

RESEARCH ARTICLE

Pharmacokinetics and biodistribution of extracellular vesicles administered intravenously and intranasally to *Macaca nemestrina*

Tom Driedonks¹ | Linglei Jiang¹  | Bess Carlson¹ | Zheng Han^{2,3} | Guanshu Liu^{2,3} | Suzanne E. Queen¹ | Erin N. Shirk¹ | Olesia Gololobova¹  | Zhaohao Liao¹ | Lyle H. Nyberg¹ | Gabriela Lima¹ | Liliia Paniushkina¹ | Marta Garcia-Contreras¹ | Kayla Schonvisky¹ | Natalie Castell¹ | Mitchel Stover¹ | Selena Guerrero-Martin^{1,4} | Riley Richardson¹ | Barbara Smith⁵ | Vasiliki Mahairaki⁸ | Charles P. Lai⁶ | Jessica M. Izzi¹ | Eric K. Hutchinson¹ | Kelly A. M. Pate^{1,4} | Kenneth W. Witwer^{1,7,8} 

¹Department of Molecular and Comparative Pathobiology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

²Russell H. Morgan Department of Radiology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

³F.M. Kirby Research Center, Kennedy Krieger Institute, Baltimore, Maryland, USA

⁴Division of Comparative Medicine and Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA

⁵Department of Cell Biology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

⁶Institute of Atomic and Molecular Sciences, Academia Sinica, Taipei, Taiwan

⁷Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

⁸The Richman Family Precision Medicine Center of Excellence in Alzheimer's Disease, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

Correspondence

Kenneth W. Witwer, Department of Molecular and Comparative Pathobiology, Johns Hopkins University School of Medicine, Baltimore, MD, USA.
Email: kwitwer1@jhmi.edu

Funding information

Michael J. Fox Foundation for Parkinson's Research, Grant/Award Number: 00900821; National Institute of Allergy and Infectious Diseases, Grant/Award Number: AI144997; NIH Office of the Director, Grant/Award Number: U42OD013117; National Institute on Drug Abuse, Grant/Award Number: DA047807; National Cancer Institute, Grant/Award Number: CA241694; National Institute of Mental Health, Grant/Award Number: MH118164; Ministry of Science and Technology, Grant/Award Number: 109-2628-B-001-032; Academia Sinica, Grant/Award Number: AS-CDA-109-M04

Abstract

Extracellular vesicles (EVs) have potential in disease treatment since they can be loaded with therapeutic molecules and engineered for retention by specific tissues. However, questions remain on optimal dosing, administration and pharmacokinetics. Previous studies have addressed biodistribution and pharmacokinetics in rodents, but little evidence is available for larger animals. Here, we investigated the pharmacokinetics and biodistribution of Expi293F-derived EVs labelled with a highly sensitive nanoluciferase reporter (palmGRET) in a non-human primate model (*Macaca nemestrina*), comparing intravenous (IV) and intranasal (IN) administration over a 125-fold dose range. We report that EVs administered IV had longer circulation times in plasma than previously reported in mice and were detectable in cerebrospinal fluid after 30–60 min. EV association with peripheral blood mononuclear cells, especially B-cells, was observed as early as 1-min post-administration. EVs were detected in liver and spleen within 1 h of IV administration. However, IN delivery was minimal, suggesting that pretreatment approaches may be needed in large animals. Furthermore, EV circulation times strongly decreased after repeated IV administration, possibly due to immune responses and with clear implications for xenogeneic EV-based therapeutics. We hope that our findings from this baseline study in macaques will help to inform future research and therapeutic development of EVs.

Tom Driedonks and Linglei Jiang contributed equally to this study.

This is an open access article under the terms of the [Creative Commons Attribution-NonCommercial-NoDerivs](https://creativecommons.org/licenses/by-nc-nd/4.0/) License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2022 The Authors. *Journal of Extracellular Biology* published by Wiley Periodicals, LLC on behalf of the International Society for Extracellular Vesicles.

KEYWORDS

extracellular vesicles, biodistribution, drug delivery, extracellular vesicles, macaques, nanomedicine, pharmacokinetics, therapeutics

1 | INTRODUCTION

Extracellular vesicles (EVs) are nano-sized vesicles produced by most or all cell types in multicellular organisms. EVs from specific cell types may also be harnessed as treatments for a wide range of human diseases and conditions, including cancer, inflammatory diseases, and tissue damage (Allan et al., 2020; Escudé Martínez de Castilla et al., 2021; Lener et al., 2015; Reiner et al., 2017). Furthermore, EVs may be loaded with therapeutic entities such as small molecule drugs (Pascucci et al., 2014), proteins (De Jong et al., 2019), (si)RNA (O'Loughlin et al., 2017; Usman et al., 2018), and CRISPR/Cas9 (Kim et al., 2017; Yao et al., 2021). The EV lipid bilayer protects its cargo from degradation and reduces off-target effects compared with non-encapsulated therapeutics (Witwer & Wolfram, 2021). Moreover, EVs may be engineered for retention by specific sites in the body, including brain, through the display of cell-specific surface motifs, usually proteins or peptides (Alvarez-Erviti et al., 2011; Noren Hooten et al., 2020; Pham et al., 2021). Since EVs occur naturally in the bloodstream and tissues, EV administration is thought to be safe and has reportedly elicited few toxic or inflammatory effects (Saleh et al., 2019; Zhu et al., 2017). Although EVs are thus thought to be promising novel therapeutic tools, many questions remain about dosing, administration route, and pharmacokinetics.

To date, most pre-clinical studies have addressed the biodistribution and pharmacokinetics of EVs using mouse models (Betzer et al., 2017; Gupta et al., 2020; Kooijmans et al., 2016; Lai et al., 2014; Lázaro-Ibañez et al., 2021; Wiklander et al., 2015; Wu et al., 2020). Most studies have reported that EVs accumulate rapidly in the liver and spleen (Gupta et al., 2020; Kooijmans et al., 2016; Lázaro-Ibañez et al., 2021; Wiklander et al., 2015), and sometimes lung (Wu et al., 2020) and kidneys (Lai et al., 2014). Additionally, EVs were found to have short circulation times in mice (Gupta et al., 2020; Kooijmans et al., 2016; Lai et al., 2014; Lázaro-Ibañez et al., 2021; Wu et al., 2020). It is not well understood how EV administration route affects circulation time and biodistribution of EVs, since only a few studies have directly compared administration routes. In one study, EVs were administered to mice by intravenous (IV), subcutaneous, and intraperitoneal routes (Wiklander et al., 2015). Compared with IV administration, subcutaneous and intraperitoneal administration resulted in lower EV uptake in liver and spleen, and higher uptake in the gastrointestinal tract and pancreas. Another study reported that intranasal (IN) administration of EVs resulted in improved brain targeting compared with IV administration (Betzer et al., 2017). While these mouse studies provide invaluable information on the biodistribution and therapeutic effects of EVs, results obtained in rodents may have limited translatability to human physiology (Leenaars et al., 2019). Specific therapeutic effects of EV have been tested in sheep (Ophelders et al., 2016), and pigs (Pötz et al., 2018), but pharmacokinetics studies on EVs in larger animals are scarce (Escudé Martínez de Castilla et al., 2021; Kang et al., 2021).

Here, we investigated the pharmacokinetics of EVs in a non-human primate (NHP) model, the pig-tailed macaque (*Macaca nemestrina*). Large animal models allow repeated sampling from the same animal, in addition to sampling of multiple biofluids at the same time, and at volumes that cannot be obtained from small rodents. NHP are also physiologically similar to humans and are the best and in some cases only models of human disease. For example, NHP are exceptionally valuable to achieve better understanding of human immunodeficiency virus (HIV) disease progression and treatment, including assessment of HIV cure strategies and central nervous system (CNS) disease (Beck et al., 2018). Indeed, the study reported here is a prerequisite to trials of EV-associated transcriptional activators as latency-reversal agents for HIV.

We used two relatively novel EV reporters: palmGRET, which is a palmitoylated enhanced green fluorescent protein (EGFP)-Nanoluciferase fusion protein (Wu et al., 2020), and MemGlow 700, a near-infrared self-quenching lipid dye (Collot et al., 2019). PalmGRET enables highly sensitive detection of EVs by emission of bioluminescence in the presence of a furimazine substrate (Wu et al., 2020). MemGlow dye emits fluorescence in the near-infrared range, where autofluorescence is generally reduced. Furthermore, this dye has been previously used to track tumour EVs in live zebrafish (Hyenne et al., 2019).

For route of delivery, we compared IV administration, the most widely used route for systemic drug delivery (Escudé Martínez de Castilla et al., 2021), with IN administration, which has been reported to achieve EV cargo delivery to the rodent brain (Betzer et al., 2017). It has been speculated that IN-administered small particles are transported by olfactory receptor neurons, which connect the nasal cavity and olfactory bulb to the brain (Ali et al., 2010). For each administration route, we assessed how different EV doses affect the half-life of EVs in plasma and cerebrospinal fluid (CSF) of pigtailed macaques. Additionally, we measured the uptake of EVs by different subsets of peripheral blood mononuclear cells (PBMCs) shortly after administration. Furthermore, we compared the biodistribution of EVs in different organs of both macaques and mice. We found that the administration route strongly affected EV pharmacokinetics and tissue distribution. Repeated IV administration of EVs resulted in an accelerated blood clearance (ABC) phenomenon, potentially *via* EV-specific IgGs. To our knowledge, this is the first reported study on the pharmacokinetics of EVs in macaques, which we trust will inform future studies on therapeutic applications of EVs.

2 | MATERIALS AND METHODS

2.1 | Cells and plasmids

Expi293F cells (Thermo Fisher) were maintained in Expi293 medium (Gibco, Waltham, MA) in vented shaker flasks on a shaker platform maintained at 125 rpm in a humidified 37°C incubator with 8% CO₂. The pLenti-palmGRET reporter (Wu et al., 2020) was provided by C.P. Lai (Addgene 158221), and endotoxin-free plasmid DNA megapreps were prepared by Genewiz (Genewiz, South Plainfield, NJ). For each EV production batch, 3 × 1 L shaker flasks were seeded with a total of 750 ml of cell suspension at 3E6 cells/ml. Cells were transfected using Expifectamine (Thermo Fisher) according to the manufacturer's instructions, with 150 µg pDNA (0.6 µg pDNA per ml of culture) and 480 µl of Expifectamine per flask. One day after transfection, 1.2 ml Enhancer 1 and 12 ml Enhancer 2 were added to each flask. Cultures were harvested 3 days after transfection. Transfection efficiency was checked on a Nikon Eclipse TE200 fluorescent microscope, cells were counted on a hemacytometer and tested for viability by Trypan blue exclusion (Thermo Fisher). Cell densities and viability of different batches at harvest are found in Table S1.

2.2 | EV separation and fluorescent labelling

Cells were removed from conditioned medium by centrifugation at 1000 × *g* for 20 min at 4°C. Supernatant was centrifuged again at 2000 × *g* for 20 min and filtered through a 0.22-µm bottle-top filter (Corning, NY). EVs were concentrated to 75 ml by tangential flow filtration (TFF) using two 100 kDa Vivaflow 50R cassettes (Sartorius, Goettingen, Germany) run in parallel on a Cole-Parmer Masterflex L/S peristaltic pump operated at 100 rpm. The concentrated EVs were fluorescently labelled by adding 200 nM MemGlow 700 nm dye (Collot et al., 2019) (Cytoskeleton Inc., Denver, CO) and incubating at RT for 30 min. EVs were concentrated further on Amicon 15 Ultra RC 100 kDa filters (Millipore Sigma, Darmstadt, Germany), spun for 20–30 min at 4000 × *g*. The concentrate was loaded onto a qEV10 70 nm size-exclusion chromatography (SEC) column (Izon, Medford, MA) run with Dulbecco's phosphate buffered saline (DPBS, PBS) (Gibco, Waltham, MA), after discarding the void volume, 5-ml fractions were collected. EV-enriched fractions 1–4 were pooled together and were concentrated again on Amicon 15 Ultra RC 100 kDa filters, spun for 20–30 min at 4000 × *g*. EVs were aliquoted and stored in LoBind tubes (Eppendorf, Bochum, Germany) at –80°C.

