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Analyzing the influence of Legionella pneumophila outer membrane vesicles

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Abstract:	Bacteria are able to secrete a variety of molecules via various secretion systems. Gram-negative bacteria cannot only secrete molecules free into the extracellular space or directly into another cell, but they can also form outer membrane vesicles (OMVs). These membrane vesicles can deliver their cargo via long distances, and additionally, the cargo is protected from degradation by proteases and nucleases. Legionella pneumophila (L. pneumophila) is an intracellular, gram-negative pathogen which causes a severe form of pneumonia. In humans, it infects alveolar macrophages where it blocks its lysosomal degradation and forms a specialized replication vacuole. Moreover, L. pneumophila produces OMVs under various growth conditions. To understand their role 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 amount and then used for the pre-treatment of a human monocytic cell line or murine bone marrow derived macrophages. The pro-inflammatory response of those cells was addressed by ELISA. Furthermore, the reaction of macrophages to a subsequent infection with L. pneumophila 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 the downstream analysis of their pro-inflammatory potential on macrophages.
Author Comments:	We want to start filming at step 2 after the media preparation, but didn't know how to highlight this in the manuscript.

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Dear Teena Mehta,

We would like to thank you for the invitation to publish our work in JoVE. Therefore, we would like to suggest the manuscript “Analyzing the influence of *Legionella pneumophila* outer membrane vesicles” by Anna Lena Jung, Kerstin Hoffmann, Christina E. Herkt, Christine Schulz, Wilhelm Bertrams, and Bernd Schmeck for possible publication in JoVE.

We consider it to be suitable, because of the recently rising interest in outer membrane vesicles generated by Gram-negative bacteria. The manuscript describes a detailed protocol in the growth and handling of *Legionella pneumophila*, an important human pathogen causing mainly pneumonia, and the purification of the secreted outer membrane vesicles. Additionally, we describe here the following downstream analysis of the vesicles in stimulation and infection experiments of human and murine macrophages.

We could show – in conclusion – that OMVs from *L. pneumophila* are initially potent pro-inflammatory stimulators of macrophages, acting via TLR-signaling, while at later time points, OMVs facilitate *L. pneumophila* replication. OMVs might thereby pave the way for subsequent bacterial replication and hence promote spreading of *L. pneumophila* in the host.

We think that it will drive research forward because – besides the known pathways – we explored one other intriguing possibility to transfer virulence features to host cells, even over distance: Outer membrane vesicles that are released by *Legionella*. The described protocol can be adapted to the analysis of outer membrane vesicles from other Gram-negative bacteria. The standardization of protocols is required to obtain results that are comparable in different studies.

All authors have read and agreed to the manuscript. They declared to have no conflict of interest. The contribution of all authors, the original data, and the funding sources are provided.

We hope that you will find merit in our manuscript and are looking forward to your critical reviews.

Sincerely,

Anna Lena Jung Bernd Schmeck

TITLE:

***Legionella pneumophila* outer membrane vesicles: isolation and analysis of their pro-inflammatory potential on macrophages**

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KEYWORDS:
Legionella pneumophila; outer membrane vesicles; macrophage; purification; differential ultracentrifugation; pro-inflammatory response; bacterial replication

SHORT ABSTRACT:
We describe here the purification of *Legionella pneumophila* (*L. pneumophila*) outer membrane vesicles (OMVs) from liquid cultures. These purified vesicles were then used for the treatment of macrophages to analyze their pro-inflammatory potential.

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

Legionella pneumophila (*L. pneumophila*) is an intracellular, Gram-negative pathogen which causes a severe form of pneumonia. In humans, it infects alveolar macrophages, where it blocks its 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 then used for the treatment of a human monocytic cell line or murine bone marrow derived macrophages. The pro-inflammatory response of those cells was addressed 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 the downstream analysis of their pro-inflammatory potential on macrophages.

INTRODUCTION:
Bacteria can secrete virulence factors via different mechanisms [1]. Besides the well-known secretion systems, Gram-negative bacteria can exchange information and deliver virulence

factors via OMVs, which are small, spheroid vesicles of 10-300 nm in diameter 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 for e. g. proteins, adhesins, toxins and enzymes along with LPS, which is found on the OMV surface [4]. The intraluminal cargo is protected from proteolytic degradation, thereby it is able to act over long distances, and the vesicles can be found in body fluids and distant organs [5-8]. 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 which can release OMVs. In the human lung, it primarily infects alveolar macrophages, even though its natural host are freshwater amoebae [11]. *L. pneumophila* infection can cause legionnaires' disease, a severe form of pneumonia [12]. It blocks phagosome-lysosome fusion in the host cell, and it recruits host organelles, whereby a replication niche is formed, the *Legionella* containing vacuole (LCV) [13,14]. Lysosomal degradation is not only inhibited by effector protein translocation via the type IV secretion system, but also by release of OMVs [15].

The purification of OMVs from bacterial cultures is needed to analyze their effect on recipient cells. Earlier studies focused on the protein content of *L. pneumophila* OMVs and 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 direct or on a following infection.

PROTOCOL:

1) Prepare media and agar plates

1.1) Prepare 1 L of broth medium (YEB). Dissolve 10 g ACES and 10 g yeast extract in 900 mL distilled water. Adjust pH to 6.9 with KOH (5 N). Add 10 mL L-cysteine (0.4 g in 10 mL distilled water) and 10 mL $Fe(NO_3)_3x9H_2O$ (0.25 g in 10 mL distilled water). Fill up to 1 L with distilled water followed by filter sterilization (pore size: 0.22 µm). Store at 4 °C.

1.2) Prepare buffered-charcoal yeast extract (BCYE) agar plates. Dissolve 10 g ACES and 10 g yeast extract in 900 mL distilled water. Adjust pH to 6.9 with KOH (5 N). Add 15 g agar and 2.5 g activated charcoal. Fill up to 1 L with distilled water and autoclave.

