Validation data for *E. coli* OMVs

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Outer membrane vesicles (OMVs) are secreted, small, non-replicative, immunogenic spherical bodies that contain many pathogen-associated molecular patterns (PAMPs) from the parent bacterium such as DNA, RNA, peptidoglycan, lipoproteins, toxins, and lipopolysaccharides (LPS). Invivogen has isolated OMVs with a diameter between 40-60 nm from late-stationary phase cultures of the commonly used *Escherichia coli* BL21 strain (**Figure 1**). In particular, LPS one of the most abundant constituents of OMVs, is recognized by and activates caspase-11 (human homologs caspase 4/5) forming a non-canonical inflammasome, which in turn can also activate the canonical inflammasome pathway. These events are characterized by the release of the alarmin HMGBI upon membrane rupture (**Figure 2)** and the release of pro-inflammatory cytokines such as IL-1β (**Figure 3**). Concomitantly, the activation of the caspase 11-4/5 inflammasome complex by *E. coli* OMVs leads to pyroptotic cell death through the action of gasdermin D (GSDMD) (**Figure 4**).

Characterization of *E. coli* OMVs

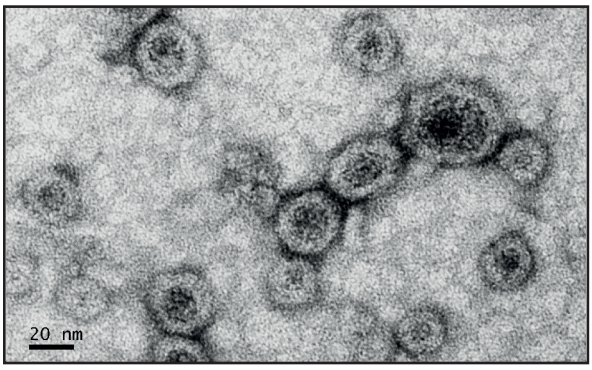

Figure 1: *E. coli* OMVs were prepared for electron microscopy using conventional negative staining procedures. *E. coli* OMVs (20 µl) were absorbed onto Formvar carbon-coated grids for 2 min, blotted, and then negatively stained with 1% uranyl acetate for 1 min. Grids were then examined with a transmission electron microscope (Jeol JEM-1400, JEOL Inc, Peabody, MA, USA) at 80 kV. Images were acquired with a digital camera (Gatan Orius, Gatan Inc, Pleasanton, CA, USA) using a range of magnifications (20 nm magnification seen to the left).

Evaluation of the efficiency of *E. coli* OMVs on a pyrotosis reporter cell line

THP1-HMGB1-Lucia™ cells are designed to study inflammasome-mediated cell death, characterized by the release of cytoplasmic components upon cell membrane rupture. THP1-HMGB1-Lucia™ cells stably express a fusion protein, in which the C-terminus of the alarmin HMGB1 (High Mobility Group Box-1) is fused to Lucia luciferase. Levels of the fusion protein in the supernatant can be readily monitored using the detection reagent QUANTI-Luc™. When these cells are activated with *E. coli* OMVs there is a dose-dependent response seen when measuring luciferase activity. The optimal concentration of *E. coli* OMVs is approximately 20 µg/ml.

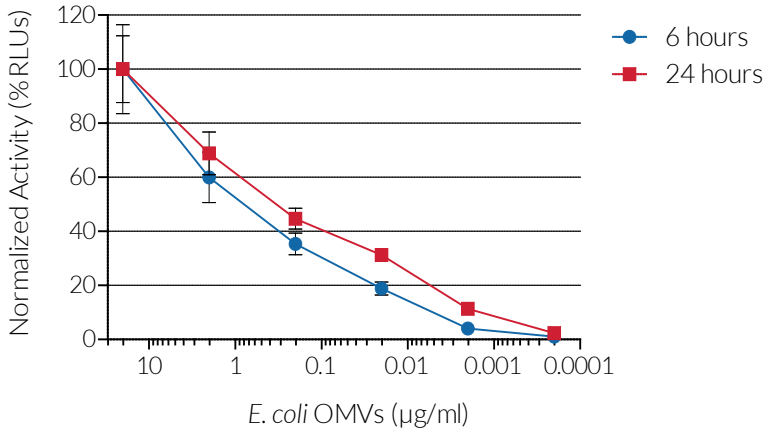

Figure 2: THP1-HMGB1-Lucia™ cells were incubated with 0.2 ng/ ml-20 µg/ml of *E. coli* OMVs for 6 (blue) and 24 hrs (red). After the incubation, Lucia luciferase activity was determined by measuring relative light units (RLUs) in a luminometer using the QUANTI-Luc™ detection reagent. Results are presented as normalized activity (percentage % RLUs).

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Evaluation of *in vitro* induction of the caspase 11-4/5 inflammasome

After endocytosis of OMVs into the cytosol, LPS on the surface of the OMVs activates the caspase 11-4/5 inflammasome leading to pyroptotic cell death and the release of cytokines such as IL-1β. Treatment of murine bone-marrow derived macrophages (BMDMs) with *E. coli* OMVs shows a clear dose-dependent response for the release of the biological marker for cellular cytotoxicity lactate dehydrogenase (LDH) (**Figure 3A**) and the pro-inflammatory cytokine IL-1β (**Figure 3B**). Additionally, knockout (KO)-caspase11 murine BMDMs show very minimal release of LDH and IL-1β, highlighting that caspase 11-4/5 inflammasome -induced cell death by OMVs is highly specific.