2.3 | Trichloroacetic acid (TCA) precipitation

For CD47 immunoblotting, EVs were precipitated using TCA. 230 µl Expi293F EVs were thawed from storage at –80°C, after which 2 µl of 2% sodium deoxycholate (Sigma Aldrich) was added. Tubes were vortexed and incubated at room temperature for 15 min. 23 µl of 100% TCA (Sigma Aldrich) was then added, samples were centrifuged at 14,000 rpm for 15 min at 4°C, and the supernatant was aspirated. Precipitated pellets were washed once with 500 µl ice-cold acetone and centrifuged again at 14,000 rpm for 15 min at 4°C. After removal of supernatant, samples were air-dried for 10 min and pellets dissolved in sample buffer for immunoblotting.

2.4 | Immunoblotting

Transfected Expi293F cell pellets were lysed in PBS + 1% Triton-X100 and Complete protease inhibitor tablets (Roche, Mannheim, Germany) for 15 min on ice. Nuclei were spun down for 15 min at 14,000 rpm in a tabletop centrifuge at 4°C. Cell lysate, final EV isolate and individual SEC fractions were mixed with 4× TGX sample buffer (Bio-Rad, Hercules, CA) under non-reducing conditions (except for CD47 blotting, under reducing conditions), boiled for 5 min at 100°C, and subjected to PAGE gel electrophoresis on a 4%–15% Criterion TGX Stain-Free Precast gel (Bio-Rad). Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane using the iBlot2 system (Thermo Fisher) run on program p0. After 1 h of blocking in 5% Blotting-Grade Blocker (Bio-Rad) in PBS + 0.05% Tween-20 (PBS-T), blots were incubated overnight at 4°C with the following primary antibodies in blocking buffer: Rabbit-anti-Calnexin (1:1000, ab22595, Abcam), mouse-anti-CD63 (1:3000, #556016, BD Biosciences), mouse-anti-CD9 (1:3000, #312102, BioLegend), rabbit-anti-Tsg101 (1:2000, ab125011, Abcam), or rabbit-anti-human CD47 (1:500, Cusabio Technology, CSB-PA005993). Blots were washed 3x with PBS-T and incubated for 1 h at room temperature with secondary antibodies mouse-IgGk-BP-HRP (sc-516102, SantaCruz) or mouse-anti-rabbit-IgG-HRP (sc-2357, SantaCruz) diluted 1:10,000 in blocking buffer. After washing 3× with PBS-T and 2× with PBS, SuperSignal West Pico PLUS Chemiluminescent Substrate (Pierce, Rockford, IL) (or, for CD47, SuperSignal West Femto Maximum Sensitivity) was used for detection on an iBright FL1000 (Thermo Fisher) in chemiluminescence mode.

2.5 | Nanoparticle tracking analysis

ZetaView QUATT-NTA Nanoparticle Tracking Video Microscope PMX-420 and BASIC NTA-Nanoparticle Tracking Video Microscope PMX-120 (ParticleMetrix, Inning am Ammersee, Germany) were used for particle quantification in scatter mode. The system was calibrated with 100 nm PS beads, diluted 1:250,000 before each run. Capture settings were sensitivity 75, shutter 100, minimum trace length 15, cell temperature was maintained at 25°C for all measurements. Samples were diluted in 0.22 μ m filtered PBS to a final volume of 1 ml. Samples were measured by scanning 11 positions twice, recording at 30 frames per second. Between samples, the system was washed with PBS until no particles remained. ZetaView Software 8.5.10 was used to analyse the recorded videos with the following settings: minimum brightness 20, maximum brightness 255, minimum area 5, and maximum area 1000.

2.6 | Transmission electron microscopy

10 μ L sample was adsorbed to glow-discharged carbon-coated 400 mesh copper grids by flotation for 2 min. Grids were quickly blotted and rinsed by flotation on three drops (1 min each) of Tris-buffered saline. Grids were negatively stained in two consecutive drops of 1% uranyl acetate (UAT) with tylose (1% UAT in deionized water (diH_2O), double filtered through a 0.22- μ m filter), blotted, then quickly aspirated to cover the sample with a thin layer of stain. Grids were imaged on a Hitachi 7600 transmission electron microscopy (TEM) operating at 80 kV with an AMT XR80 CCD (8 megapixel).

2.7 | Single Particle Interferometric Reflectance Imaging Sensing (SP-IRIS)

EVs diluted 1:100 in DPBS were diluted 1:1 in incubation buffer (IB) and incubated at room temperature on ExoView R100 (NanoView Biosciences, Brighton, MA) chips printed with anti-human CD81 (JS-81), anti-human CD63 (H5C6), anti-human CD9 (HI9a) and anti-mouse-IgG1 (MOPC-21). After incubation for 16 h, chips were washed with IB 4 times for 3 min each under gentle horizontal agitation at 500 rpm. Chips were then incubated for 1 h at RT with fluorescent antibodies anti-human CD81 (JS-81, CF555) and anti-human CD63 (H5C6, CF647) diluted 1:200 in a 1:1 mixture of IB and blocking buffer. Anti-human CD9 was not used, since the third wavelength was needed for the palmGRET reporter protein. The chips were subsequently washed once with IB, three times with wash buffer, and once with rinse buffer (all washes 3 min at 500 rpm agitation). Chips were immersed twice in rinse buffer for 5 s and removed at a 45° angle to remove the liquid from the chip. All reagents and antibodies were obtained from NanoView Biosciences (#EV-TETRA-C). All chips were imaged in the ExoView scanner (NanoView Biosciences) by interferometric reflectance imaging and fluorescent detection. Data were analysed using ExoView Analyzer 3.0 software. Fluorescent cutoffs were as follows: CF555 channel 300 a.u., CF488 channel 410 a.u., CF647 channel 300 a.u., allowing < 1% of particles above background in the isotype control. Fluorescent counts from multiple measurements were normalized against the total fluorescent particle count.

2.8 | Imaging flow cytometry

EVs diluted 1:10 in DPBS were quantified by imaging flow cytometry on an Amnis ImageStreamX MkII instrument (Amnis Corp, Seattle, WA) on low flow speed, using a 60x objective and extended depth of field (EDF) option enabled. EGFP signal was collected in channel 2 (480–560 nm filter), MemGlow 700 nm signal was collected in channel 5 (642–745 nm filter), and sideward scatter (SSC) was collected in channel 6 (745–800 nm filter). Negative controls recommended by the MiFlowCyt-EV consortium (Welsh et al., 2019) were included in all measurements: buffer only control, free dye control (200 nM MemGlow700 in PBS), single stained EVs (palmGRET only, and MemGlow700 labelled EV from untransfected Expi293F cells). Serial dilutions were included to ensure measurement in the linear range of the instrument, and to rule out swarm effects. Data were analysed using Amnis IDEAS software v6.2.

2.9 | MACSPlex surface marker characterization

EV surface markers were characterized *via* MACSPlex (Miltenyi Biotec, #130-122-209), following manufacturer's instructions. In brief, 120 μ L EV sample was mixed with 15 μ L MACSPlex capture beads and incubated in a shaker at room temperature overnight. Next, 500 μ L MACSPlex buffer was added, centrifuged at 3000 $\times$ g for 5 min, and 500 μ L supernatant was removed. 15 μ L MACSPlex detection reagent cocktail was added (anti-CD9, -CD63 and -CD81, as included in kit), and samples were incubated for 1 h. After two washes in 500 μ L assay buffer, data were collected on a BD LSR Fortessa flow cytometer. For flow cytometric data

analysis, median signal intensity was used for relative quantification of surface markers. PBS (negative control) signal was subtracted from the median signal intensity of each sample. The median signal intensity of CD9 beads was used to normalize the abundance of other surface markers.

2.10 | In vivo administration

2.10.1 | Mice

Balb/cJ mice (Jackson Laboratories, 8–12 weeks, female) were injected intraperitoneally with 100 μ l fluorofurimazine (Su et al., 2020) under sedation with isoflurane. Subsequently 1.5E11 EVs were administered by tail-vein injection or intranasal instillation. Bioluminescent imaging was performed on a Caliper IVIS SpectrumCT *In Vivo* Imaging System (Caliper Life Sciences, Hopkinton, MA, USA). Images were taken every 30 s (exposure time = 30 s) in luminescence mode. Mice were perfused with PBS *via* cardiac puncture 40 min after EV administration, organs were harvested and imaged *ex vivo* in fluorescence mode (ex 689, em 713), and in bioluminescence mode while immersed in NanoGlo substrate (diluted 1:50, Promega #N1110). Tissues were homogenized in 1 ml N-PER (brain) or T-PER (other organs) + Complete Mini protease inhibitor cocktail tablet (Roche, Mannheim, Germany) in FastPrep Lysing Matrix D tubes on a FastPrep homogenizer. Homogenates were centrifuged for 5 min at $10,000 \times g$ at 4°C, supernatant was taken off and used in NanoGlo and BCA assays (Pierce, Rockford, IL). Mice experiments were performed under approval of the Johns Hopkins University Animal Care and Use Committee (ACUC), study number M018M145.

2.10.2 | Macaques

Juvenile pigtailed macaques (*M. nemestrina*, male, 3–4 years old) were obtained from the JHU pigtail colony. EVs (7E10 EVs, with five-fold increments for all subsequent doses) were administered by intravenous injection into the small saphenous vein or by intranasal instillation through a catheter under ketamine sedation (10 mg/kg body weight). Macaques remained sedated during blood/CSF collection for the first hour by administering ketamine in 10–20 mg increments, and were sedated again with 10 mg/kg body weight at the 4 and 24 h timepoints. After each EV dose and biofluid collection, macaques were given 2 weeks to recover until the next EV administration, for a total of five doses. To assess the turnover rate of EVs in CSF, we administered 3E10 EVs *via* intrathecal injection into the subarachnoid space, and collected blood and CSF after 0, 0.5, 1, 3, 6, and 24 h. To assess retention of EVs in the nasal cavity, nasal lavage was performed 1 h after intranasal EV administration using 10 ml PBS, spun down at $2000 \times g$ for 10 min. Macaque experiments were performed under approval of the Johns Hopkins University Animal Care and Use Committee (ACUC).

At each timepoint, 500 μ l blood was collected by venipuncture into tubes containing 100 μ l ACD. Blood was processed within 1.5 h after collection by centrifugation for 5 min at $800 \times g$, plasma was taken off and stored directly at -80°C. To collect PBMC from the same sample, the blood cell pellet was reconstituted to 1 ml with DPBS, carefully layered onto a 1 ml Histopaque-1077 cushion (Sigma, St. Louis, MO), and centrifuged for 30 min at $400 \times g$ without brake. After discarding the supernatant layer, the PBMC-containing interphase was transferred to a new tube. PBMCs were washed twice by adding 1 ml PBS, centrifuging for 10 min at $250 \times g$, and discarding the supernatant. The final PBMC pellet was taken up in 200 μ l lysis buffer (PBS + 1% Triton-X100 + Complete Mini protease inhibitor cocktail tablet (Roche, Mannheim, Germany)), lysed on ice for 15 min. Nuclei were removed by centrifuging 15 min at $16,000 \times g$ at 4°C. Protein concentration was determined by BCA assay (Pierce, Rockford, IL). Per timepoint, 500 μ l CSF was collected which was centrifuged for 10 min at $2000 \times g$ to remove cells. The supernatant was taken off and stored directly at -80°C. After the final dose, macaques were euthanized using Nembutal (20–30 mg/kg) and perfused with PBS. Organs were excised and snap-frozen at -80°C. Parts of the spleen and bronchial lymph nodes were processed directly for flow cytometry (see below for details).

2.11 | Flow cytometry

PBMCs were immunolabeled directly in whole blood with fluorescent antibodies. 100 μ l whole blood was added to antibody cocktails (mo-anti-CD159a-PE, Beckman Coulter, cat# IM3291U, dil 1:30; mo-anti-CD4-PerCP/Cy5.5 BD Biosciences 552838 dil 1:7.5; mo-anti-CD20-e450 Thermo Fisher, cat# 48-0209-42 dil 1:60; mo-anti-CD3-V500 BD, cat# 560770, dil 1:30; mo-anti-CD8-BV570, BioLegend, cat# 301038, dil 1:60; mo-anti-CD14-BV650, BioLegend, 563419, dil 1:30), briefly vortexed and incubated at room temperature for 20 min. Next, red blood cells were lysed by addition of 2 ml RBC lysis buffer (ACK lysing buffer 0.83% NH_4Cl , 0.1% KHCO_3 , 0.03% ethylenediaminetetraacetic acid, EDTA) and incubation at room temperature for 10 min. Tubes were centrifuged at $400 \times g$ for 5 min and supernatant was discarded. Next, 2 ml PBS was added and tubes were centrifuged again at $400 \times g$ for 5 min. Supernatant was discarded, labelled PBMCs were carefully resuspended in 500 μ l PBS and measured

directly on a BD LSR Fortessa flow cytometer. As negative controls, we included whole blood collected before the injection of EVs, and fluorescence minus one (FMO) controls for CD159a and CD4 to allow for accurate gating of GFP, PE and PerCP/Cy5.5 fluorescence. Cells from the spleen and bronchial lymph nodes were mechanically isolated from freshly excised tissues using 18-gauge needles in cold RPMI and passed through a 100- μ m cell strainer. Spleen cells were lysed using RBC lysis buffer. 10^6 cells were resuspended in 100 μ l of PBS 2% FBS solution for antibody staining using the same antibody cocktail as the PBMCs.