1.2)1. Add 10 mL L-cysteine (0.4 g in 10 mL distilled water) and 10 mL $Fe(NO_3)_3x9H_2O$ (0.25 g in

10 mL 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*

2.1) Spread *L. pneumophila* strain Corby (wild type, WT) on BCYE agar plates and incubate at 37 °C for 3 d. Inoculate 10 mL YEB at an OD_{600} of 0.3 with *L. pneumophila* from the pre-culture plate and incubate the bacteria at 37 °C on a rotating shaker (150 rpm) for 6 h.

2.2) Verify the purity of the liquid culture by spreading 100 µL of the suspension on a blood agar plate. Incubate overnight at 37 °C.

2.3) Add the remaining liquid culture to 90 mL of fresh YEB medium and incubate on a rotating shaker (37 °C, 150 rpm) to reach an OD_{600} of 3.0-3.5, which takes approximately 16 to 20 h.

3) Prepare and quantify *L. pneumophila* OMVs

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

3.1) Centrifuge the liquid culture at 4,000 x g for 20 min to pellet bacteria. Transfer the supernatant in fresh centrifuge tubes, discard the bacterial pellet and repeat centrifugation (4,000 x g for 20 min). Repeat this step once.

3.2) Sterile-filter the remaining supernatant twice (pore size: 0.22 µm). Transfer bacteria-free supernatant to ultracentrifuge tubes and ultracentrifuge at 100,000 x g for 3 h.

3.3) Decant supernatant and discard it. Resuspend OMV pellet in sterile phosphate-buffered saline (PBS) and ultracentrifuge (100,000 x g for 3 h) to remove contaminating proteins and LPS.

3.4) Discard supernatant and resuspend OMV pellet in 500 µL sterile PBS. Streak 20 µL on a blood and on a BCYE agar plate to exclude bacterial contamination of the prepared vesicles. Incubate blood agar plate overnight and BCYE agar plate for 3 d (both at 37 °C).

3.5) Quantify protein amount of obtained OMV preparation with bicinchronic acid assay according to the manufacturer's instructions.

NOTE: The concentration from 100 mL *L. pneumophila* culture is usually 1 µg/µL. Store prepared and quantified OMVs at -20 °C.

4) Pre-treat macrophages

4.1) Prepare THP-1 cells.

NOTE: THP-1 is a monocytic cell line derived from a leukemia patient.

4.1.1) Add $2x10^5$ THP-1 cells per 24 well and differentiate them by addition of 20 nM phorbol 12-myristate 13-acetate (PMA) into macrophage-like cells. Incubate for 24 h at 37 °C.

4.1.2) Replace the medium with 500 µL 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.

4.2) Isolate murine bone marrow derived macrophages (mBMDM) as described in [20].

4.3) Treat THP-1-derived macrophages or mBMDM with OMVs.
4.3.1) Thaw OMVs prepared in section 3 and add them according to their protein amount (0.1, 1 and 10 µg/mL) to the human or murine macrophages. Incubate macrophages with OMVs at 37 °C for at least 20 h. Use supernatant for ELISA or move on with section 5.

5) Infect macrophages and assess bacterial replication by colony forming unit (CFU) assay
5.1) Use *L. pneumophila* from section 2.1 and pre-treated THP-1 cells or mBMDM from section 4.3 or not pre-treated macrophages as control (each $2 \cdot 10^5/24\ well$). Don't exchange medium.

5.2) 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; $1 \cdot 10^5 L.pneumophila/24\ well$) and incubate for 24 and 48 h, respectively. Both *L. pneumophila* Corby (WT or flagellin-lacking mutant) need to be prepared as described in section 2.1.

5.3) Lyse the cells in their medium by addition of saponin (final concentration: 0.1 %) and incubate at 37 °C for 5 min.

5.4) 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.

5.5) Streak 50 µL of the required dilutions on BCYE agar plates and incubate for 3 d at 37 °C.

5.6) Visually count formed colonies at sight. Calculate CFU ($\frac{CFU}{mL} = counted\ colonies \cdot dilution\ factor$). Normalize CFU count result to not pre-treated but infected macrophages, those are set to 100%.

REPRESENTATIVE RESULTS:
The experimental setup to prepare *L. pneumophila* OMVs and to analyze their influence on pro-inflammatory response of macrophages and a 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 the use of mBMDM from different genetic backgrounds, as presented by 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 infection, while 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 and following 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 LEGENDS:

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 fresh YEB medium after 6 h. A small volume is additionally 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 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. Scale bar represents 2.5 cm. **(C)** Human or murine macrophages are stimulated with the quantified OMVs. Cell culture supernatant can be used for ELISA, or macrophages can additionally be infected with *L. pneumophila* to determine bacterial replication by CFU assay on 10 cm BCYE agar plates.

Figure 2: Pro-inflammatory activation of THP-1 cells by *L. pneumophila* OMVs. (A) The monocytic THP-1 cell line is used as a model for alveolar macrophages here. 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. Cell-free supernatant was used for IL-8 ELISA. Shown are mean values of three independent experiments ± SEM. 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. Supernatant was collected after 24 and 48 h of incubation, and released IL-6 was measured in the supernatant via ELISA. Mean values of three independent experiments are shown ± SEM. THP-1 cells secreted significant amounts of IL-6 already with the lowest dose of OMVs (0.1 µg/mL). The secretion of IL-6 increased with increasing OMV doses and with prolonged incubation time. Re-print with permission from [20].
Statistics: Mann-Whitney test; *p<0.05 and **p<0.01 compared to corresponding 0 µg/mL OMV.

