DETAILED MATERIAL AND METHODS SECTION

Extracellular Vesicle Production and Isolation

All media and reagents for tissue culture were purchased from GIBCO (Life technologies Inc.). MLP29 cells are a murine liver-derived progenitor cell line¹. For EV production, MLP29 cells were plated to non-collagenized 150-mm dishes, at 15-30 million cells per dish. Cells were cultured in complete DMEM medium [Dulbecco's modified Eagle medium supplemented with 10% (v/v) foetal bovine serum (FBS), 0.1 mg/ml streptomycin and 100 units/ml penicillin (GIBCO, Life technologies Inc.)] for 24 h (MLP29 cells) at 37°C and 5% of CO₂, washed two times with Dulbecco's modified phosphate-buffered saline (PBS) and incubated 48 h (MLP29 cells) in 25 mM HEPEScontaining complete DMEM medium previously depleted of contaminating vesicles by overnight centrifugation at 110,000 x g². After incubation, media were collected and EVs were isolated as previously described ³; in brief, culture supernatant was centrifuged at 1500 x g for 10 min to remove lifted cells and cellular debris. The resultant supernatant was centrifuged at 10,000 x g and 100,000 x g for 30 min and 75 min, respectively. The resulting pellet was suspended in PBS, pooled, and again centrifuged at 100,000 x g for 75 min. The final pellet of EVs was suspended in PBS at 1/2000th of the original volume of the culture supernatant, and aliquots were stored at -80°C.

Neuraminidase treatment

To perform the neuraminidase treatment (generation of EVs termed "MLP-Neu), MLP29 EVs (100 ug) diluted in 1 ml of PBS, were treated 12 hours at 37°C with 2,3-2,6-2,8-neuraminidase (N2876-6U, Sigma Aldrich) at a concentration of 0.8 U.I./ ml. Another fraction of EVs was treated in the same conditions but without the addition of the enzyme (generation of EVs termed "MLP-No Treat"). Finally, the enzyme was removed by re-

isolation of EVs through a sucrose cushion purification 4 and ultracentrifugation at 100,000 x g. The resulting EVs (both MLP-Neu and MLP-No Treat) were suspended in 150 ul of PBS, and were submitted to subsequent radioactive labeling.

Characterization of EVs

Characterization of EVs were performed as previously ⁵. Briefly, cryo-electron microscopy were performed directly on EV preparations directly adsorbed onto glowdischarged holey carbon grids (QUANTIFOIL, Germany). Grids were blotted at 95% humidity and rapidly plunged into liquid ethane with the aid of VITROBOT (Maastricht Instruments BV, The Netherlands). Size distribution within EV preparations was analyzed by Nanoparticle Tracking Analysis (NTA) measuring the rate of Brownian motion using a NanoSight LM10 system, which is equipped with a fast video capture and particle-tracking software (Nanoof EVs - Sight, Amesbury, U.K.). For Western blotting, samples were incubated for 5 min at 37°C, 65°C and 95°C, and separated on 4-12% precasted gels (Life Technologies, Thermo Fisher Scientific). All proteins were detected under non-reducing conditions. Mouse monoclonal antibodies were purchased from the indicated vendors: anti-flotillin (clone 18) from BD Biosciences (Mountain View, CA, USA), anti-TSG101 (clone 4A10) from Abcam (Cambridge, UK). Mouse anti-Cox IV (clone 4D11-B3-E8) was purchased to Cell Signaling Inc (Massachusetts, USA), and Rat anti-Lamp I (clone 1D4B) was purchased through Developmental Studies Hybridoma Bank (Iowa, USA).

Radiochemistry

The radioiodination of EV preparations was carried out by electrophylic aromatic substitution on the tyrosine residues of proteins contained at the surface of the EVs. In brief, solutions of the EVs (concentration of protein = 400 ng /20 μ L) were incubated

with Na[¹²⁴I]I (37 MBq) (Perkin Elmer) in PBS (20 μ L, 0.5 M, pH = 7.4) for 2 hours at 25°C into a Iodination Tube (PierceTM Pre Coated Iodination Tubes (Thermo Scientific, USA)). Gentle periodic shaking was applied. When the reaction was finished, the crude was diluted with PBS solution (250 μ L, 0.01 M, containing NaCl 1 M, pH=7.4) and the resulting solution was transferred to a vial containing Na₂S₂O₃ (50 μ L, 0.1 M). Finally, the resulting solution was purified on a SephadexTM Column G-25 DNA grade (GE Healthcare, USA) using PBS (0.01 M, containing NaCl 1 M, pH=7.4) as the mobile phase. The eluted product was collected in 100 μ L fractions and those containing the highest concentration of radioactivity (see **Figure S1** for example of elution profile) were pooled, diluted with physical saline solution and used for *ex vivo/in vivo* experiments (*vide infra*). A sample of this solution was allowed to completely decay and the concentration of protein was determined using the Bradford test to quantify the eventual loss of EVs during the labeling process.

Labeling efficiency and radiochemical purity were determined by radio-thin layer chromatography (radio-TLC) using iTLC-SG chromatography paper (Agilent Technologies, CA, USA) and aqueous ethanol solution (85%) as the stationary and mobile phases, respectively. TLC plates were analyzed using a TLC-reader (MiniGITA, Raytest). Radiochemical stability of the radiolabelled EVs was assessed in vitro by incubation in physiologic saline solution at 37 °C for 72 hours. At this time point, samples were withdrawn and analyzed by radio-TLC using the same conditions as described for quality control. Radiochemical stability was directly calculated from chromatographic profiles.