2.12 | Serum stability assay

3 ml blood was collected from the vein into a serum collection tube. The blood was placed at room temperature for 15 min. The coagulated blood was spun at $1000 \times g$ for 15 min at 4°C. EVs were spiked into the serum at a dose similar to that achieved in the *in vivo* experiments, scaled down from an estimated 500 ml of macaque plasma to 50 μ l serum. Thus, 4.7 μ l palmGRET EVs (dose 1:7.0E+06 EVs, dose 2:4.0E+07 EVs, dose 3:2.0E+08 EVs, dose 4:9.0E+08 EVs) were spiked into 50 μ l macaque serum ($n = 2$), and incubated for 0, 1, 4 and 24 h at 37°C. EV stability was measured by nanoluciferase assay as described below.

2.13 | Nanoluciferase assays

Purified EV samples and SEC fractions were diluted 20-fold in PBS and were loaded into a white plastic 96 well plate, 50 μ l per well, in duplicates. Biofluid samples, PBMC lysates and tissue homogenates, were loaded undiluted at 50 μ l per well in duplicates. Nano-Glo substrate (furimazine, Promega, Madison, WI) was diluted 1:50 in assay buffer according to the manufacturer's instructions. 50 μ l diluted Nano-Glo reagent was added per well, and bioluminescence was measured immediately on a Fluoroskan Ascent plate reader (software v6.2) in bioluminescence mode, integration time 20 ms.

2.14 | Antibody and cytokine measurements

Total IgG levels were measured in macaque plasma using a human IgG ELISA kit (Abcam, ab195215), which is cross-reactive with macaque IgG. Plasma samples, collected 24 h after EV administrations, were diluted 70,000 $\times$ in sample diluent, and IgG levels were measured following the manufacturer's instructions. The plate was measured on a BioRad plate reader at 450 nm.

EV-specific antibody levels were measured by ELISA. PalmGRET EVs were lysed in RIPA buffer (Thermo Fisher # 89900) + protease inhibitors on ice for 30 min. ELISA plates were coated with EV lysates (equivalent of 1.8×10^8 EVs/well) overnight at 4°C. Plates were aspirated and blocked with 1 $\times$ SuperBlock T20 blocking buffer (Thermo Fisher #37536) for 1 h at room temperature. After four washes with Wash buffer (PerkinElmer, from p24 ELISA kit NEK 050/050A/050B), 100 μ l of macaque plasma, diluted in 1 $\times$ Assay buffer (Thermo Fisher DS98200), was added to the wells and incubated at 37°C for 2 h. After four washes, diluted anti-monkey-IgG-HRP (1:10,000, LifeSpan Biosciences LS-C56745) or anti-monkey-IgM-HRP (1:65,000, LifeSpan Biosciences LS-C61207) were added and incubated at 37°C for 1 h. After four washes, 100 μ l 1-Step Ultra-TMB-ELISA solution (Thermo Fisher # 34028) was added to the plate and incubated in the dark for 6 min, after which the reaction was stopped using 0.5N H_2SO_4 . The plate was measured on a BioRad plate reader at 450 nm.

Cytokine levels in macaque plasma were measured using the LEGENDplex NHP Inflammation Panel in filter plates (BioLegend #740332). Plasma samples were diluted 1:4 in assay buffer. Cytokine levels were measured following manufacturer's instructions using a BD LSR Fortessa flow cytometer.

2.15 | Statistics and EV half-life calculation

Statistical differences were determined by one-way ANOVA with Tukey's post-hoc test in GraphPad Prism 9.1, differences with $p < 0.05$ were considered to be statistically significant. EV half-life in biofluids was determined by linear regression of the nanoluciferase signal versus time on a log-lin chart. EV half-life was calculated from the slope of the regression line: $t_{1/2} = \log(2)/\text{slope}$

2.16 | Availability of protocols

Procedural details have been submitted to the EV-TRACK knowledgebase and are available under EV-TRACK ID: EV210210 (Van Deun et al., 2017). High resolution flow cytometry experiments were performed following the MIFlowCyt-EV guidelines (Welsh et al., 2019).

3 | RESULTS

3.1 | Production, separation, and general characterization of labelled EVs

To study the pharmacokinetics and biodistribution of EVs in larger animals such as macaques, highly sensitive reporters are required that can be detected with a high signal-to-background ratio. Therefore, we used two state-of-the-art EV reporters: the near-infrared, self-quenching membrane dye MemGlow 700 (Collot et al., 2019), which was previously used to track tumour EV in live zebrafish (Hyenne et al., 2019), and the dual reporter protein palmGRET (palmitoylated EGFP-Nanoluciferase), which was previously used to track tumour EVs in mice (Wu et al., 2020). We transiently expressed palmGRET in Expi293F suspension cells (Figure S1A) and harvested the conditioned culture medium 3 days later. Cells and debris were removed from culture medium by centrifugation and filtration. The EV-containing culture medium was concentrated tenfold by TFF, a technique which is increasingly used for volume reduction during large-scale processing of EVs (Busatto et al., 2018; McNamara et al., 2018). The EV concentrate was subsequently labelled with MemGlow 700, concentrated further by ultrafiltration, and subjected to SEC to separate EVs from free dye and non-EV-associated proteins (Figure S1B).

To satisfy the MISEV criteria (Théry et al., 2018), we extensively characterized individual SEC fractions and pools by microBCA, SDS-PAGE and Western blot. A small protein peak was observed in SEC fractions 1–4 (Figure S1C and D) which was positive for EV markers CD63, CD9, and TSG101 but devoid of ER marker calnexin (Figure 1a), indicating that EVs were isolated with minimal contamination by other cellular material. TEM and nanoparticle tracking analysis (NTA) of pooled fractions 1–4 confirmed the presence of EVs with expected morphology and an average diameter (by NTA) of 122.6 nm (SD $\pm$ 9.9 nm) (Figure 1b and c). We performed further EV characterization using a bead-capture flow cytometry assay (Wiklander et al., 2018), which enables profiling of 37 common EV surface markers (Figure 1d). Beside the major tetraspanins CD9, CD63 and CD81, the surface markers CD29 (integrin beta 1), CD146 (MCAM) and CD326 (EpCAM) were detected at considerable levels. Such integrins and adhesion molecules may steer the organotropism of EVs (Hoshino et al., 2015). The surface protein CD47, which may prolong EV circulation times *in vivo* by reducing uptake by macrophages (Kamerkar et al., 2017), was additionally detected by Western blot, albeit at low levels (Figure 1e).

3.2 | Characterization of label incorporation

We used a nanoluciferase assay to determine the presence of palmGRET in different SEC fractions (Figure 1f). Nanoluciferase was present in EVs (fractions 1–4) but was also highly abundant as free protein in the later protein fractions (7–11), highlighting the importance of size-based separation of EVs from non-EV proteins. An overview of the characteristics of different EV batches can be found in Table S1. To confirm the incorporation of MemGlow 700, we used the Amnis Imagestream ISX imaging flow cytometer, which allows near-infrared detection and is suited to characterize small EVs (Erdrügger et al., 2014; Görgens et al., 2019) (Figure S2). To set gates for double-positive EVs, we used control EVs that contained only palmGRET or only MemGlow 700 (Figure S2A). We performed serial dilutions to rule out coincidence events (Figure S2B). Detergent treatment resulted in strongly reduced event counts, confirming that the measured events were indeed membrane particles (Figure S2C) in accordance with the MIFlowCyt-EV recommendations (Welsh et al., 2019). On average, 30% of the EVs were double-positive for both reporters (Figure S2D), while hardly any double-positive events were detected in unlabelled EVs, PBS, or free dye controls. Next, we used SP-IRIS to investigate the colocalization of palmGRET with the major EV tetraspanins CD9, CD63 and CD81 (Figure 1e). We observed the most palmGRET signal in EVs captured by anti-CD63 antibodies, and slightly less in EVs displaying CD81 and CD9, indicating that each of these tetraspanins was present in the palmGRET-labelled EV population. Next, we performed detergent/protease protection assays to confirm that palmGRET is enclosed within a lipid bilayer (Figure 1f). Protease K treatment of palmGRET EVs alone did not affect the nanoluciferase signal, whereas addition of detergent lead to a reduction in signal. This confirmed that palmGRET was enclosed within the lumen of EVs, as previously reported (Wu et al., 2020).

3.3 | Stability and detection in blood plasma

Prior to *in vivo* studies, we sought to determine detectability of labelled EVs in the biological matrix of blood plasma. This was done not only to assess assay sensitivity, but also because factors in blood might reduce stability of signal or contribute to background. palmGRET EVs were spiked into plasma, and serial dilutions were prepared over a 100,000-fold range (Figure 1g). Nluc signal was detected above background levels (macaque plasma without EVs) over the entire dilution range. We compared the Nluc signal with the number of particles per well (calculated from NTA particle counts and dilution factor), which suggested that the limit of detection was approximately around 200 EVs/ μ l (detection limit: 10,000 EVs in 50 μ l = 200 EV/ μ l). We additionally tested the stability of different doses of palmGRET EVs in macaque serum for up to 24 h (Figure S2E). More than 60% of the initial dose was detectable in serum after 24 h, with 80% for the two highest doses, indicating that palmGRET EVs are relatively stable