Figure 3: Pro-inflammatory activation of macrophages depends on TLR2/4. (A) 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. Shown are mean values ±SEM of three independent experiments. 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. Re-print with permission from [20].

Statistics: Mann-Whitney test; *p<0.05 compared to corresponding 0 µg/mL OMV; #p<0.05 compared to equally treated WT sample.

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), LPS/IFN-γ (200 ng/mL each) or 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 addition of saponin and bacteria were plated on BCYE agar plates. CFUs were calculated relative to 0 µg/mL OMV after every time point. Bars represent 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 corresponding 0 µg/mL OMV. (B) mBMDM from mice with different genetic backgrounds (WT, TLR2$^{-/-}$, TRIF/MyD88$^{-/-}$) were pre-incubated with 0.1 µg/mL *L. pneumophila* OMVs for 20 h and then infected with a flagellin-deficient mutant of *L. pneumophila* Corby (MOI 0.5) for 48 h. mBMDM were lysed by addition of saponin and *Legionella* were plated on BCYE agar plates. CFU were calculated relative to 0 µg/mL OMV, indicated by the solid line. Bars represent mean values of three independent experiments ±SEM each performed in duplicates. mBMDM from WT mice showed an increase in *L. pneumophila* replication after OMV pre-treatment. TLR2$^{-/-}$ macrophages showed a significantly reduced *Legionella* replication, which was comparable to TRIF/MyD88$^{-/-}$ mBMDM. Statistics: Mann-Whitney test; p<0.05 compared to WT sample. Re-print with permission from [20].

DISCUSSION:

OMVs of bacterial pathogens and the impact of these membrane vesicles on their target cells are currently studied intensively. For example, *Clostridium perfringens*-derived OMVs induce cytokine secretion in macrophages, B lymphocytes can be activated by OMVs from *Borellia burgdorferi*, and *Helicobacter pylori*-released membrane vesicles can act on gastric epithelial cells [21-23]. *L. pneumophila*, an intracellular pathogen which 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* OMV from liquid culture to study the potential role of OMVs in pneumonia. It is critical to work under sterile conditions and to rule out contaminations with other bacteria, to obtain a pure *L. pneumophila*-derived OMV preparation. The isolation of OMVs contains a filtration step through 0.22 µm pores to exclude the contamination of the obtained OMV pellet with *L. pneumophila*, even though this is reducing 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 additionally infected the cells with *L. pneumophila* to more closely approximate

the situation in *Legionella* pneumonia, where OMVs are released inside the LCV and 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 [20]). For the stimulation of other recipient cells or *in vivo* experiments, other OMV doses might be necessary and need to be established. The analysis of the effect of L. pneumophila OMVs represents an advancement to the protocol described by Jager and Steinert **[24].**

PMA-differentiated THP-1 cells serve as a model for alveolar macrophages here, due to the limited availability of primary human material. The addition of PMA differentiates the monocytic THP-1 cells in 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 to study the effects of *L. pneumophila* [27-29]. The possibility of using genetic knockouts for different TLRs or other proteins make them a valuable tool for studying OMV effects. mBMDM instead of alveolar macrophages are used, due to the limitation of alveolar macrophages to lower the amount of mice per experiment. Key experiments might require alveolar macrophages for validation.

Besides the herein described protocol of ultracentrifugation to purify the OMVs, it is possible to additionally 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 eletron microscopy or nanoparticle tracking analysis, as a supplementary step in quality control. This can additionally provide a means of quantification, besides the here presented protein measurement. Optionally, the LPS concentration can be analyzed by 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 as expected, OMVs were discarded.

As part of the ongoing efforts to elucidate the biological mechanisms and functions behind OMVs, the influence of different stress conditions on the 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 [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. The basic research in OMVs will help in the future to develop new vaccine strategies, as already possible 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 other host cells, as it 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 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 occurring during *in vivo* infections, while ensuring a low rate of cytotoxicity. By this, the pathological role of OMVs, their impact on inter-species communication and the host-pathogen interaction could be addressed.

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 to isolation procedures and to extent OMV studies to other Gram-negative bacteria and other host cells. Furthermore, research will benefit from the detailed *in vitro* knowledge to additionally extent experiments to *in vivo* settings. In the future, the protocols should be extended to 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.

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DISCLOSURES:
The authors have nothing to disclose.