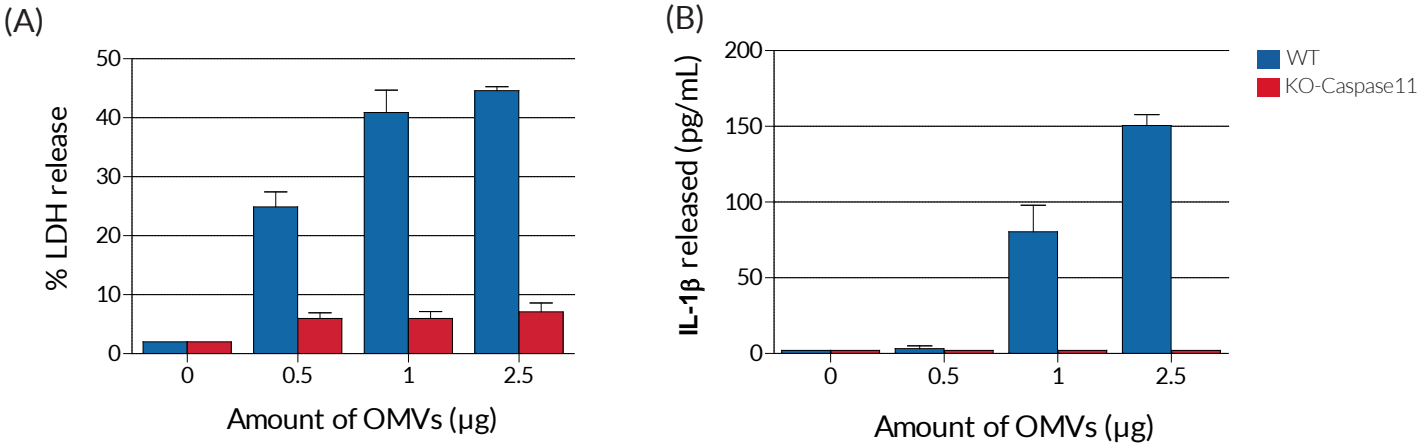

Figure 3: Bone marrow-derived macrophages (BMDMs) from wild type (WT) (blue) and caspase-11 knockout (KO) (red) mice were treated with a range of different concentrations (from 0.5 to 2.5 µg/250 000 cells) of *E. coli* OMVs (prepared in PBS) for 16-20 hours. After the incubation, the cell supernatants were used for both a **(A)** lactate dehydrogenase (LDH) assay and an **(B)** ELISA for measuring the release of IL-1β.

Evaluation of *in vivo* induction of the caspase 11-4/5 inflammasome

E. coli OMVs can also be used for *in vivo* studies that have been designed to mimic pathogenic bacterial infection of a host. These studies are essential to elucidate the role of the caspase 11-4/5 inflammasome in the outcome of the infection. We recommend to use *E. coli* OMVs InvivoFit™ grade, as these have been thoroughly tested to ensure sterility. Treatment of mice intraperitoneally with *E. coli* OMVs InvivoFit™ shows a dose-dependent response. Additionally, this response is completely dependent on the presence of gasdermin-D, a key player in pyrototic cell death.

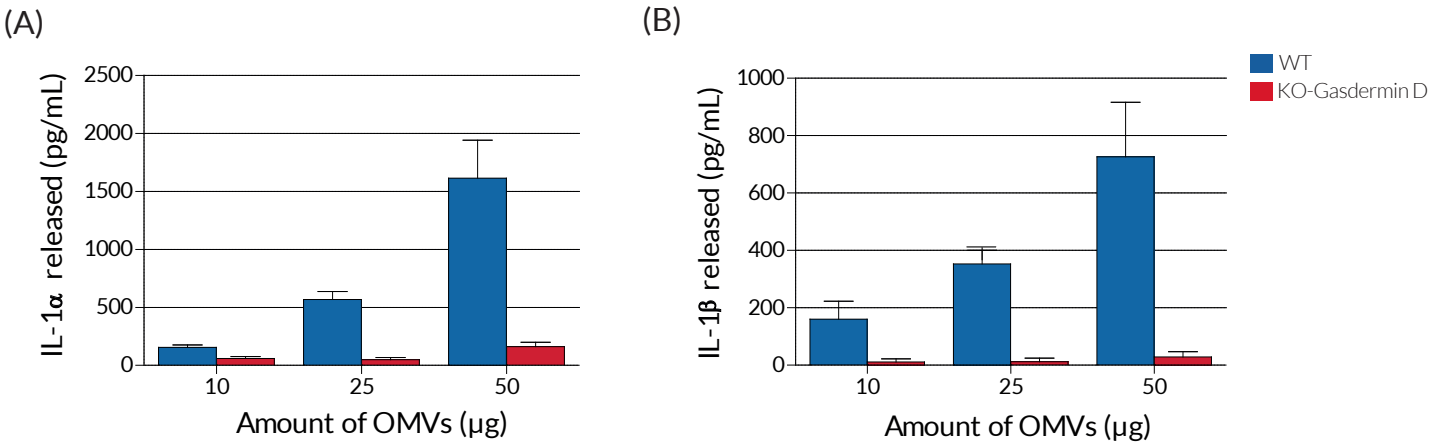

Figure 4: Wild type (WT) (blue) and Gasdermin-D knock out (KO) (red) mice (20-25g) were injected with 150 µg of poly IC (LMW) for the priming stage of inflammasome activation, and then left for 6 hours. Following this, a range of different concentrations from 10-50 µg of *E. coli* OMVs (prepared in PBS -/-) were injected intraperitoneally into the mice. After an additional 5 hours, peritoneal **(A)** IL-1α and **(B)** IL-1β cytokine levels were assayed using ELISA kits.

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