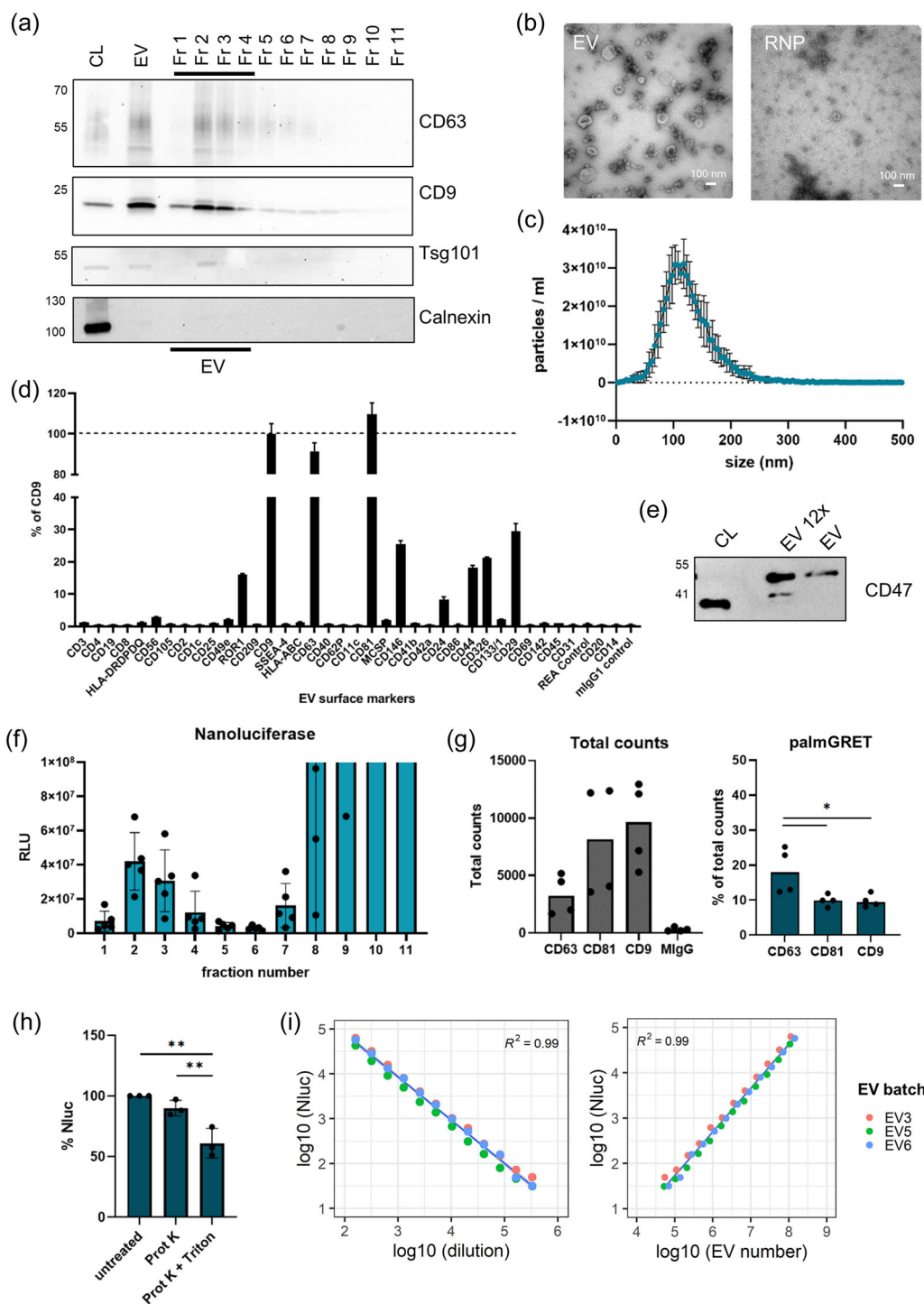


FIGURE 1 Characterization of palmGRET EVs. (a) EVs produced by Expi293F cells were concentrated by TFF and ultrafiltration, followed by size-exclusion chromatography (SEC). Equal volumes of individual SEC fractions, pooled EV (fractions 1–4) and cell lysate were analysed by SDS-PAGE and Western blot for EV markers CD63, CD9, TSG101, and ER marker (i.e., cytosol contaminant marker) Calnexin. Plot is representative of $n = 6$ repeats. (b) Pooled EV and protein fraction 10 (RNP) were imaged by negative stain transmission electron microscopy. Scale bars = 100 nm. Image is representative of $n = 6$ batches. (c) Particle size and concentration of pooled EV preparations were determined by NTA. Average of $n = 5$ EV batches is shown. (d) EV surface markers were profiled by MACSplex assay. Expression of surface markers is shown as percentage of CD9 expression. (e) Western blot detection of CD47 in Expi293F cell lysates, TCA-precipitated EVs (12 $\times$), and EVs. (f) SEC fractions 1–11 were diluted 20 $\times$, and the presence of the palmGRET reporter was validated by Nano-Glo assay. Measurement of $n = 5$ EV batches is shown. (g) SP-IRIS was used to determine the co-localization of palmGRET with CD63, CD81, and CD9. Total counts is the number of total fluorescent spots of $n = 4$ EV batches that are captured by anti-tetraspanin and isotype antibodies. (h) Pooled EVs were incubated with protease K and Triton X-100, protease K alone, or without additives. (i) Pooled EVs were spiked into plasma of a healthy macaque, diluted in twofold serial dilutions from 160 $\times$ to 32,768 $\times$, and measured by Nano-Glo assay. Nano-Glo data were plotted against the dilution factor (left) and theoretical EV concentration from NTA (right).

in serum and not rapidly degraded by serum factors. These findings supported further use of the model to evaluate palmGRET EV pharmacokinetics and biodistribution.

3.4 | Design and dosing: In vivo study

We next compared intravenous (IV or i.v.) and intranasal (IN or i.n.) administration of different amounts of EVs, tracking the abundance of EVs in plasma and CSF over 24 h after each administration (Figure 2a). The starting dose was based on a previous study in which EVs from 4E7 mesenchymal stem cells were administered into sheep fetuses (Ophelders et al., 2016). We used a comparable number of cells to produce EVs for our starting dose. Initial measurements showed that Expi293F cells produced about 1.7×10^3 EV/seeded cell under the culture conditions we used. This set the starting dose to approximately 7×10^3 EVs (4×10^7 cells $\times 1.7 \times 10^3$ EV/cell $\approx 7 \times 10^3$ EVs). Three subsequent EV doses were administered at five-fold greater concentration each time, with several weeks between doses. The fourth and highest dose was then administered a second time.

3.5 | Detection of EVs in blood plasma

Intranasal administration of EVs resulted in little if any nanoluciferase detection in plasma at any timepoint and after any dose (Figure 2b). This suggested that Expi293F EVs might have remained in the nasal cavity, mucosa, or lungs after administration, and did not enter the bloodstream. In contrast, intravenously administered EVs could be reliably detected in plasma at all doses (Figure 2c). For the three lowest doses (7×10^3 , 4×10^4 and 2×10^5 EVs), nanoluciferase signal could be detected in plasma up to 24 h (1440 min) after administration. Unexpectedly, the fourth and highest dose (9×10^5 EVs, magenta data points) was cleared more rapidly from plasma than dose 3, and was hardly detectable above background after 4 h (360 min). We double-checked the particle concentration and nanoluciferase signal in this particular EV dose, to rule out any issues with storage or handling, but we did not observe any abnormalities that could explain the observed lower signal and increased clearance. When we repeated administration of this highest dose (black data points), we observed a comparable clearance pattern.

3.6 | Half-life of EVs in blood plasma

Next, we used these data to calculate the half-life of EVs in plasma. The data followed a biphasic decay profile on a log-lin chart, with rapid decay shortly after administration followed by slower decay at a later timepoint, described by a two-compartment pharmacokinetic model (Morishita et al., 2015). We calculated the half-life from the data points during the first 60 min, which corresponds to the rapid decay phase in the model (Figure 2d). We observed an EV half-life between 36 and 42 min for the three lowest doses. In contrast, the half-life of signal after administration of the highest dose was approximately 11 min. Because we traced the clearance of the repeat of the highest dose for 15 min, the half-life of repeat dose 4 could not be reliably determined.

3.7 | ABC: Magnitude or number of doses?

The accelerated clearance observed at the highest dose could be due to the dose itself or to the repeated EV administration. We thus administered EVs to a naïve macaque in a different order, starting with the highest dose (9×10^5 EVs), then followed by the lowest dose (7×10^3 EVs) and increasingly higher doses again (Figure 2e). As before, EVs were detected in plasma after 1 min and were cleared over time. Some signal remained detectable in plasma 24 h following the highest dose, but not the other doses. However, the highest dose, now administered first, was cleared the slowest, while accelerated clearance was observed starting with the third EV administration (Figure 2f). Half-life decreased to ~ 9 min for dose 4 (2×10^5 EVs), and further to ~ 6 min for a repeat administration of the same dose. These results suggest that repeated administration, rather than the absolute EV dose, contributed predominantly to accelerated clearance.

3.8 | Accelerated clearance and inflammatory responses

We next asked whether the immune system and inflammatory responses might be involved in the ABC phenomenon. Inflammatory cytokines/chemokines were measured in plasma collected at different timepoints after administration of 9×10^5 EVs, for both the first and second subjects (Figure S3A and B). We did not observe induction of key inflammatory cytokines such as IL-6, TNF α and IL-1 β after EV administration, consistent with earlier reports (Saleh et al., 2019). IL-8 was detected at all timepoints

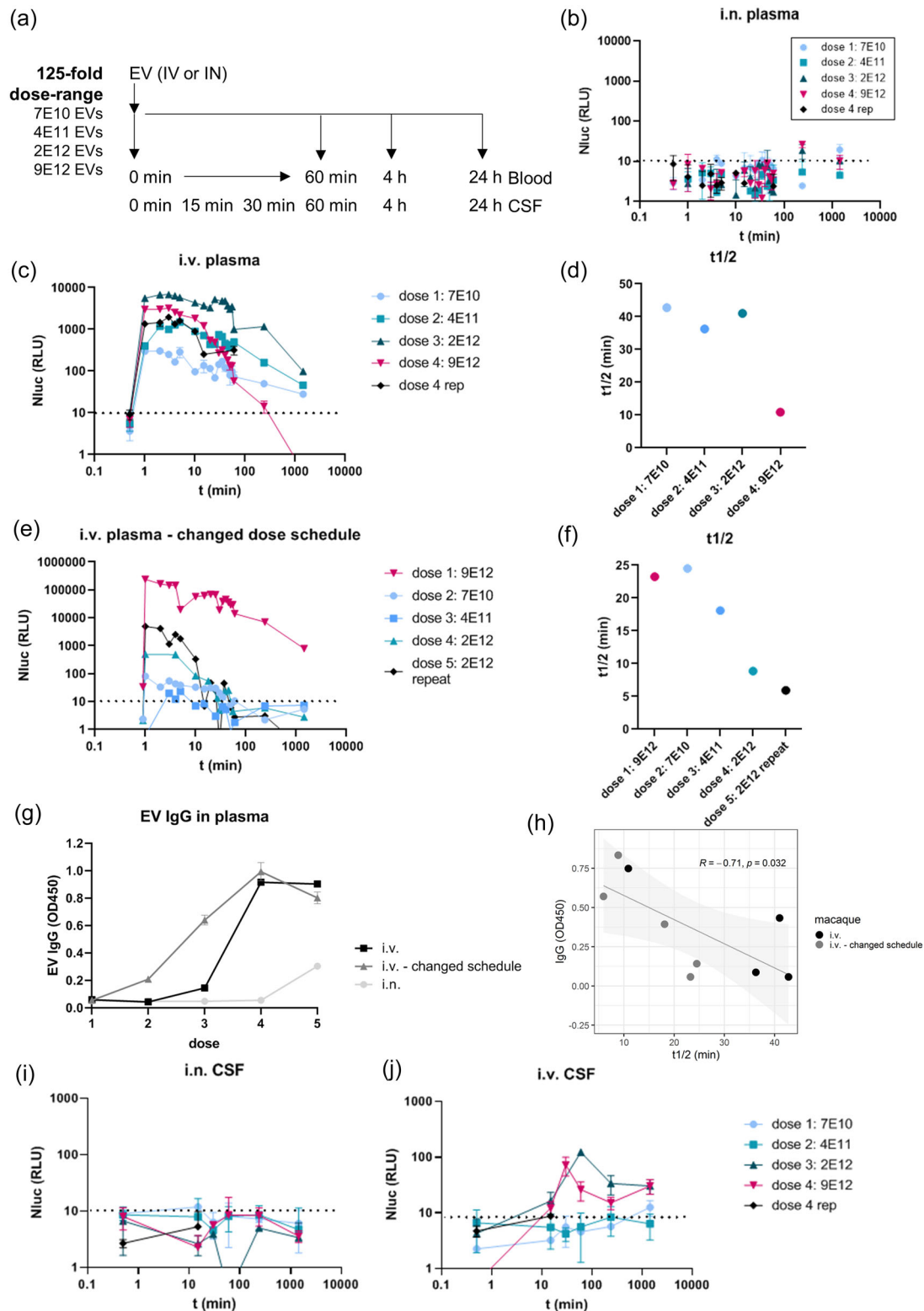


FIGURE 2 Pharmacokinetics of palmGRET EV administered intravenously and intranasally to macaques. (a) Schematic of the study setup. EVs were administered intravenously (IV) or intranasally (IN) to macaques over a 125-fold dose range. Blood and CSF were sampled before administration, and at timepoints indicated in the chart. (b) Detection of palmGRET EVs in plasma at different timepoints after intranasal administration to the first subject. (c) Detection of palmGRET EVs in plasma after intravenous administration. (d) EV half-life versus EV dose, from the data in (c). Nluc was plotted on a log-axis and t on a linear axis. The half-life was calculated from the slope between $t = 2$ and $t = 60$, using the formula $t_{1/2} = \log(2)/\text{slope}$. (e) Detection of palmGRET EVs in plasma after IV administration to a second subject with a different dosing schedule. (f) EV half-life for each of the i.v. EV doses in (e), calculated as for (d). (g) EV-specific IgGs were measured by ELISA in plasma collected after doses 1 to 5. (h) Linear correlation between $t_{1/2}$ and EV-specific IgGs. (i) Detection of palmGRET EVs in CSF after intranasal administration to the second subject. (j) Detection of palmGRET EVs in CSF after intravenous administration to the second subject. palmGRET EVs were detected by Nano-Glo assay throughout. Error bars indicate standard error of the assay.