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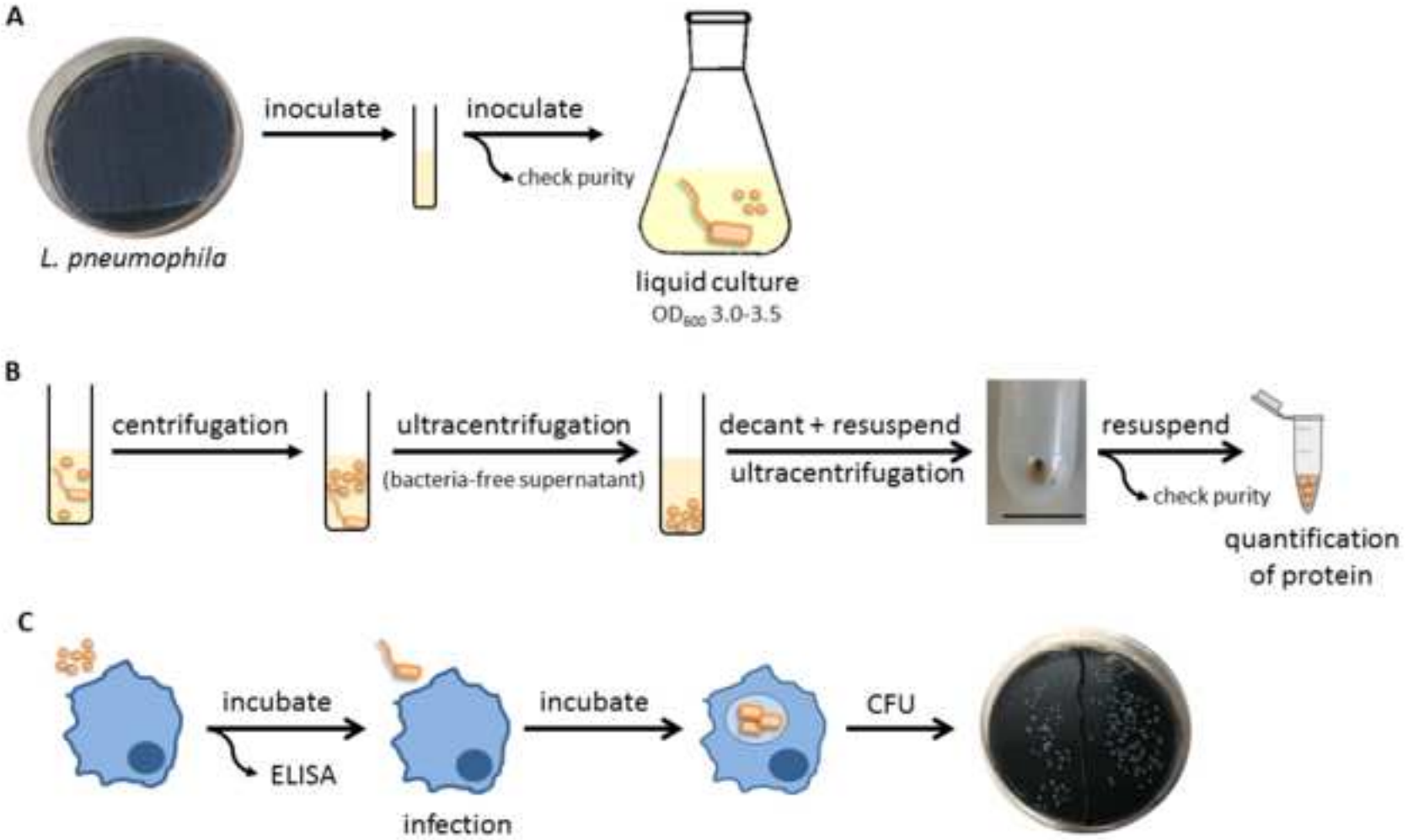

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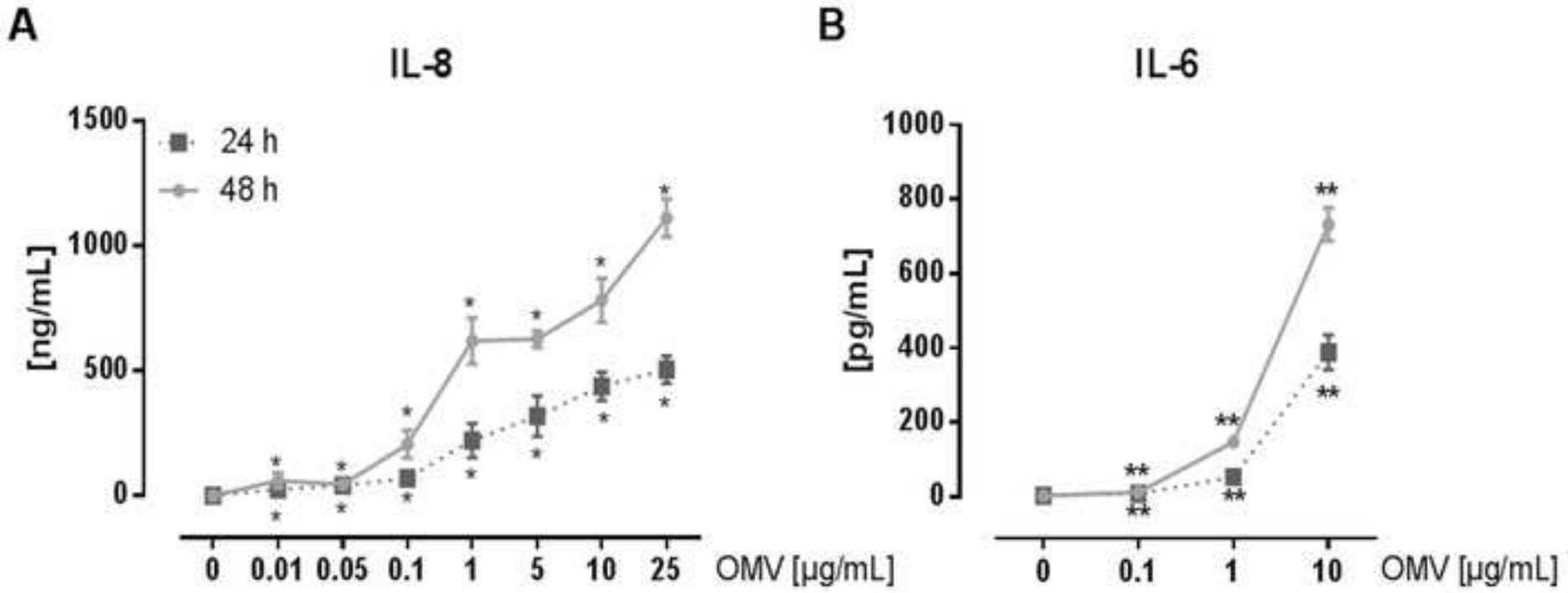

