Online Supplement

Extracellular vesicles in sputum of children with cystic fibrosis pulmonary exacerbations

Ben-Meir E.[1,2], Antounians L.[3,4], Eisha S.[2], Ratjen F.[1,2], Zani A.[3,4,5], Grasemann H.[1,2]

1 Division of Respiratory Medicine, Department of Paediatrics, The Hospital for Sick Children, Toronto, Canada.

2 Programs in Translational Medicine, and

3 Developmental and Stem Cell Biology, Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada.

4 Division of General and Thoracic Surgery, The Hospital for Sick Children, Toronto, Ontario, Canada.

5 Department of Surgery, University of Toronto, Toronto, Ontario, Canada.

Methods

Nanoparticle tracking analysis (NTA) To quantify EVs and determine size distribution, volumes of 300 μL of homogenized sputum EVs were analyzed by the NanoSight LM10 system (NanoSight Ltd, Salisbury, UK). 30-second videos of EVs (number of experiments n = 3, number of videos n = 3) were collected, averaged, and analyzed using LM10 NTA equipped with a 65 mWatt 405 nm violet laser (NanoSight Ltd, Salisbury, UK). For size calibration, 100 nm polystyrene beads (Malvern Instruments, Saint-Laurent, Canada) were used as previously described [1]. At capture with sCMOS camera on NTA 3.1 (machine) Build 3.1.46 (software), the sample temperature was 22 °C.

Transmission electron microscopy (TEM) To assess EV morphology, samples were mixed with equal volumes of 4% paraformaldehyde in PBS and adhered to formvar-carbon coated copper grids as described below. These grids were formed with 0.5% formvar solution (Electron Microscopy Sciences, Hatfield, PA) from powder in ethylene dichloride. A glass slide was dipped in the formvar, allowed to dry, its edges scored then pushed into a water bath to float the film off the slide. The grids were then placed on the film and flipped out of the water with parafilm. The sheet of formvar coated grids were then placed in a Cressington carbon evaporator. EV samples were fixed again with 2% glutaraldehyde in PBS, contrasted with uranyl-oxalate (Electron Microscopy Sciences, Hatfield, PA) and embedded in methyl cellulose-UA. AFSC-EVs were visualized on a Tecnai 20 (FEI, Hillsboro, OR) at 25 kX to 100 kX magnification.

Expression of EV protein markers and protein cargo Western blot analyses of canonical EV markers [2] in protein extracts of EVs isolated from the sputum homogenates were performed, as well as from human mesenchymal stem cells as positive control. EV proteins were extracted by incubation of isolated EVs with tissue extraction buffer and proteinase inhibitor for 30 minutes

on ice. Samples were then centrifuged at 10,000 X g for 20 minutes, and supernatants containing the EV protein collected. Total protein content in the extracts was quantified by the Pierce Bradford assay (ThermoFisher Scientific, Waltham, MA). Western blotting was performed using antibodies directed against EV-specific proteins, as recommended [2], directed to CD63 and CD9 (both from System Biosciences, Palo Alto, CA; primary antibody: rabbit anti-human, 1:1,000 dilution; secondary antibody: goat anti-rabbit HRP, 1:10,000 dilution), and Flotillin-1 (FLOT1; BD Transduction Laboratories, San Jose, CA; primary antibody mouse anti-human, 1:1000 dilution; secondary antibody: goat anti-mouse HRP, 1:1,000 dilution). To ensure that the EV preparations did not include nuclear proteins (indicative of cellular contamination), we assessed anti-H3K27me3 (Diagenode, Denville, NJ; primary antibody rabbit anti-human, 1:1000 dilution; secondary antibody: goat anti-rabbit HRP, 1:1,000 dilution) expression as a control. Western blot analysis of proteins involved in CF airway inflammation included NE (R&D systems, Minneapolis, MN; primary antibody: mouse anti-human, 1:1,000 dilution; secondary antibody: goat anti-mouse HRP, 1:1,000 dilution), MPO (R&D systems, Minneapolis, MN; primary antibody: goat anti-human, 1:1,000 dilution; secondary antibody: rabbit anti-goat HRP, 1:1,000 dilution), and IL-8 (Santa Cruz Biotechnology, Dallas, TX; primary antibody: mouse anti-human, 1:500 dilution; secondary antibody: goat anti-mouse HRP, 1:1,000 dilution). 40µL of each EVs protein extract was reduced with 4X reducing agent and 10X sample buffer and loaded onto SDS-PAGE gels. PageRuler™ plus prestained protein ladder (ThermoFisher Scientific, Waltham, MA) was used. Proteins were transferred to polyvinylidene difluoride membranes, which were pre-blocked in 5% non fat milk in tris-buffered saline and Tween (TBST, Sigma Aldrich, St. Louis, MO), washed in TBST and incubated with primary and

secondary antibodies. Blots were visualized by enhanced chemo-luminescence (Pierce™, ECL Western Blotting Substrate, ThermoFisher Scientific, Waltham, MA).

Figure legends:

Figure E1. Neutrophil elastase (NE) and myeloperoxidase (MPO) EV content. Western blot with anti-MPO, anti-NE and anti-FLOT1 antibodies.

References:

1 McVey MJ, Spring CM, Kuebler WM. Improved resolution in extracellular vesicle populations using 405 instead of 488 nm side scatter. J Extracell Vesicles 2018; 7(1): 1454776. doi: 10.1080/20013078.2018.1454776.

2 Welsh JA, Goberdhan DCI, O'Driscoll L, et al. Minimal information for studies of extracellular vesicles (MISEV2023): from basic to advanced approaches. J Extracell Vesicles 2024;13(2): e12404. doi: 10.1002/jev2.12404.