(including baseline) after repeated dosing, and was induced after 4 h in the naïve macaque, albeit with a high standard deviation. Additionally, MCP-1 was detected but did not show a trend consistent with induction. These results appear to be inconsistent with an acute inflammatory response to EV administration. In addition, total IgG levels in plasma, collected 24 h after each dose, did not show a clear trend across the different doses (Figure S3C). However, EV-specific ELISA showed detectable IgG in plasma after three intravenous administrations (Figure 2g), while IgM levels were not elevated (Figure S3D). In the other macaque, where we changed the dosing schedule, high IgG levels were already detectable after two intravenous administrations. Interestingly, increased IgG levels correlated with decreased EV half-life (Figure 2h). This suggests that repeated intravenous administration with EVs may elicit EV-specific antibody responses that lead to accelerated clearance.

3.9 | Detection of EVs in CSF

In addition to EV clearance from plasma, we tracked the uptake of EVs into the CSF after intranasal or intravenous administration. After intranasal administration (Figure 2i), EVs could not be reliably detected above background in CSF at any timepoint and after any dose. In contrast, signal was observed in CSF after intravenous administration at the higher doses (Figure 2j). EV signal in CSF peaked after 30 min for dose 4 and at 60 min for dose 3, and remained detectable above background for up to 24 h. Intravenous doses 1 and 2 did not lead to detectable CSF at any of the timepoints. Intravenously administered EVs may thus migrate from plasma into CSF, at least at higher doses.

3.10 | Half-life of EVs in CSF and detection in plasma after intrathecal injection

We next questioned whether EV half-life in CSF is comparable to that in plasma. To achieve initial levels of EVs in CSF that were similar to those administered into blood, we introduced 3.2×10^{10} EVs directly into the CSF of a previously untreated subject *via* intrathecal injection, followed by collection of CSF and plasma at regular intervals (Figure S4). When injected into an estimated 15 ml of CSF, the EV concentration would be largely comparable to that of 2×10^{12} EVs into 500 ml plasma (in the $\sim 1 \times 10^9$ EV/ml range). Strong nanoluciferase signal in CSF remained detectable up to 6 h. Based on data from the first hour post-treatment, EV half-life in CSF was approximately 12.5 min, considerably shorter than half-life in plasma. Meanwhile, nanoluciferase signal was detected above background in plasma only at later timepoints, suggesting that EVs might be able to diffuse from CSF into plasma, but at relatively low levels. Organs including brain were harvested 24 h after administration, but nanoluciferase was not detectable at this late timepoint.

3.11 | Association of EVs with PBMCs

Since circulating white blood cells contribute to clearance of EVs from blood (Verweij et al., 2019), we investigated association of EV signal with PBMCs at different timepoints following EV administration. Specifically, we isolated PBMCs from the blood samples collected in the 24 h after administration, lysed them and measured the amount of nanoluciferase. After intranasal administration (Figure 3a), no nanoluciferase could be detected in the PBMCs at any of the doses, consistent with the absence of intranasally delivered EVs in blood plasma. In contrast, intravenous administration at all doses led to detectable EV signal in PBMCs as soon as 1 min after injection (Figure 3b). At the highest doses, nanoluciferase was detectable in PBMCs up to 1 h. There was also a linear relationship between total nanoluciferase signal (all time points combined) and dose for the highest three doses (Figure 3c). The repeat dose 4 was not included since blood samples could be collected over only 15 min.

3.12 | Association of EVs with PBMC subtypes

We next sought to determine the PBMC subtype(s) in blood to which EVs associated. Flow cytometry was performed with PBMCs from whole blood samples collected during the first 10 min after administration of the highest EV dose (Figure 3d), using an antibody panel that identifies several PBMC subtypes: monocytes (CD159- CD3- CD20- and CD14+ or CD14-), T cells (CD3+ and CD4+ or CD8+), B cells (CD3- CD20+), and NK cells (CD159+). Granulocytes were gated based on their unique forward scatter/side scatter properties. EVs were detected based on the internal GFP label as well as by presence of the Mem-Glow self-quenching lipid dye. The gating strategy is depicted in Figure S5A. We observed EV signal in granulocytes, monocytes, CD3+ lymphocytes and CD20+ B cells already at 1 min after administration (Figure 3e), consistent with nanoluciferase results from PBMC lysates (Figure 3b). Monocytes, CD3+ cells, and B cells differed in their percentage of EV association: 80.8% of

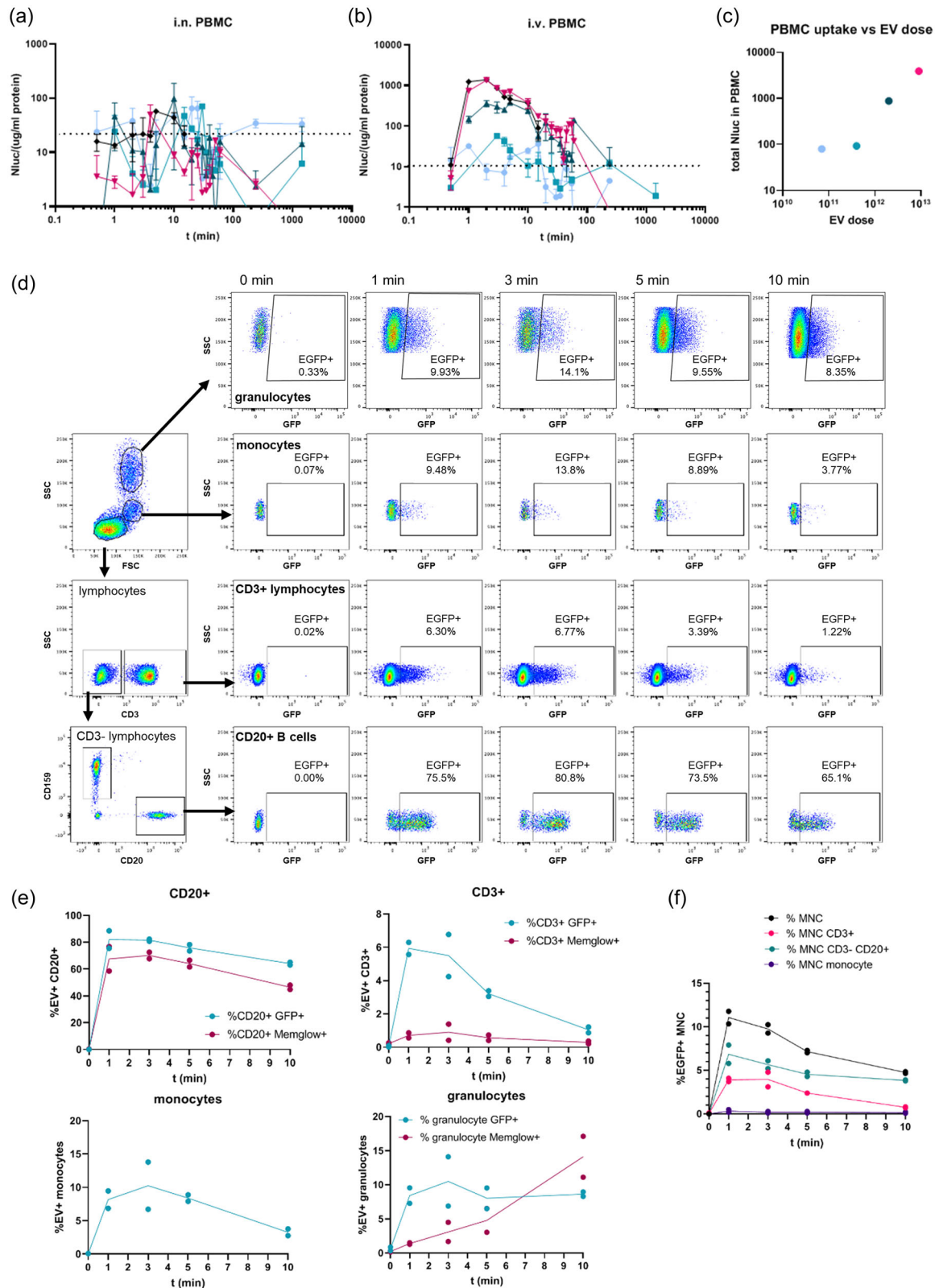


FIGURE 3 EV association with peripheral blood mononuclear cell (PBMC) subsets quantified by flow cytometry. (a-b) Detection of palmGRET EVs in PBMC lysates at different timepoints after IN (a) and IV (b) administration by Nano-Glo assay. (c) Total nanoluciferase signal detected in PBMC lysates during the first 60 min after administration was calculated from (b), and plotted against the EV dose. (d) Whole blood collected after intravenous administration of the highest dose (dose 4) was immunolabeled, after which palmGRET (GFP)-containing EVs in PBMC subsets was measured by flow cytometry. The full gating strategy is found in Figure S4A. EVs associated with granulocytes, monocytes, CD3+ lymphocytes and CD20+ B cells (bottom), presented as GFP+

(Continues)

FIGURE 3 (Continued)

cells as a percentage of each cell population. Plots are representative of $n = 2$ EV administrations into the same animal, 2 weeks apart. (e) Quantification of % GFP+ and % MemGlow+ PBMC subsets from (d), as percentage of each cell population. Data from $n = 2$ EV administrations are shown. (f) Quantification of GFP+ mononuclear cells (MNC), CD3+ lymphocytes, CD20+ B cells, and monocytes, relative to the total pool of MNC. Data from $n = 2$ EV administrations are shown.

B cells became GFP+, while 14.1% of granulocytes, 13.8% of monocytes and 6.7% of CD3+ cells became GFP+. Of the CD3+ cells, both CD4+ and CD8+ T cells became GFP-positive to a similar extent (Figure S5B). EV signal was not found in NK cells (Figure S5C). We found similar percentages of EV association after repeating administration of the highest EV dose (Figure 3e). Gating of GFP+ or MemGlow+ cells showed largely similar kinetics for both EV markers. However, while both GFP-containing and MemGlow-containing EVs associated with B cells, CD3+ cells seemed to be less associated with the MemGlow signal. Granulocytes became GFP+ in the first 5 min, and MemGlow+ after 10 min. Since monocytes were less abundant than B cells and T cells, the percentage of MemGlow-positive monocytes could not be reliably determined. Taking into account both the percentage of GFP positivity and cell type contribution to the overall PBMC population, B cells were the largest positive population (~7% of all GFP+ mononuclear cells were B cells), followed by T cells (~4%), while monocytes were less than 1%. Overall, 11% of mononuclear cells associated with GFP-containing EVs (Figure 3f). Thus, intravenous EV administration leads to rapid association with various PBMC subsets.

3.13 | Organ biodistribution in mouse

We also investigated how intranasal and intravenous administration affected distribution of our labelled EVs to different organs. For this part of the study, we performed initial experiments in mice, since their small size makes them suitable for *in vivo* and *ex vivo* imaging. For *in vivo* imaging, we used a modified nanoluciferase substrate that is more water-soluble than regular Nano-Glo, fluorofurimazine (FFz), allowing better distribution of the substrate throughout the whole animal (Su et al., 2020). After interperitoneal injection of FFz, we administered 1.4E11 EVs by intravenous or intranasal routes and measured bioluminescence (Figure S6A). After intranasal administration, we observed bright signal in the nasal cavity and in some cases also in the lungs. After intravenous administration, we observed most signal in the liver. After 40 min, we perfused the mice, harvested the organs and measured bioluminescence (Figure S6B) and near-infrared fluorescence *ex vivo* (Figure S6C). Most signal was observed in lungs (intranasal) or liver (intravenous), in line with our *in vivo* imaging observations. Intravenous administration additionally gave *ex vivo* bioluminescence in lung, spleen and kidney (Figure S6B), which was not observed in fluorescence mode (Figure S6C). Next, we prepared tissue homogenates from all harvested organs and measured EV uptake by nanoluciferase assay (Figure 4). Intranasal administration did not result in strong nanoluciferase signal in most organs, although we observed high but variable nanoluciferase signal in lung, in line with our *in vivo* imaging results. Intravenous administration gave strong nanoluciferase signal in the liver and spleen, consistent with earlier reports (Gupta et al., 2020; Kooijmans et al., 2016; Lázaro-Ibañez et al., 2021; Wiklander et al., 2015; Wu et al., 2020), and in kidney and lung, consistent with *ex vivo* bioluminescent imaging. Heart, colon and brain showed the lowest amount of nanoluciferase. EV uptake in brain was lower for intranasal administration than for intravenous administration. We also expressed the measured nanoluciferase signal as percentage of input dose per organ (Figure S7A), and found that only a small percentage of the administered EVs was detected in the organs at the 40-min timepoint.