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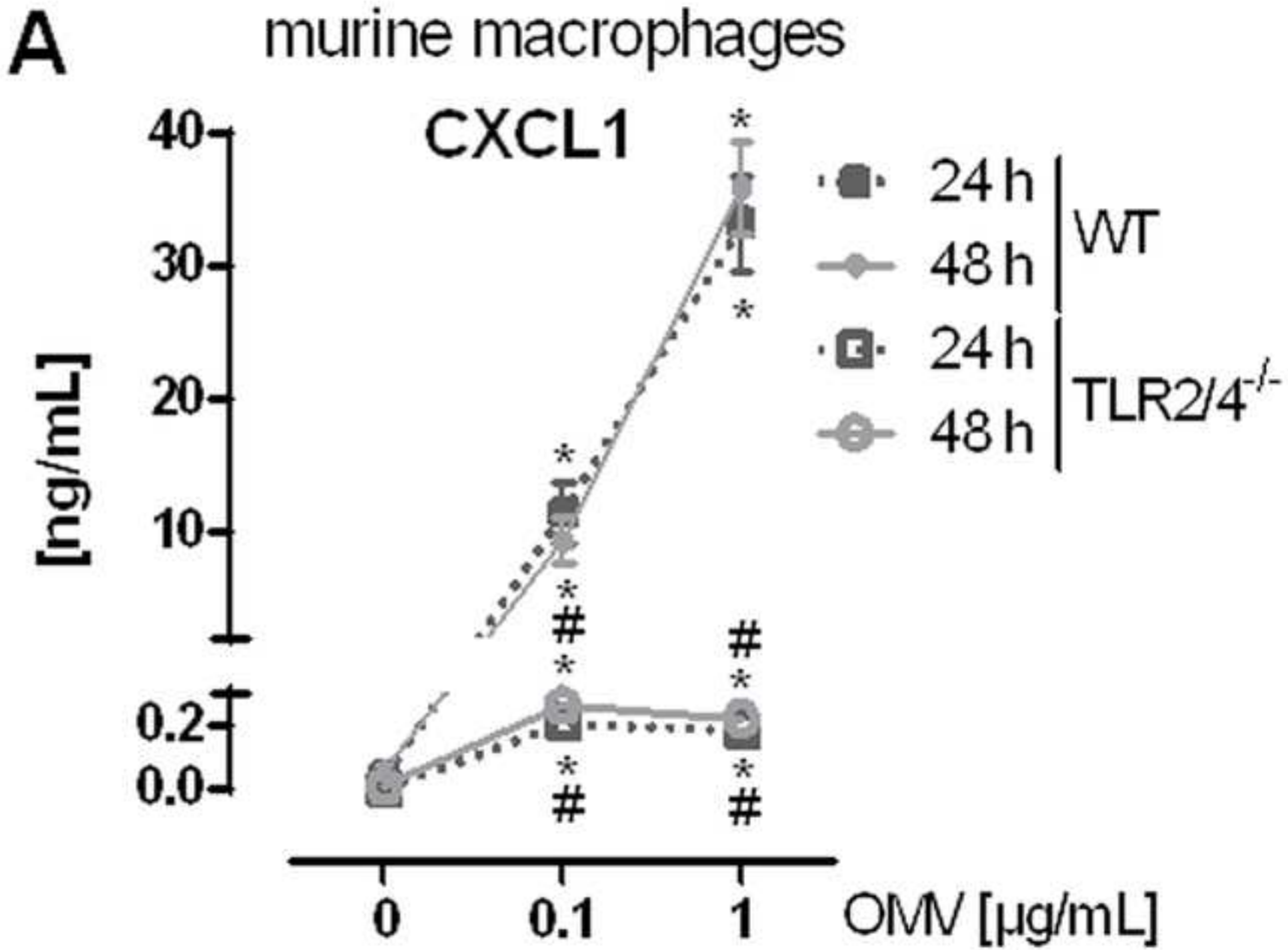

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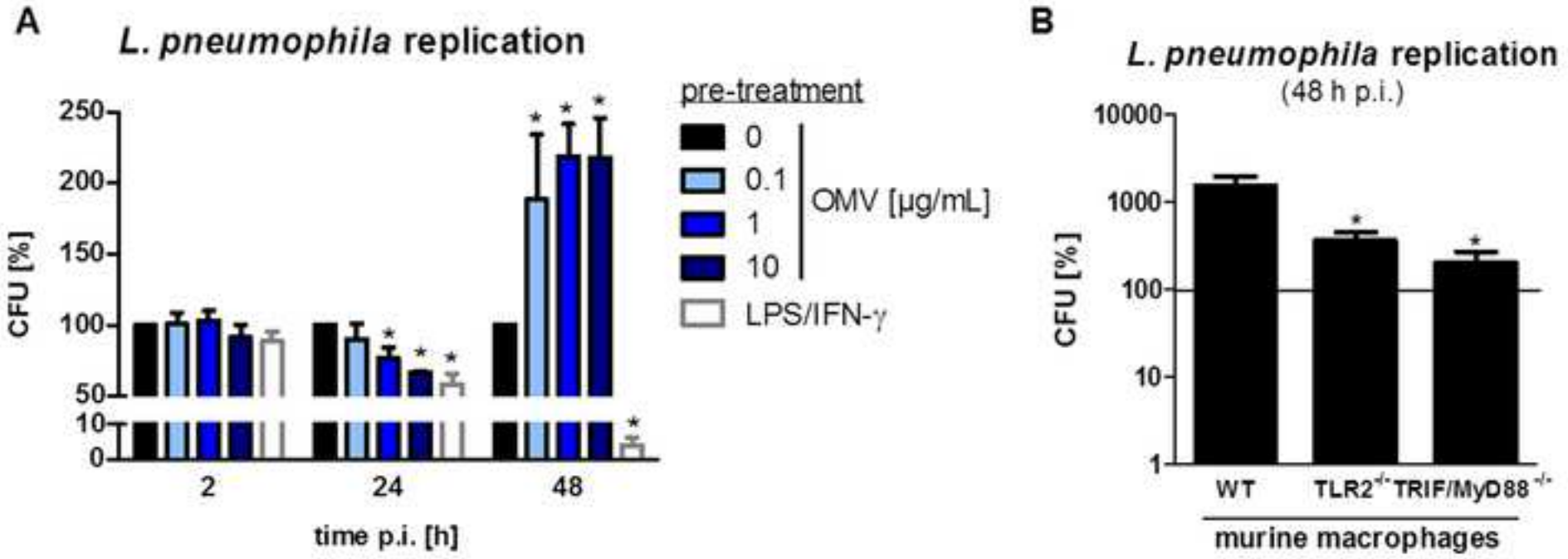

