Pseudomonas aeruginosa membrane vesicles cause endothelial barrier failure and lung injury

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To the Editor:

Sepsis is a common cause of lung hyperinflammation and barrier failure, resulting in acute respiratory distress syndrome (ARDS). Despite antibiotics, mortality from bacterial sepsis increases in the developed world, suggesting injurious mechanisms beyond live bacteria. In addition to bacterial toxins, membrane vesicles (MVs) may present potential mechanisms of organ failure in sepsis. Bacterial MVs are extracellular vesicles formed from bacterial membranes [1] that can elicit systemic inflammatory responses, *e.g.* by inflammasome activation [2, 3]. Here, we tested whether MVs from a relevant sepsis pathogen, *Pseudomonas aeruginosa*, were sufficient to cause characteristic signs of acute lung injury (ALI), the preclinical analogue to ARDS, *in vitro* and *in vivo*.

MVs were collected from different bacterial strains. Equal colony forming units (CFUs) of *Pseudomonas aeruginosa* (PA14), *Escherichia coli* (Nissle 1917), *Staphylococcus aureus* (ATCC 6538) or *Lactobacillus casei* (MET-1) were inoculated into cultures for 24 h, with the exception of *L. casei*, which was cultured to 48 h. PA14 was additionally cultured for 6 h. Bacterial MVs were isolated by differential centrifugation and filtered [4]. MV size and number were determined by transmission electron microscopy (TEM) and flow cytometry [5]. PA14 lipopolysaccharide (LPS) content in culture (<1–3 $\mu g \cdot mL^{-1}$) and from isolated MVs (2 $\mu g \cdot mL^{-1}$) was measured (ToxinSensor Endotoxin Detection System, GenScript; Piscataway, NJ, USA). Passage 6 primary human microvascular pulmonary endothelial cells (HMPECs; C-12281 PromoCell, Heidelberg, Germany) were treated with 0–3.1×10^2 PA14 MVs per cell or LPS (2 $\mu g \cdot mL^{-1}$ of L9143; Sigma Aldrich, Oakville, ON, Canada) and trans-endothelial electrical resistance (TEER) was measured over 4 h [5]. MVs (8×10^{10} PA14 MVs per kg) in 200 µL of normal saline (NS) were transfused by tail vein into 12-week-old male BALB/c mice (Charles River, Canada) primed for 2 h with intraperitoneal NS or 2 $mg \cdot kg^{-1}$ LPS and MV-induced ALI was assessed after 4 h by a composite of lung wet-to-dry weight ratio, bronchoalveolar lavage fluid (BALF) protein, and a histological score [5]. For inflammasome activation, lung protein expression of NLRP3 and cleaved caspase-1 and plasma IL-1β levels were assessed. Animal experiments were approved by St. Michael's Hospital Animal Care Committee (#590).

Cultured bacteria yielded MVs of 20–200 nm in size (figure 1a and b). PA14 MVs were selected for further analysis based on PA14's known pathogenicity and the relative abundance of MVs produced by PA14 (figure 1c). Titration of PA14 MVs from 6 or 24 h culture to HMPECs revealed a loss of endothelial barrier integrity at 3.1×10^2 MVs per cell, while LPS at concentrations comparable to those in 3.1×10^2 MVs per cell caused no change in TEER (figure 1d and e). Loss of barrier function was not due to cytotoxicity, as neither LPS nor MVs caused lactate dehydrogenase release from HMPECs (figure 1f). Next, we probed whether PA14 MVs could elicit septic ALI in mice primed with a small dose of LPS. Mice exposed to LPS then MVs showed increased lung wet-to-dry weight ratio, BALF protein content and histological characteristics of ALI, while LPS or MVs alone had little or no effect (figure 1g–j). Plasma IL-1β levels, as well as lung protein expression of NLRP3 and cleaved caspase-1, were elevated in LPS+MV treated mice, indicating inflammasome activation (figure 1k–m).