3.14 | Organ biodistribution in macaque

EV biodistribution was also assessed by nanoluciferase assay of macaque tissues harvested 60 min after administration of the last, highest dose (9E12 EVs; Figure 5a) given to the first subject. After intravenous administration, we observed strong signal in liver and spleen, in line with the rodent results. Some uptake in lung was observed after intravenous, but not intranasal administration. EV uptake by kidney, heart, colon and brain was limited for both administration routes. We again calculated the nanoluciferase signal as percentage of the input dose per organ (Figure S7B), and observed that, as in mice, only a small percentage of administered EVs could be detected in organs at the 60-min timepoint. In addition to these peripheral organs, a faint nanoluciferase signal was observed in the medulla in brain after intravenous administration (Figure 5a, bottom right), suggesting this region might be the most accessible to EVs from the bloodstream. Nevertheless, the signal in medulla was low compared with signal in organs such as liver and spleen. Next, we used flow cytometry to identify which cell types in the spleen took up EVs (Figure 5b). After intravenous administration, we observed GFP+ monocytes and CD3+ lymphocytes, but the most EVs were associated with B cells, consistent with the PBMC results reported above. Interestingly, after intranasal EV administration, we also observed a small percentage of GFP+ CD3+ lymphocytes in the spleen (Figure 5c).

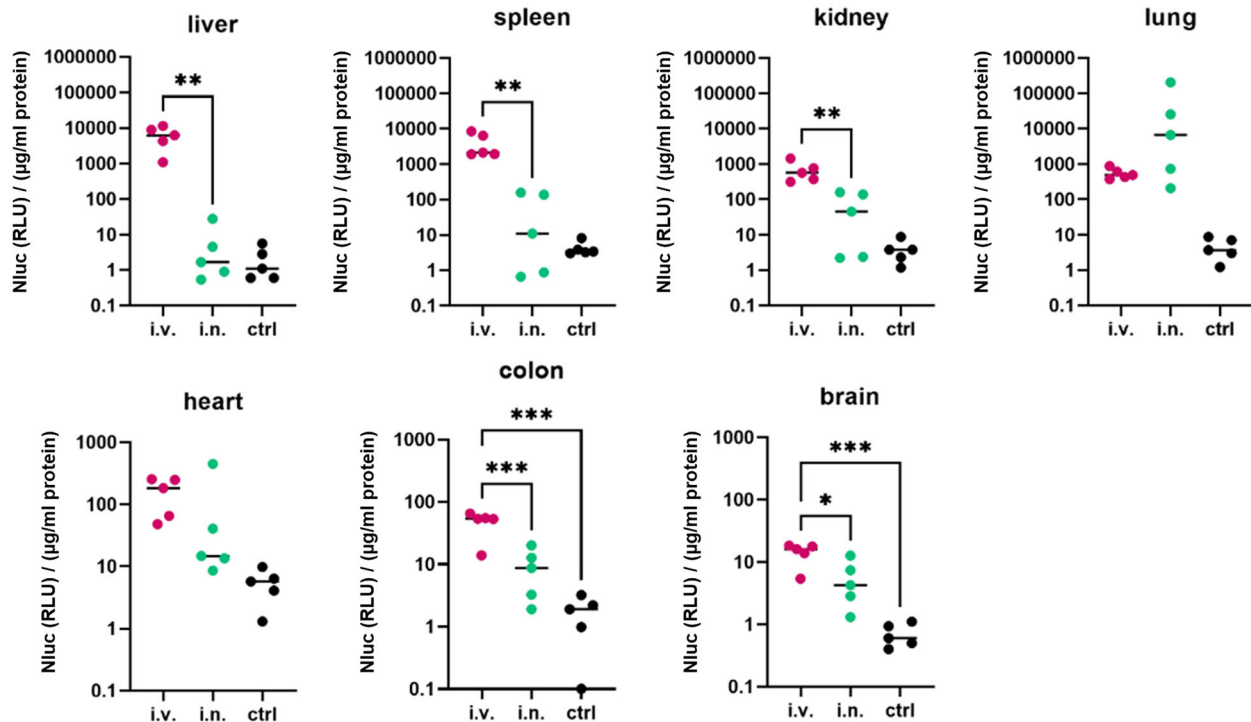


FIGURE 4 Biodistribution of palmGRET EVs administered intravenously or intranasally into mice. EV uptake into mouse tissues was determined by Nano-Glo assay on tissue homogenates. Datapoints show the amount of nanoluciferase signal (RLU) normalized to the protein content of the lysates. Data from $n = 5$ animals are shown. Statistical comparisons: one-way ANOVA with Tukey's post-hoc test, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3.15 | Barriers to intranasal uptake?

We next queried why intranasal administration did not result in systemic uptake of EVs. The *in vivo* bioluminescent images in mice showed a prominent signal in the nose of the animals, suggesting that EVs may be retained in the nasal cavity. To investigate this possibility in macaque, we administered 9E12 EVs intranasally into a naïve macaque. After 1 h, the nasal cavity was lavaged with 10 ml PBS. We measured nanoluciferase activity in the nasal lavage fluid and observed a very strong signal compared with signal in plasma and CSF collected at the same time (Figure 5d), consistent with the nasal mucosa preventing EVs from being distributed to other locations.

4 | DISCUSSION

4.1 | Half-life of EVs in plasma

To date, most preclinical studies on EVs have used mice, rats and zebrafish (Escudé Martinez de Castilla et al., 2021; Kang et al., 2021). Remarkably, we measured the circulation time of EVs in NHP ($t_{1/2}$ approx. 40 min) to be much longer than that reported in mice ($t_{1/2}$ approx. 5 min) (Gupta et al., 2020; Kooijmans et al., 2016; Lai et al., 2014; Lázaro-Ibañez et al., 2021; Wu et al., 2020) and zebrafish (Verweij et al., 2019). While most of these studies used different EV producer cells, which may have different clearance kinetics, one study reported that Expi293F EVs have a half-life of 10 min in mice (Lázaro-Ibañez et al., 2021). In contrast, our EVs remained detectable in macaque plasma up to 24 h after IV administration, suggesting that EV clearance may differ between different animal species. Comparing our results with the clearance of HIV-1 virions in macaques (Igarashi et al., 1999), the half-life of our EVs was in the range of that of HIV-1 virions infused into naïve macaques, namely 13.0–19.3 min based on viral RNA, and 22–29 min based on pelletable Gag p24 in plasma (Igarashi et al., 1999). Considering that HIV-1 actively fuses with and infects target cells, it is not surprising that EVs, which are thought to lack a consistent fusion mechanism like those of enveloped viruses, may have slightly longer circulation times. It has been suggested that species differences between the EV source (producer cell) and recipient animal model may affect circulation times. For example, human lentiviral vectors may be less stable in serum from evolutionary distant species (DePolo et al., 1999). Further study is required to determine if similar principles apply to the stability of EVs. However, we have shown *in vitro* that our EVs remained stable in macaque serum for at least 24 h at 37°C.

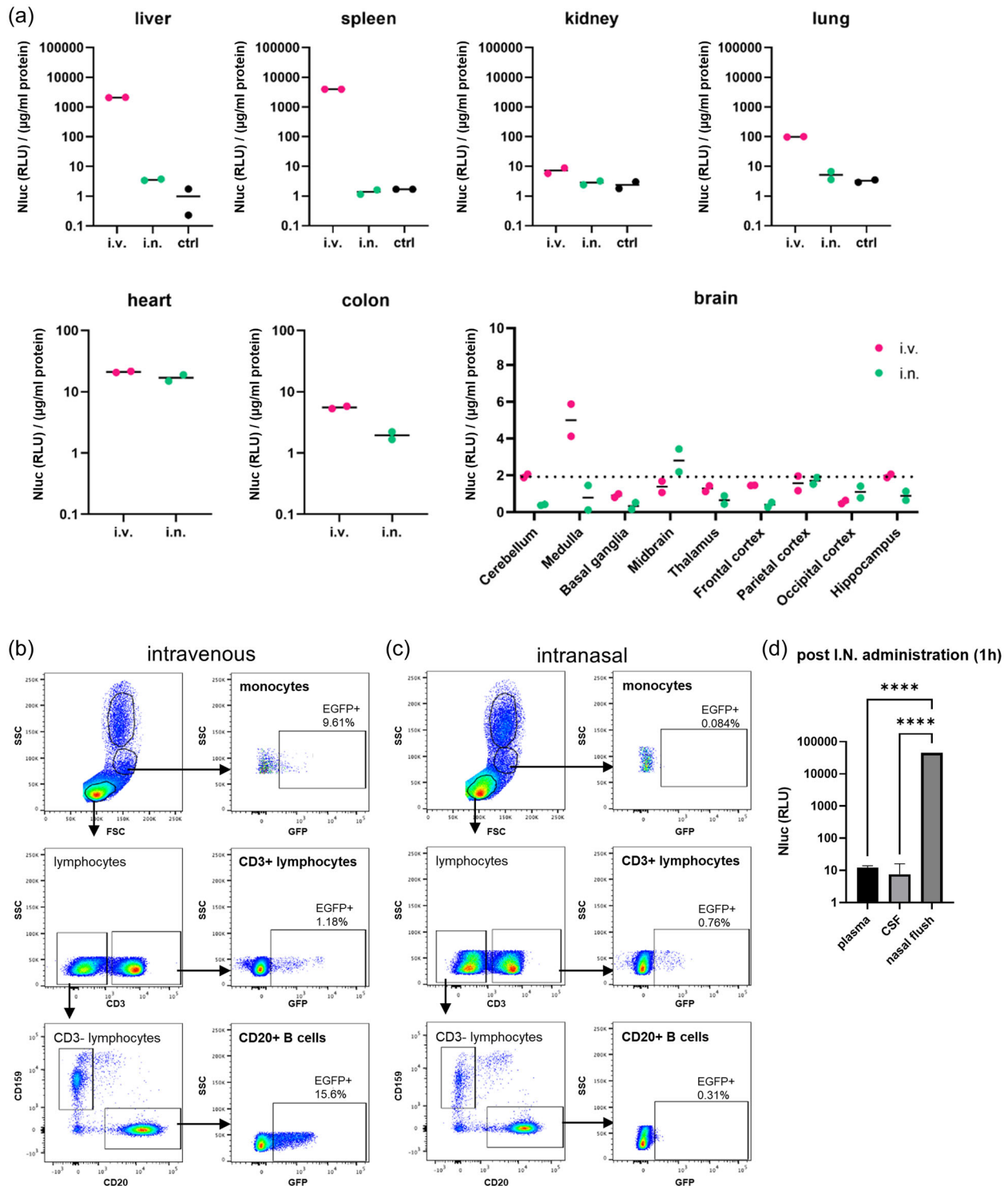


FIGURE 5 Biodistribution of palmGRET EVs administered intravenously or intranasally to macaques. (a) Organs were collected 60 min after IV or IN administration of the highest dose of EVs (dose 4 repeat). EV uptake into macaque tissues was determined by Nano-Glo assay of tissue homogenates. Datapoints show the amount of nanoluciferase signal (RLU) normalized to the protein content of the lysates. Data from 1 animal per group is shown. Dots indicate replicate measurements of the same sample. (b) Spleen was homogenized and immunostained directly for flow cytometric analysis of EV uptake by peripheral blood mononuclear cells (PBMCs) in the spleen after intravenous administration. Gating strategy was the same as for whole blood PBMCs (see Figure S4A). Dot plots show the % GFP+ cells as a percentage of the total B cells, CD3+ lymphocytes, or monocytes, respectively. (c) Similar to (b), EV uptake by PBMCs in the spleen after intranasal administration. Dot plots show the % GFP+ cells as percentage of the total B cells, CD3+ lymphocytes, or monocytes, respectively. (d) The nasal cavity was flushed with PBS 1 h after IN administration. Presence of EVs was measured by Nano-Glo assay, and compared with plasma and CSF of the same subject. **** $p < 0.001$ as determined by one-way ANOVA with Tukey's post-hoc test.