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Name of Reagent/ Equipment	Company	Catalog Number
10 cm petridish	Sarstedt AG & Co KG (Nuembrecht, Germany)	82.1473
70 Ti rotor	Beckman Coulter Incorporation (California, USA)	337922
ACES	Carl Roth GmbH & Co KG (Karlsruhe, Germany)	9138.2
activated charcoal	Carl Roth GmbH & Co KG (Karlsruhe, Germany)	X865.2
agar-agar, Kobe I	Carl Roth GmbH & Co KG (Karlsruhe, Germany)	5210.2
Columbia agar with 5 % sheep blood	Becton Dickinson GmbH (Heidelberg, Germany)	254005
cuvettes	Sarstedt AG & Co KG (Nuembrecht, Germany)	67.742
ELISA (human)	BD OptEIA™; Becton Dickinson GmbH (Heidelberg, Germany)	IL-8: 555244 IL-6: 550799
ELISA (murine)	DuoSet, R&D (Minneapolis, USA)	CXCL1: DY453-05
$Fe(NO_3)_3x9H_2O$	Carl Roth GmbH & Co KG (Karlsruhe, Germany)	5632.1
Fetal calf serum (FCS)	Life Technologies GmbH (Darmstadt, Germany)	10270-106
Heracell 240i CO_2 incubator	Thermo Fisher Scientific Germany BV & Co KG (Braunschweig, Germany)	40830469
Heraeus Multifuge X3R	Thermo Fisher Scientific Germany BV & Co KG (Braunschweig, Germany)	75004515
Inoculation loop	Sarstedt AG & Co KG (Nuembrecht, Germany)	86.1567.010
KOH	Carl Roth GmbH & Co KG (Karlsruhe, Germany)	6751.1
L. pneumophila Corby	---	---
L. pneumophila Corby ΔflaA	---	---
L-cystein	Carl Roth GmbH & Co KG (Karlsruhe, Germany)	
mBMDM	---	---
PBS	Biochrom GmbH (Berlin, Germany)	L 1825
phorbol 12-myristate 13-acetate	Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany)	P8139-1MG
rotating shaker (MaxQ 6000)	Thermo Fisher Scientific Germany BV & Co KG (Braunschweig, Germany)	SHKE6000
RPMI 1640 high glucose	Life Technologies GmbH (Darmstadt, Germany)	11875-093
saponin	Carl Roth GmbH & Co KG (Karlsruhe, Germany)	9622.1
Ultrospec 10	Biochrom Ltd (Cambridge, England)	80-2116-30

sterile filter (pore size: 0.22 µm)	Corning Incorporated (new York, USA)	431096
THP-1	Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany)	88081201-1VL
Sorvall Discovery 100 SE	Thermo Fisher Scientific Germany BV & Co KG (Braunschweig, Germany)	
yeast extract	Carl Roth GmbH & Co KG (Karlsruhe, Germany)	2363.2
Pierce BCA protein assay kit	Thermo Fisher Scientific Germany BV & Co KG (Braunschweig, Germany)	23225

Comments/Description

kindly provided by Prof Dr Antje Flieger (RKI, Berlin, Germany)
kindly provided by Prof Dr Klaus Heuner (RKI, Berlin, Germany)

kindly provided by Prof Dr Markus Schnare (Philipps Univeristy Marburg, Marburg, Germany) and Prof Dr Carsten Kirschning (University Duisb

ɔurg Essen, Essen, Germany)

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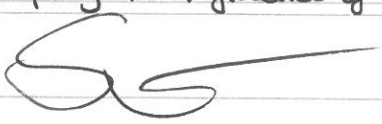
Signature: 

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>First and foremost we would like to extend our gratitude to you for the constructive criticisms given by the reviewers. We have addressed all issues that were brought up by the reviewers and we feel that our manuscript has greatly profited from these modifications.<

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript by Jung et al. summarizes a protocol of their recently published study in PLoS Pathogens 2016, in which the effect of outer membrane vesicles (OMVs) isolated from Legionella pneumophila (L. pneumophila) on monocyte-derived cells as a model of interaction of this pathogen with alveolar macrophages in the lung was studied. The topic is very important, the concept of OMVs as bacterial messengers and modulators of host-microbe interactions is fascinating, and the protocol is indeed timely as it further alerts scientific community about this important problem and provides a straight-forward way to approach it. However, there are a number of questions, which should be addressed in order to make this protocol a meaningful resource and high quality reference material for other investigators.

Major Concerns:

1. More attention should be paid to definition of the macrophage (considering recent advancements in macrophage nomenclature). THP-1 cells and THP-1-derived cells are unlikely macrophages in this classical sense, unless the authors provide evidence that they have features of alveolar macrophages, the target cell for this pathogen. It is unclear why mouse bone marrow-derived macrophages were proposed to be used? Do they represent a model for L. pheumophila host-pathogen interactions? Are they similar in any way to alveolar macrophages? Given than alveolar macrophages are the primary target cell for L. pneumophila relevant to disease pathogenesis, and these cells can be easily isolated from the lung by bronchoalveolar lavage, it is unclear why the authors did not use these primary cells? Description of this methodology will significantly increase the impact and clinical relevance of this protocol.