Sepsis affects an estimated 49 million people and causes 11 million deaths annually [6]. Despite antibiotics, sepsis may advance to multi-organ failure and ARDS even after blood cultures become negative, suggesting excessive inflammation and/or circulating bacterial products as drivers of disease

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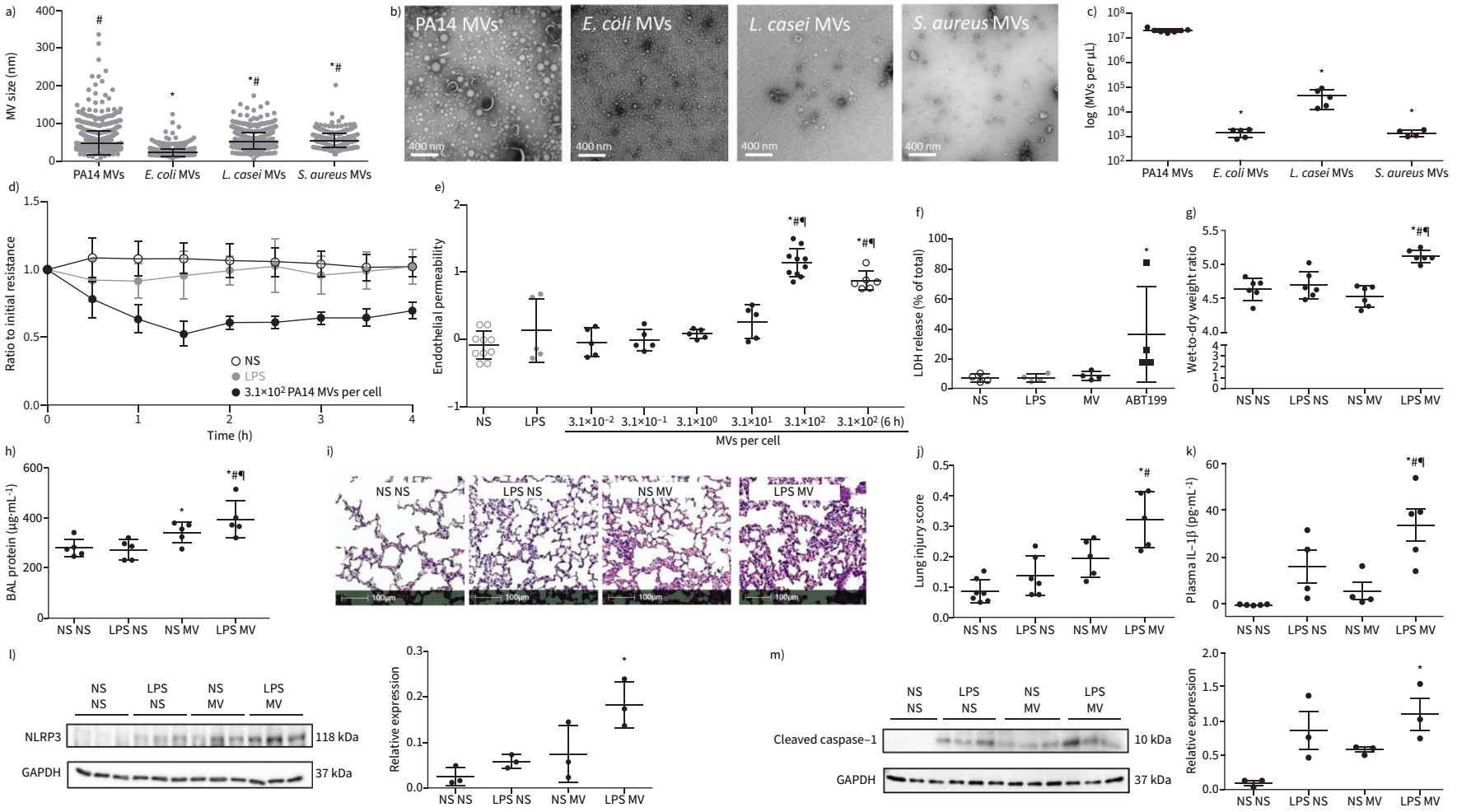