4.2 | Accelerated clearance after repeated administration of EVs

An important consideration in potential EV therapeutics is whether repeated administration will provoke immune responses. If so, EVs might need to be prepared from autologous or allogeneic cells, as opposed to the much easier and cheaper alternatives of non-autologous or even xenogeneic materials (Somiya et al., 2018; Urzì et al., 2021). In our study, EVs were cleared markedly more rapidly after the 4th and 5th administrations (repeated administration of the highest dose) than after administrations at lower doses and weeks earlier. By changing the order of the doses in follow-up experiments, we have shown that repeated dosing, and not the magnitude of the dose itself, led to accelerated clearance. We did not observe a strong induction of inflammatory cytokines or total IgG levels, in line with previous findings in other models (Alvarez-Erviti et al., 2011; Saleh et al., 2019; Zhu et al., 2017). However, we did observe increased levels of EV-specific IgGs, which negatively correlated with EV circulation times. This was not expected due to the high species similarity between macaques and the human cells that produced the EVs. Accelerated clearance after repeat dosing is a known issue with PEGylated synthetic nanoparticles (Dams et al., 2000; Kooijmans et al., 2016), mediated by PEG-specific IgM (Ishida et al., 2006). It was recently shown that a similar principle applies to PEGylated EVs (Emam et al., 2021). Whether autologous EVs are also subject to accelerated clearance should be addressed in the future. Various studies have shown that repeated administration of EVs gives strong therapeutic effects, such as a reduction in tumour burden (Kamerkar et al., 2017; Mendt et al., 2018; Usman et al., 2018). The accelerated clearance that we observed is also not necessarily a roadblock to therapeutic applications of EVs, which, for many applications, must leave the circulation and enter tissues.

4.3 | Association with and uptake by PBMC subtypes

Early after EV administration, we observed that EVs were associated with, and potentially taken up by, several PBMC subtypes. Our results differ somewhat from previous findings in that the EVs were predominantly associated with B cells. However, from our flow cytometric experiments, the type(s) of association cannot be deduced: simple surface binding, internalization (endocytosis) and EV-cell fusion. In support of our observation, a previous study showed that human plasma EVs can be taken up by B cells (Eitan et al., 2017). Even so, in that study, the majority of uptake was observed in monocytes (Eitan et al., 2017). Previous *in vivo* work, for example, with clodronate-depleted monocyte/macrophage populations (Imai et al., 2015), and with blockade of scavenger receptor class A (Watson et al., 2016), also implicated monocytes/macrophages in EV clearance. Possibly, the biological source of EVs, reporter system, species differences, or age of the subject contribute to these apparent differences. The rapid depletion of EV-associated signal from these cells is also worth noting, and could be due to degradation of EVs/label, to clearance of cells, or to dissociation of EVs from cells. Interestingly, within 1 h of IV administration, we detected GFP+ B cells and monocytes in the spleen, suggesting rapid trafficking of blood B cells and monocytes to the spleen (and possibly elsewhere) after interacting with EVs.

4.4 | Uptake into CSF, but not into brain

A striking result of our study was the low level of EV uptake into central nervous system compartments, regardless of administration route. First, intravenous administration: acceptance of the ability of EVs to cross the blood-brain barrier (BBB) in both directions has become so widespread that statements to this effect are often not even referenced in the literature. Yet much of the evidence for EV transfer across the blood-brain barrier is indirect. After intravenous administration, EVs might enter brain tissue directly across the BBB. They might also traverse the choroid plexus epithelium, a more permeable counterpart to the BBB (Praetorius & Damkier, 2017). We detected low levels of EVs in brain tissue (especially medulla) and in CSF following IV administration, albeit at much lower levels than in peripheral compartments. In CSF, signal remained detectable at 24 h. This result is similar to findings of a study of IV insulin in dogs, in which rapid clearance from plasma was followed by detection in CSF, peaking around 90 min (Schwartz et al., 1991). However, we are not convinced that entry into CSF is an efficient precursor to brain entry. We did not observe any EV uptake into brain even after injecting EVs directly into the CSF (Driedonks and Witwer, data not shown). This suggests that reaching the CSF may not be enough to gain entry to the brain. Overall, the low levels of signal in both tissue and CSF suggest that the EV blood-brain route in our model is more of a precarious footpath than a superhighway.

4.5 | Negligible brain uptake after IN administration

Our results suggest that IN delivery of EVs to the brain in large animals should not be a foregone conclusion. Previously, studies of intranasally administered recombinant vesicular stomatitis viral vectors (Johnson et al., 2007) and a nanogel pneumococcal vaccine formulation (Fukuyama et al., 2015) in macaques found no brain uptake. Certainly, numerous studies report that IN EVs or their presumed cargo enter the brain parenchyma (see, for example (Hayes et al., 2021; Hu et al., 2018; Pathipati et al., 2021;

Thomi et al., 2019; Upadhyia et al., 2020; Zhuang et al., 2011)). As a result, intranasal delivery of EVs is thought to be a promising way to treat central nervous system (CNS) disease (reviewed in (Pauwels et al., 2021)). However, the studies with positive results that we are aware of have all been performed in mouse or rat models, and predominantly with EVs sourced from mesenchymal stem cells (MSCs) or other stem cells, which seem to perform well in targeting the brain (Pauwels et al., 2021). Physiology of the recipient species or characteristics of the source cells could explain disparate results. For example, many studies relied on various brain injury models (tumours, stroke, brain injury, morphine treatment) which may enhance the capacity to take up EVs compared with healthy animals (Pathipati et al., 2021; Thomi et al., 2019; Zhuang et al., 2011). Additionally, EV uptake and signal uptake may not overlap completely depending on EV separation technique. For example, one study finding efficient intranasal administration used MSC EVs that were incubated with gold nanoparticles (GNPs) and then ultracentrifuged for 2 h at $100,000 \times g$ (Betzer et al., 2017). Although these procedures were meant to label EVs and remove free GNPs, it is unclear how efficient the labelling was and also doubtful that ultracentrifugation would separate EV-associated GNPs from free particles. Free GNPs might thus have contributed to or fully explained these results, with little or no EV uptake.

Intranasal delivery factors may influence outcome. Alternatives to instillation such as nebulization or aerosolization should be tested in large animals, in addition to instillation, and repeated short-term administrations might also be useful. Infusion of larger volumes leads to more entry into the lungs, instead of just the nasal cavity (Southam et al., 2002), and could perhaps also influence brain uptake. Peptides or other adhesion molecules might be used on the EV surface to encourage uptake. One study used hyaluronidase treatment of the nasal cavity (Upadhyia et al., 2020), which may degrade extracellular matrix and enhance diffusion capacity (Buhren et al., 2016), remedying the nasal mucosa retention we observed. In any case, our findings suggest that different modes of intranasal delivery of different types of EVs should now be assessed in multiple models and perhaps with pre-treatments to determine if this route is a feasible EV delivery option for large animals including humans.

4.6 | EV uptake by peripheral organs

In both macaques and mice, IV-administered EVs were most efficiently taken up by the liver and spleen, followed by lung. This is in accordance with many other EV biodistribution studies in mice (Gupta et al., 2020; Kooijmans et al., 2016; Lázaro-Ibañez et al., 2021; Wiklander et al., 2015; Wu et al., 2020). EVs were prominently detected in B cells in spleen after IV administration. It was recently reported that EVs may be taken up in the liver by Kupffer cells, hepatocytes and liver sinusoidal epithelial cells (Németh et al., 2021), likely mediated by scavenger receptors (Watson et al., 2016). However, we were unable to assess this in our macaque model. In mice, we observed low levels of uptake by kidney and, to a lesser extent, heart and colon. Thus, peripheral biodistribution of IV EVs was largely comparable between macaques and mice, with the exception of differences in kidney and lung. In mouse lungs, large EVs might be retained in the narrow microcapillaries ($1\text{--}2\ \mu\text{m}$) (Kang et al., 2021). Wider capillaries of macaques might explain the relatively reduced retention of EVs. It is important to note that EV biodistribution studies based on reporter proteins cannot show how many EVs cumulatively reached an organ, since protein-based reporters may be degraded over time (Gupta et al., 2020). Instead, these data can be used to reliably compare the relative uptake of EVs by different organs at a certain timepoint. Furthermore, we cannot account for EVs that have been taken up by endothelial cells, for example those of the vascular system.

Factors beyond administration route that affect EV biodistribution may include differences in tetraspanins or other surface proteins, EV labelling strategy (Gupta et al., 2020; Lázaro-Ibañez et al., 2021), and the type of EV donor cells (Luo et al., 2020; Wiklander et al., 2015). Illustrating this, endogenous CD63-Nluc EVs from cardiomyocytes were shown to target different organs than CD63-Nluc EVs from Expi293F cells (Lázaro-Ibañez et al., 2021; Luo et al., 2020). We have investigated the biodistribution of EVs from only one cell source, which may not be representative of EVs from, for example, MSCs or red blood cells. Additionally, the disease status of the recipient may affect tissue accumulation. Uptake of MSC-EVs into kidneys was increased in mice with acute kidney injury compared to healthy controls (Grange et al., 2014; Han et al., 2021). Future research will likely highlight other factors that control EV tissue retention (or even true “homing” or “targeting,” if this is possible for EVs (Noren Hooten et al., 2020)), and will give clues on how to better control the fate of EVs *in vivo*.

Taken together, nanoluciferase-based reporters allowed sensitive tracking of EVs in larger animals for pharmacokinetic measurements. We show that EVs from human cells had a longer circulation time in macaques compared with mice, but that CNS penetration was low for both IV and IN administration. Repeated administration led to more rapid clearance, which may have implications for EV-based therapies against cancer and immune diseases. We hope that our findings from this baseline study in macaques will help to inform future research and therapeutic development of EVs.

AUTHOR CONTRIBUTIONS

Tom Driedonks, Kelly Pate and Kenneth Witwer conceived the research and designed experiments. Tom Driedonks, Linglei Jiang, Zheng Han, Guanshu Liu, Erin Shirk, Olesia Gololobova, Zhaohao Liao, Lyle Nyberg, Liliia Paniushkina, Marta

Garcia-Contreras, Gabriela Lima and Barbara Smith performed experiments. Bess Carlson, Suzanne Queen, Zheng Han, Guanshu Liu, Kayla Schonvisky, Natalie Castell, Mitchell Stover, Selena Guerrero-Martin, Riley Richardson, Jessica Izzi, Eric Hutchinson and Kelly Pate performed animal experiments. Charles Lai designed and provided the palmGRET reporter construct. Tom Driedonks and Kenneth Witwer wrote the manuscript. All authors reviewed and revised the manuscript.

ACKNOWLEDGEMENTS

The authors thank W. Zhang and T. Nilles for assistance with the imaging flow cytometry measurements. This study was supported by the US National Institutes of Health through AI144997 (to K.W.W.) and U42OD013117 (to E.K.H.). The Witwer lab is also supported in part by the NIH through DA047807, MH118164, and CA241694, and by the Michael J. Fox Foundation (Grant 00900821). C.P.L. was supported by the Ministry of Science and Technology (MOST) grants (109-2628-B-001-032) and Academia Sinica Career Development Award (AS-CDA-109-M04).

CONFLICT OF INTEREST

The authors report no conflicts of interest.