>Thank you very much for putting forth your concerns. We took great care to address these issues adequately by including a paragraph in the discussion section to address these questions. Isolation of alveolar macrophages is only easily done in mice and even there the obtained amount of cells is limited. We wanted to keep the amount of mice per experiment to a minimum, so we refused to do it in this experimental setup.<

2. Since interaction of L. pneumophila-derived OMVs with macrophages is the focus of this protocol, could the Authors provide more insights into how these vesicles are detected by macrophages prior to engulfment. Does this process involve innate immune recognition? Can this be studied and, most importantly, modulated therapeutically.

>We appreciate the reviewer's indication and included information in the introduction and comments in the discussion section. Therapeutically, OMVs might be used for vaccine development in the future, as it is already possible for *Neisseria meningitides* OMVs (Boxsero, Novartis, https://www.novartis.com/news/media-releases/novartis-bexsero%C2%AE-vaccine-approved-fda-prevention-meningitis-b-leading-cause). <

3. The protocol will benefit from adding notes related to the specific purpose of each step (i.e., what is the goal of each specific procedure in terms of generation, isolation,

purification, quality control, quantification of OMVs). If some steps are original or represent modification of previously published protocols, please more emphasis on those steps and explain the innovation. If the procedures have been previously described, please specify and provide references. Since some major OMV methodological papers have been published (Jäger J and Steinert M., Methods Mol Biol 2013; Chutkan H et al, Methods Mol Biol 2013; Klimentova et al, Microbiol Res, 2015), it would be important to mention these protocol, as well as discuss what similarities and differences (particularly with Jäger J and Steinert M., Methods Mol Biol 2013).
>We appreciate the reviewer's indication and included information in discussion section.<

4. Discussion of naturally secreted (native) and induced (bioengineered) OMVs would be important, and different procedures are needed to obtain these different types of OMVs. Moreover, they may have different composition and biological properties. Have the Authors compared these two types of OMVs? Even if they haven't, this should be discussed in the protocol. How closely stress-induced OMVs mimic in vivo lung environment?
>Following the reviewer's suggestion, we added a comment on that in the discussion section. As it is very challenging to obtain a bronchoalveolar lavage from a patient with Legionnaire's disease, we unfortunately could not investigate the effects of in vivo generated OMVs so far despite the indisputably great scientific interest. An alternative might be to obtain OMVs from patients in other disease circumstances.<

5. Given that expected size of OMVs is up to 300 nm, a 0.22 µm sterilization filter (step 3.2) might capture larger OMVs and lower the yields. Have the Authors consider this possibility and, if so, is there any way to overcome this potential problem?
>Thank you very much for putting forth your concerns. We addressed this in the discussion section. We know that this filtration step leads to the loss of the largest OMVs (>0.22µm), but we wanted to ensure that the obtained OMV preparation is bacteria free. A bacterial contamination of the OMV pellet would reduce the comparability and accuracy of the performed experiments.<

6. It is possible that a total amount of OMV in the media is low. Would a subsequent pre-concentration step be required?
>Thank you very much for putting forth your concerns. We addressed this in the discussion section. We know that an additional concentration step is possible, even though we never performed it. When the OMV yield was lower as expected, we did not use it for further downstream experiments, as we considered this preparation to have failed. Changes in *Legionella pneumophila* metabolism due to e.g. media composition, temperature changes, etc might have caused the low OMV yield, but for the moment we chose not to analyze this in our experiments.<

7. It is unclear to a non-experienced reader how unwanted extracellular materials that may potentially contaminate OMV preparations (pili, flagella, fimbria, pili, large protein complexes or aggregates) will be removed to ensure that they do not contribute to unexpected immunomodulatory effect of OMVs, such as pro-inflammatory response in macrophages that the Authors propose to evaluate. Would any additional purification steps be necessary (such as density gradient centrifugation or gel filtration)?
>Thank you for your suggestion. We changed the manuscript accordingly, by adding a paragraph in the discussion section.<

8. OMV quantification requires more details. What methods the Authors would suggest? What yields that would consider satisfactory in order to proceed with the protocol? Would any troubleshooting be needed depending on quantification results?
>Thank you very much. We added the method of choice (bicinchronic acid assay) in the manuscript, but it is also possible to perform a Bradford assay to quantify the protein amount. The expected yield is added in the "NOTE" at the end of paragraph 3. As mentioned above, a lower yield was expected as a failed experiment and OMVs were discarded.<

9. Step 4. Pre-treat macrophages. Appropriate terminology should be used to accurately describe these cells. Note that some readers (particularly students and junior investigators) would consider this article as a "gold standard" textbook protocol and their future understanding of science might be altered if the terminology is used improperly. The authors should explain that this a leukemia monocytic cell line, used as a model; explain the reason why PMA is used; how to validate they are macrophages after PMA stimulation; can PMA affect the ability of cells to respond to OMVs; why these cells can be utilized as a model. References to previously published literature containing evidence-based answers to these questions may be used, but explanations are needed.
>Thank you very much for putting up your concerns. We changed the manuscript accordingly and provided literature in the discussion section stating that THP-1 cells are an appropriate model cell line for *L. pneumophila* studies and also for the use of mBMDM.<

10. Step 4.1.2. Why the media should be high-glucose and contain 10% FBS? Does this mimic the environment of alveolar macrophages? Have the Authors observed similar effects using serum-free medium?
>Thank you for this indication. We used RPMI 1640 supplemented with FCS as this is the suggested media by the provider. THP-1 cells do not proliferate without FCS. We did not perform stimulation experiments without FCS, as it might be that the change in media composition is altering the ability of THP-1 cells to adequately respond to the applied stimuli. The restriction of growth factors by serum-free media was not in the scope of this study.
The composition of the alveolar environment is of course different from commercially available media. It is not trivial to find the exact same composition as the alveolar lining fluid. Currently we are trying to mimic the composition of the alveolar lining fluid to better recapitulate the *in vivo* setting.<