Animals

Male mice weighing 22 ± 2 g (BALB/cJRj, 9 weeks, Janvier; see below for number of animals) were used. The animals were maintained and handled in accordance with the Guidelines for Accommodation and Care of Animals (European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes) and internal guidelines. All experimental procedures were approved by the ethical committee and the local authorities before conducting experimental work (Code: PRO-AE-SS-059).

PET scan procedures in mice

PET experiments (n=3 per administration route and EV preparation) were performed using an eXploreVista-CT small animal PET computerized tomography (PET-CT) system (GE Healthcare). Anesthesia was induced with 3% isoflurane and maintained by 1.5-2% of isoflurane in 100% O₂. ¹²⁴I-labeled EVs were administered using two different routes: intravenously (1.8 ± 0.5 MBq, 150μ L, corresponding to ca. 120 ng of protein; injected *via* one of the lateral tail veins) or into the hock (the lateral tarsal region just above the ankle in the left limb, 30μ L, 0.6 ± 0.2 MBq, corresponding to 40 ng of protein). A solution of [¹²⁴I]NaI in buffered saline solution was administered to a different set of animals (n=3 per i.v. administration and n=2 per intradermal administration) to obtain quantitative data on the biodistribution of the "free" radionuclide. In all cases, 40 min dynamic PET scans (frames: 4 x 10 s, 4 x 20 s, 4 x 2 min, 4 x 3 min, 3 x 6 min) were started immediately after administration of the labeled EVs. Static images were also acquired at t= 8, 24, 48 and 72 hours after administration in order to obtain complete time-activity-profiles. Two beds were defined in all cases to acquire whole body images. After each PET acquisition, a CT scan (X-Ray energy: 40 kV, intensity: 140 μ A) was performed for a later attenuation correction application in the image reconstruction. Random and scatter corrections were also applied to the reconstructed image (filtered back projection reconstruction algorithm), generating a 175x175x118 dimension image, with a 2 mm axial FWHM spatial resolution in the centre of the Field Of View (FOV).

For intravenous injection, PET-CT images of the same animal were co-registered and analyzed using PMOD image processing tool. Volumes of interest (VOIs) were placed on selected organs (namely: brain, lungs, liver, kidneys, bladder and thyroid gland), as well as, the heart in order to get an estimation of the concentration of radioactivity in blood. Time–activity curves (decay corrected) were obtained as cps/cm³ in each organ. Curves were transformed into real activity (Bq/cm³) curves by using a calibration factor, obtained from previous scans performed on a phantom (micro-deluxe, Data spectrum Corp.) under the same experimental conditions (isotope, reconstruction algorithm and energetic window).

Ex-vivo biodistribution in mice

After finishing the last imaging session, and without recovering from anesthesia, animals were sacrificed by perfusion using saline solution. Selected organs, including the lungs, heart, kidneys, spleen, testicles, liver, small intestine, brain, and thyroid gland were quickly removed, rinsed with purified water (18.2 M Ω -cm, obtained using a Thermo Scientific Barnstead NANOpure Diamond Water System, Thermo Fisher Scientific, Waltham, MA, USA) and measured in an automatic gamma counter (2470 Wizard, PerkinElmer). Urine and blood samples were also obtained just before perfusion. Part of the blood was processed to separate the plasma. A fraction of the plasma was counted in the gamma counter. For animals injected in the hock, left and right axilar, popliteal, and

inguinal lymph nodes (LNs) were counted. Complementary autorradiographic images of

the LNs were obtained using a BetaIMAGERTM system (Biospace Lab, Paris, France).

References:

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Figure S1. Radiolabelling profile of MLP29-secreted EVs. Amount of radioactivity in different fractions collected after purification of the radiolabelled EVs. Each fraction contains ca. 100 μ L. Notice that the radioactivity associated to EVs (named EVs) appears in different fractions that free iodine. Below the graph, we show the results of cryo-EM imaging and Western-blotting analysis of Cd81 of the pooled F6 to F11 fractions.



Figure S2. Characterization of EVs. Panel A shows NTA (the red line represents +/-SEM of three measurements).and cryo-electron micrograph (the bar represents 100 nm) of EVs obtained from MLP29 EVs Non treated, after cushion and before the labelling process. **Panel B** shows NTA and cryo-electron micrograph of EVs obtained from MLP29 EVs Neu (treated with neuroaminidase), after cushion and before the labelling process. The bar represents 100 nm. The **Panel C** shows some characteristic markers from EVs (Lamp1, Flotillin-1, Tsg101, Cd81) and non-EV markers (mitochondria marker, CoxIV). Notice that neuramidase treatment change the mobility of glycosylated Lamp1 protein during the electrophoresis.



Figure S3. Radio TLC analysis of crude reaction mixture (**a**) and purified ¹²⁴I-labeled EVs (**b**). Example shown for MLP29; all EVs presented similar chromatogram.





Figure S4. Accumulation of [¹²⁴I]NaI (white bars; named Control), [¹²⁴I]MLP29 neuraminidase treated-EVs (dark grey bars; named MLP-Neu) and [¹²⁴I]MLP29 EVs (light grey bars; named MLP-No treat), in different organs 72 hours after hock administration, measured *ex vivo* by dissection and gamma counting. Results are expressed as % of injected dose per gram of tissue.





Figure S5. PET-CT coronal images obtained at different time points after hock administration of [¹²⁴I]MLP-Neu EVs (a), [¹²⁴I]MLP-No Treat EVs (b) and [¹²⁴I]NaI (c). PET images have been co-registered with CT images of the same animals for accurate location of the radioactive signal.