protocol of ultracentrifugation to purify OMVs, it is possible to perform a density gradient centrifugation, which is included in the protocol by Chutkan *et al.*[30]. This could improve the purity of the obtained OMV preparation and reduce the amount of co-purified protein aggregates, flagellin, and LPS. The purity of the obtained OMV preparation can be analyzed by transmission electron microscopy or by nanoparticle tracking analysis as a supplementary step in quality control. This can provide an additional means of quantification, beyond the protein measurement procedure presented here. Optionally, the LPS concentration can be analyzed by a limulus amebocyte lysate test. If the OMV yield is low, an additional concentration step via centrifugal filters could be performed, which was not done here. If the yield was lower than expected, the OMVs were discarded.

As part of the ongoing effort to elucidate the biological mechanisms and functions behind OMVs, the influence of different stress conditions on OMV production could be tested. Nutrient deprivation, changes in incubation temperature, or exposure to harmful agents might have an impact on OMV secretion[31]. Possible stress conditions are discussed in the protocol by Klimentova and Stulik[32]. Moreover, hyper- or hypovesiculating *L. pneumophila* mutants could be generated. The different OMV preparations could then be analyzed in infection experiments with macrophages, human lung tissue explants (described in Reference 17), or even in *in vivo* models. Besides the role of OMVs in innate immune signaling, their influence in bacterial communication can be addressed experimentally. Furthermore, the impact of various innate immune signaling cascades might be analyzed by the use of murine knockout cells or the generation of CRISPR/Cas9 knockouts in human cell lines. This basic research in OMVs will assist in the development of new vaccine strategies, which already exist for meningitis B transmitted by *Neisseria meningitides*[33].

Starting from the protocol on OMV isolation and characterization, one can apply this to other Gram-negative bacteria and to other host cells; it only needs to be adjusted to the growth of the bacteria in liquid culture. The protocol published by Chutkan *et al*. provides detailed information on the generation of OMVs from *Escherichia coli* and *Pseudomonas aeruginosa*[30]. The culture should not reach the late stationary phase in order to avoid increases in lysed bacteria and contaminating proteins and membranes. Additionally, the OMV dose used for stimulation of the host cells needs to be determined according to the amount of OMVs present during *in vivo* infections, while still ensuring a low rate of cytotoxicity. In this way, the pathological role of OMVs, their impact on inter-species communication, and host-pathogen interactions could be examined.

To further study the role of *L. pneumophila* OMVs in pneumonia, standardized OMV preparations with sufficient yields and comparable infection experiments are needed. This protocol will help to standardize isolation procedures and to extend OMV studies to other Gram-negative bacteria and to other host cells. Furthermore, research will benefit from the detailed *in vitro* knowledge, which can be used to extend experiments to *in vivo* settings. In the future, this protocol could be extended to the isolation of OMVs from primary biological material, such as serum or bronchoalveolar lavage fluid, to gain insight into the composition of OMVs released under physiological conditions. This will help to determine key parameters of OMV composition and to understand the properties of *in vitro*-generated OMVs.

Disclosures

The authors have nothing to disclose.

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