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FIGURE 1 Bacterial *Pseudomonas aeruginosa* (PA14) membrane vesicles (MVs) mediate acute lung injury (ALI). a) Group data show bacterial MV size as determined by transmission electron microscopy (TEM). MVs were sized from 15 (*P. aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*) or 40 (*Lactobacillus casei*) TEM images from three biological replicates of each bacterium by measuring MV diameters using ImageJ. *: statistical significance compared with PA14 MVs; #: statistical significance compared with *E. coli* MVs (n=479–1015 MVs each). b) Representative TEM images for MVs isolated from (left to right) *P. aeruginosa* (PA14), *E. coli*, *L. casei* and *S. aureus.* Images were acquired using a FEI Tecnai F20 G2 microscope operating at 200 kV equipped with a 4 K Gatan camera. c) Group data for concentration of bacterial MVs determined by high sensitivity flow cytometry. MV samples were analysed by a BD FACS ARIA III SORP equipped with a 55 mW 405-nm laser, a 100-µm nozzle, a Fournier optical transformation unit, and a small particle detection module. Gating involved biexponential forward and side scatter plots in conjunction with light scatter; for side scatter polystyrene 800 nm beads served as upper limit and instrument noise as lower limit. MVs were enumerated by calculating the gravimetric consumption of MVs. *: statistical significance compared with PA14 MVs (n=4–7). d) Trans-endothelial electrical resistance (TEER) is shown as a ratio relative to baseline over 4 h in human primary microvascular pulmonary endothelial cells (HMPECs; 32 168±3337 cells per well) exposed to normal saline (NS), lipopolysaccharide (LPS) without MVs (at concentrations identical to those measured in 3.1×10^{2} PA14 MVs per cell), and 3.1×10^{2} PA14 MVs per cell. e) Group data for area under the curve of TEER measurements over 4 h for HMPEC treated with NS, LPS or 3.1×10^{-2}, 3.1×10^{-1}, 3.1×10^{0}, 3.1×10^{1} and 3.1×10^{2} MVs per cell from PA14 bacteria grown for 6 h (open black circles) or 24 h (solid black circles). Absolute TEER values (Ω cm^2) for NS, LPS as well as 3.1×10^{-2}, 3.1×10^{-1}, 3.1×10^{0}, 3.1×10^{1} and 3.1×10^{2} MVs per cell at time zero h were comparable (14.2±0.8, 13.8 ±0.4, 15.1±1.1, 14.1±1.6, 15.0±0.9, 13.3±1.4 and 13.3±1.0 for 24 h cultured PA14 MVs and 13.4±1.0 for 6 h cultured (3.1×10^{2} MVs per cell) PA14 MVs respectively; mean±SD from n=5 independent MV batches). *: statistical significance compared with NS; #: statistical significance compared with LPS; ¶: statistical significance compared with 3.1×10^{-2} to 3.1×10^{1} PA14 MVs per cell (n=5–10 each). f) HMPEC cell viability (lysis) monitored as lactate dehydrogenase (LDH) release into the supernatant (CyQUANT LDH Cytotoxicity Assay kit, Invitrogen, C20301). HMPECs were co-incubated for 4 h with NS, LPS (2 µg·mL^{-1}), MV (3.1×10^{2} PA14 MVs per cell) or the BCL-2 inhibitor ABT199, which served as positive control for cell death (25 µmol·L^{-1}, Tocris, Bristol, UK). *: statistical significance compared with NS, LPS and 3.1×10^{2} PA14 MVs per cell. g–m) Mice primed with intraperitoneal NS or LPS 2 h prior to tail vein infusion of 8×10^{10} PA14 MVs per kg or NS sham were monitored for 4 h prior to ALI endpoint analysis (n=3–7). g) Group data for lung wet-to-dry weight ratio from right lower lung lobes. *: statistical significance compared with NS/NS; #: statistical significance compared with LPS/NS; ¶: statistical significance compared with NS/MV. h) Group data for bronchoalveolar lavage (BAL) fluid total protein content from left lungs. *: statistical significance compared with NS/NS; #: statistical significance compared with LPS/NS; ¶: statistical significance compared with NS/MV. i) Representative images of haematoxylin- and eosin-stained sections from right upper lung lobes. j) Group data for semi-quantitative analysis of histological evidence for lung injury based on 20 fields per lung scored from 0 (no injury) to 1 (maximal injury). *: statistical significance compared with NS/NS; #: statistical significance compared with LPS/NS. k) Plasma IL-1β levels determined by ELISA. *: statistical significance compared with NS/NS; #: statistical significance compared with LPS/NS; ¶: statistical significance compared with NS/MV. l) Western blot and corresponding densitometric analysis of mouse lung tissue for NLRP3 (1:1000 AG-20B-0014-C100; Adipogen San Diego, CA, USA) with GAPDH (1:1000 sc-25778; Santa Cruz, Dallas, TX, USA) as loading control. *: statistical significance compared with NS/NS. m) Western blot and corresponding densitometric analysis of mouse lung tissue for cleaved caspase-1 (1:1000 recombinant anti-pro Caspase-1 + p10 + p12 antibody (EPR16883) (ab179515); Abcam, Cambridge, UK) with GAPDH as loading control. *: statistical significance compared with NS/NS. Group data are depicted as mean±SD. Statistical analysis used one-way analysis of variance followed by Tukey's test (GraphPad Prism; San Diego, CA, USA).