11. If LPS is deactivated in the media containing OMVs, would THP1-derived cells show comparable pro-inflammatory response to OMVs?
>Thank you for this comment. We do not expect that we co-purified free LPS shed by the Legionella, as the purification of this LPS includes several concentration steps of the bacteria-free media. Additionally, the OMVs present there are discarded. The flow-through of 100kDa cut-off filters is used for further concentration in 10kDa cut-off filters. The concentrated retentate from the 100kDa filters contains the OMVs (described in Lück, C and Helbig, JH; Methods Mol Biol 2013). Furthermore, accidentally co-purified LPS might again be lost in the second round of ultracentrifugation (step 3.3). This PBS washing steps is improving the purity of the obtained OMV pellet, as protein aggregates and LPS are once more depleted.<

12. What is the source of flagellin-lacking L. pneumophila mutant?

>We apologize for this inconvenience and added this information in the included table.<

13. Apart from the parameters of host-pathogen interactions described by the Authors in the original paper in PLoS Pathogens, what other important aspects of innate immunity and microbial pathogenesis can be studied?
>Thank you for your suggestion. We included this in the discussion section.<

14. Discussion of limitations of the protocol and alternative approaches is missing.
>Thank you for this indication. We have added this in the discussion section.<

15. Discussion of therapeutic relevance of studies using this protocol would significantly increase the impact of this paper.
>Thank you for this indication. We have included information on this.<

Minor Concerns:
1. Title: Influence on what? (perhaps, should be specified; or re-phrased)
>Thank you. We have changed this accordingly.<

2. Abstract (2nd sentence) and Introduction (line 92): using the phrases "cannot only" and "also" makes it difficult to understand the meaning. "Their role" should be changed to "the role of OMVs"
>Thank you. We have changed this accordingly.<

3. Abstract: "Furthermore, the reaction of macrophages to a subsequent infection with L. pneumophila was studied by colony forming unit assays" ("the reaction of macrophages" - too broad term for such a specific assay; please specify)
>Thank you. We have changed this accordingly.<

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
Manuscript describes the method of isolation of OMV of Legionella pneumophila, and analysis of the pro-inflammatory effect on certain macrophages

Major Concerns:
N/A

Minor Concerns:
Title: Analyzing the influence of Legionella pneumophila outer membrane vesicles Suggestion: Legionella pneumophila outer membrane vesicles: isolation and analysis of their proinflammatory potential on macrophages.
>Following the reviewer´s suggestions, we have changed the title.<

SHORT ABSTRACT:
We describe here the purification of Legionella pneumophila (L. pneumophila) outer membrane vesicles (OMVs) from liquid cultures. These purified vesicles were then used for the treatment of macrophages to analyse their proinflammatory potential.
>Thank you. We have changed this accordingly.<

L68 L. pneumophila > Legionella pneumophila
>Thank you. We have changed this accordingly.<

L96 macrophages, even though its natural host are freshwater amoebae. L. pneumophila > Legionella pneumophila
>Thank you. We have changed this accordingly.<

L123 Fill up to 1 L with distilled water and sterile-filter it; followed by filter sterilization (
>Thank you. We have changed this accordingly.<

L130 Add 10 mL L-cystein (0.4 g in 10 mL distilled water) and 10 mL Fe(NO3)3x9H2O (0.25 g in
10 mL distilled water) to cooled BCYE. How were these solutions sterilized? Explain. L-cystein >L- cysteine
>Thank you. We have changed this accordingly.<

L133 Spread L. pneumophila strain Corby (wildtype) on BCYE agar plates > (wild type) also at other places
>Thank you for this indication. We have changed the manuscript accordingly.<

L138 agar plate. Incubate over-night at 37 °C. > overnight
>Thank you. We have changed this accordingly.<

L148 supernatant in fresh centrifuge tubes, discard the bacterial pellet and centrifuge again (4,000 x g for 20 min). Transfer supernatant, discard bacterial pellet and repeat centrifugation (4,000 x g 150 for 20 min).
Better supernatant in fresh centrifuge tubes, discard the bacterial pellet and centrifuge again (4,000 x g for 20 min). Repeat this step once.
>Thank you. We reworded this accordingly.<

L204 Count formed colonies by eye > Count formed colonies at sight
>Thank you for this indication. We have changed this.<

L209 to analyze their influence on macrophages > to analyze their influence on pro-inflammatory response of macrophages
>Thank you for this comment. We have changed the sentence.<

L215 L. pneumophila (figure 4 A). The impact of TLR signaling explain abbreviation TLR > Toll like receptor (TLR) signaling

>Thank you, we have changed this.<

L226 Scale bar represents 2.5 cm. There is no scale bar in this figure
>Following a suggestion by the editorial office, we included a scale bar at figure 1B to highlight the size of the ultracentrifugation tube and the obtained OMV pellet.<

280 DISCUSSION:
281 OMVs of Gram-negative pathogens and the impact of these membrane vesicles on their target cells are currently studied intensively. For example, Clostridium perfringens-derived OMVs: remark Clostridium perfringens is not a Gram-negative bacterium
>Thank you for this comment. We rephrased this sentence.<

L284 remark: Borrelia burgdorferi is not classified as a gram positive or gram negative bacterium
>Thank you for this comment.<

Fig. 2, 3 and 4 have been used in another paper of the same authors (PLoS Pathog 12(4): e1005592). Reference has been given in the text but not at the figure itself. No direct reference to this figure in the current manuscript. Copyright??
>The figures 2-4 are already published in PLoS Pathog 12(4):e1005592 as you correctly mentioned. The figure legends have this reference included. (Re-print with permission from [19].)<