progression. Based on the emerging recognition of MVs as parts of the bacterial secretome and as carriers of virulence [7], we considered that MVs may play a direct role in the induction of ARDS/ALI in the absence of live bacteria. In line with previous studies, MVs of different bacterial species were on average ~50 nm in size [1, 8, 9]. MV release was not related to lysis of bacteria grown to the stationary phase as 1) supernatant analyses showed no release of bacterial RNA polymerase, 2) TEM revealed no indication of bacterial lysis or debris, and 3) TEER data could be replicated with MVs from the log phase of bacterial growth (6 h in figure 1e). PA14 produced considerably more MVs than other less-pathogenic bacteria grown under similar conditions. Considering the abundance of MVs formed by PA14 and the documented pathogenicity of this strain, we examined the potential contribution of PA14 MVs to ALI. Previously, studies have implicated MVs as inducers of immune cell activation and lung inflammation [2, 10] that may contribute to the induction of ALI. Similarly, bacterial MVs have been reported to reduce monolayer integrity in extrapulmonary epithelia [11]. Here, we show that PA14 MVs cause a dose-dependent loss of endothelial barrier integrity *in vitro*, and induce characteristics of ALI and inflammasome activation in LPS-primed mice *in vivo*. Although our analyses focused specifically on ALI, one may speculate that similar mechanisms may promote extra-pulmonary organ failure in sepsis. Consequentially, means to reduce or prevent MV release *e.g.* by peptidylarginine deaminase inhibitors, membrane peptidogylcans or cannabidiol [8, 9, 12], or to attenuate systemic inflammation in response to MVs [13], may prove beneficial for treating septic patients. The recognition that certain MVs can act as potential inducers of microvascular barrier failure and ALI should further be considered as potential caveat in current attempts to exploit MVs as vaccine candidates against pulmonary infections [7]. This concern relates especially to patients with pre-existing inflammatory conditions mimicking the scenario of our two-hit model, and to specific bacterial species such as PA14, while MVs from other species seem to be harmless, as indicated by the fact that approved MV-related vaccines are not associated with ARDS [14].

The present study has limitations. First, while the concentrations of MVs delivered in our model are in keeping with levels of circulating extracellular vesicles *in vivo*, their relevance for clinical sepsis remains to be shown. Second, we only examined the effects of PA14 MVs, yet in polymicrobial sepsis various MVs may have additive or even synergistic interactions which may change ALI outcomes. Additionally, our MVs were derived from cultured cells as opposed to generated within human hosts. Future studies are required to address the implications of where and how MVs are formed. Lastly, in our *in vivo* model MVs only caused modest ALI in mice primed by prior LPS. Such LPS priming generates a pro-inflammatory milieu on which ALI develops once a second hit, such as transfused blood products, mechanical ventilation or bacterial infection occurs, reflecting the multifactorial pathogenesis of ARDS. Yet *in vitro*, even MVs alone sufficed to induce endothelial barrier failure. Notably, MVs may themselves shuttle LPS and activate toll-like receptors (TLRs) on target cells, triggering immune responses such as inflammasome activation [1, 3]. In addition to such TLR-dependent effects [2, 15], MVs may also elicit TLR-independent responses [15], indicating that mechanisms other than LPS contribute to MV pathogenicity. Consistently, in the present study TEER effects of MVs could not be replicated by equal doses of LPS alone. Exact mechanisms of MV pathogenicity in addition to MVs acting as LPS shuttles/chaperones remain to be elucidated but may comprise 1) proteolysis as described for MVs of *Serratia grimesii*, 2) epigenetic regulation by transfer of small RNAs, 3) MVs containing the barrier-disruptive biolipid ceramide, and 4) inflammasome activation by MVs.

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