ORCID

Linglei Jiang  <https://orcid.org/0000-0002-8304-5911>

Olesia Gololobova  <https://orcid.org/0000-0002-0392-268X>

Kenneth W. Witwer  <https://orcid.org/0000-0003-1664-4233>

REFERENCES

- Ali, J., Ali, M., Baboota, S., Kaur Sahni, J., Ramassamy, C., Dao, L., & Bhavna (2010). Potential of nanoparticulate drug delivery systems by intranasal administration. *Current Pharmaceutical Design*, 16(14), 1644–1653. <https://doi.org/10.2174/138161210791164108>
- Allan, D., Tieu, A., Lalu, M., & Burger, D. (2020). Mesenchymal stromal cell-derived extracellular vesicles for regenerative therapy and immune modulation: Progress and challenges toward clinical application. *Stem Cells Translational Medicine*, 9(1), 39–46. <https://doi.org/10.1002/sctm.19-0114>
- Alvarez-Erviti, L., Seow, Y., Yin, H., Betts, C., Lakhal, S., & Wood, M. J. A. (2011). Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nature Biotechnology*, 29(4), 306–309. <https://doi.org/10.1038/nbt.1807>
- Beck, S. E., Queen, S. E., Metcalf Pate, K. A., Mangus, L. M., Abreu, C. M., Gama, L., Witwer, K. W., Adams, R. J., Zink, M. C., Clements, J. E., & Mankowski, J. L. (2018). An SIV/maaque model targeted to study HIV-associated neurocognitive disorders. *Journal of NeuroVirology*, 24(2), 204–212. <https://doi.org/10.1007/s13365-017-0582-4>
- Betzer, O., Perets, N., Angel, A., Motiei, M., Sadan, T., Yadid, G., Offen, D., & Popovtzer, R. (2017). In vivo neuroimaging of exosomes using gold nanoparticles. *ACS Nano*, 11(11), 10883–10893. <https://doi.org/10.1021/acsnano.7b04495>
- Buhren, B. A., Schruppf, H., Hoff, N. P., Bölke, E., Hilton, S., & Gerber, P. A. (2016). Hyaluronidase: From clinical applications to molecular and cellular mechanisms. *European Journal of Medical Research*, 21(1), 1–7. <https://doi.org/10.1186/s40001-016-0201-5>
- Busatto, S., Vilanilam, G., Ticer, T., Lin, W.-L., Dickson, D. W., Shapiro, S., Bergese, P., & Wolfram, J. (2018). Tangential flow filtration for highly efficient concentration of extracellular vesicles from large volumes of fluid. *Cells*, 7(12), 273. <https://doi.org/10.3390/cells7120273>
- Collot, M., Ashokkumar, P., Anton, H., Boutant, E., Faklaris, O., Gallii, T., Mély, Y., Danglot, L., & Klymchenko, A. S. (2019). MemBright: A family of fluorescent membrane probes for advanced cellular imaging and neuroscience. *Cell Chemical Biology*, 26(4), 600–614.e7. <https://doi.org/10.1016/j.chembiol.2019.01.009>
- Dams, E. T. M., Laverman, P., Oyen, W. J. G., Storm, G., Scherphof, G. L., Van Der Meer, J. W. M., Corstens, F. H. M., & Boerman, O. C. (2000). Accelerated blood clearance and altered biodistribution of repeated injections of sterically stabilized liposomes. *Journal of Pharmacology and Experimental Therapeutics*, 292(3), 1071–1079.
- De Jong, O. G., Kooijmans, S. A. A., Murphy, D. E., Jiang, L., Evers, M. J. W., Sluijter, J. P. G., Vader, P., & Schiffelers, R. M. (2019). Drug delivery with extracellular vesicles: From imagination to innovation. *Accounts of Chemical Research*, 52(7), 1761–1770. <https://doi.org/10.1021/acs.accounts.9b00109>
- DePolo, N. J., Harkleroad, C. E., Bodner, M., Watt, A. T., Anderson, C. G., Greengard, J. S., Murthy, K. K., Dubensky, T. W., & Jolly, D. J. (1999). The resistance of retroviral vectors produced from human cells to serum inactivation in vivo and in vitro is primate species dependent. *Journal of Virology*, 73(8), 6708–6714. <https://doi.org/10.1128/jvi.73.8.6708-6714.1999>
- Eitan, E., Green, J., Bodogai, M., Mode, N. A., Baek, R., Jørgensen, M. M., Freeman, D. W., Witwer, K. W., Zonderman, A. B., Biragyn, A., Mattson, M. P., Hooten, N. N., & Evans, M. K. (2017). Age-related changes in plasma extracellular vesicle characteristics and internalization by leukocytes. *Science Reports*, 7(1342), 1–14. <https://doi.org/10.1038/s41598-017-01386-z>
- Emam, S. E., Elsadek, N. E., Abu Lila, A. S., Takata, H., Kawaguchi, Y., Shimizu, T., Ando, H., Ishima, Y., & Ishida, T. (2021). Anti-PEG IgM production and accelerated blood clearance phenomenon after the administration of PEGylated Exosomes in mice. *Journal of Controlled Release*, 334(December 2020), 327–334. <https://doi.org/10.1016/j.jconrel.2021.05.001>
- Erdbrügger, U., Rudy, C. K., Etter, M., Dryden, K. A., Yeager, M., Klivanov, A. L., & Lannigan, J. (2014). Imaging flow cytometry elucidates limitations of microparticle analysis by conventional flow cytometry. *Cytometry Part A*, 85(9), 756–770. <https://doi.org/10.1002/cyto.a.22494>
- Escudé Martinez de Castilla, P., Tong, L., Huang, C., Marios Sofias, A., Pastorin, G., Chen, X., Storm, G., Schiffelers, R. M., & Wang, J.-W. (2021). Extracellular vesicles as a drug delivery system: A systematic review of preclinical studies. *Advanced Drug Delivery Reviews*, 175, 113801. <https://doi.org/10.1016/j.addr.2021.05.011>
- Fukuyama, Y., Yuki, Y., Katakai, Y., Harada, N., Takahashi, H., Takeda, S., Mejima, M., Joo, S., Kurokawa, S., Sawada, S., Shibata, H., Park, E. J., Fujihashi, K., Briles, D. E., Yasutomi, Y., Tsukada, H., Akiyoshi, K., & Kiyono, H. (2015). Nanogel-based pneumococcal surface protein A nasal vaccine induces MicroRNA-associated Th17 cell responses with neutralizing antibodies against streptococcus pneumoniae in macaques. *Mucosal Immunology*, 8(5), 1144–1153. <https://doi.org/10.1038/mi.2015.5>
- Görgens, A., Bremer, M., Ferrer-Tur, R., Murke, F., Tertel, T., Horn, P. A., Thalmann, S., Welsh, J. A., Probst, C., Guerin, C., Boulanger, C. M., Jones, J. C., Hanenberg, H., Erdbrügger, U., Lannigan, J., Ricklefs, F. L., El-Andaloussi, S., & Giebel, B. (2019). Optimisation of imaging flow cytometry for the analysis of

- single extracellular vesicles by using fluorescence-tagged vesicles as biological reference material. *Journal of Extracellular Vesicles*, 8(1), 1587567. <https://doi.org/10.1080/20013078.2019.1587567>
- Grange, C., Tapparo, M., Bruno, S., Chatterjee, D., Quesenberry, P. J., Tetta, C., & Camussi, G. (2014). Biodistribution of mesenchymal stem cell-derived extracellular vesicles in a model of acute kidney injury monitored by optical imaging. *International Journal of Molecular Medicine*, 33(5), 1055–1063. <https://doi.org/10.3892/ijmm.2014.1663>
- Gupta, D., Liang, X., Pavlova, S., Wiklander, O. P. B., Corso, G., Zhao, Y., Saher, O., Bost, J., Zickler, A. M., Piffko, A., Maire, C. L., Ricklefs, F. L., Gustafsson, O., Llorente, C., Gustafsson, M. O., Bostancioglu, R. B., Mamand, R., Hagey, D. W., & Görgens, A. (2020). Quantification of extracellular vesicles in vitro and in vivo using sensitive bioluminescence imaging. *Journal of Extracellular Vesicles*, 9(1), 1800222. <https://doi.org/10.1080/20013078.2020.1800222>
- Han, Z., Liu, S., Pei, Y., Ding, Z., Li, Y., Wang, X., Zhan, D., Xia, S., Driedonks, T., Witwer, K., Weiss, R. G., van Zijl, P. C., Bulte, J. W., Cheng, L., & Liu, G. (2021). Highly efficient magnetic labelling allows MRI tracking of the homing of stem cell-derived extracellular vesicles following systemic delivery. *Journal of Extracellular Vesicles*, 10(3), e12054. <https://doi.org/10.1002/jev2.12054>
- Hayes, S. H., Liu, Q., Selvakumaran, S., Haney, M. J., Batrakova, E. V., Allman, B. L., Walton, P. A., Kiser, P., & Whitehead, S. N. (2021). Brain targeting and toxicological assessment of the extracellular vesicle - packaged antioxidant catalase - SKL following intranasal administration in mice. *Neurotoxicity Research*, 39, 1418–1429. ; No. 0123456789. <https://doi.org/10.1007/s12640-021-00390-6>
- Hoshino, A., Costa-Silva, B., Shen, T.-L., Rodrigues, G., Hashimoto, A., Tesic Mark, M., Molina, H., Kohsaka, S., Di Giannatale, A., Ceder, S., Singh, S., Williams, C., Soplop, N., Uryu, K., Pharmed, L., King, T., Bojmar, L., Davies, A. E., Ararso, Y., ... Zhang, T. (2015). Tumour exosome integrins determine organotropic metastasis. *Nature*, 527(7578), 329–335. <https://doi.org/10.1038/nature15756>
- Hu, G., Liao, K., Niu, F., Yang, L., Dallon, B. W., Callen, S., Tian, C., Shu, J., Cui, J., Sun, Z., Lyubchenko, Y. L., Ka, M., Chen, X. M., & Buch, S. (2018). Astrocyte EV-induced LincRNA-Cox2 regulates microglial phagocytosis: Implications for morphine-mediated neurodegeneration. *Molecular Therapy - Nucleic Acids*, 13(December), 450–463. <https://doi.org/10.1016/j.omtn.2018.09.019>
- Hyenne, V., Ghoroghi, S., Collot, M., Bons, J., Follain, G., Harlepp, S., Mary, B., Bauer, J., Mercier, L., Busnelli, I., Lefebvre, O., Fekonja, N., Garcia-Leon, M. J., Machado, P., Delalande, F., López, A. A., Silva, S. G., Verweij, F. J., & van Niel, G. (2019). Studying the fate of tumor extracellular vesicles at high spatiotemporal resolution using the zebrafish embryo. *Developmental Cell*, 48(4), 554–572.e7. <https://doi.org/10.1016/j.devcel.2019.01.014>
- Igarashi, T., Brown, C., Azadegan, A., Haigwood, N., Dimitrov, D., Martin, M. A., & Shibata, R. (1999). Human immunodeficiency virus type 1 neutralizing antibodies accelerate clearance of cell-free virions from blood plasma. *Nature Medicine*, 5(2), 211–216. <https://doi.org/10.1038/5576>
- Imai, T., Takahashi, Y., Nishikawa, M., Kato, K., Morishita, M., Yamashita, T., Matsumoto, A., Charoenviriyakul, C., & Takakura, Y. (2015). Macrophage-dependent clearance of systemically administered B16BL6-derived exosomes from the blood circulation in mice. *Journal of Extracellular Vesicles*, 4(2015), 1–8. <https://doi.org/10.3402/jev.v4.26238>
- Ishida, T., Ichihara, M., Wang, X. Y., Yamamoto, K., Kimura, J., Majima, E., & Kiwada, H. (2006). Injection of PEGylated liposomes in rats elicits PEG-specific IgM, which is responsible for rapid elimination of a second dose of PEGylated liposomes. *Journal of Controlled Release*, 112(1), 15–25. <https://doi.org/10.1016/j.jconrel.2006.01.005>
- Johnson, J. E., Nasar, F., Coleman, J. W., Price, R. E., Javadian, A., Draper, K., Lee, M., Reilly, P. A., Clarke, D. K., Hendry, R. M., & Udem, S. A. (2007). Neurovirulence properties of recombinant vesicular stomatitis virus vectors in non-human primates. *Virology*, 360(1), 36–49. <https://doi.org/10.1016/j.virol.2006.10.026>
- Kamerkar, S., Lebleu, V. S., Sugimoto, H., Yang, S., Ruivo, C. F., Melo, S. A., Lee, J. J., & Kalluri, R. (2017). Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer. *Nature*, 546(7659), 498–503. <https://doi.org/10.1038/nature22341>
- Kang, M., Jordan, V., Blenkiron, C., & Chamley, L. W. (2021). Biodistribution of extracellular vesicles following administration into animals: A systematic review. *Journal of Extracellular Vesicles*, 10, e12085. <https://doi.org/10.1002/jev2.12085>
- Kim, S. M., Yang, Y., Oh, S. J., Hong, Y., Seo, M., & Jang, M. (2017). Cancer-derived exosomes as a delivery platform of CRISPR/Cas9 confer cancer cell tropism-dependent targeting. *Journal of Controlled Release*, 266(April), 8–16. <https://doi.org/10.1016/j.jconrel.2017.09.013>
- Kooijmans, S. A. A., Fliervoet, L. A. L., van der Meel, R., Fens, M. H. A. M., Heijnen, H. F. G., van Bergen en Henegouwen, P. M. P., Vader, P., & Schiffelers, R. M. (2016). PEGylated and targeted extracellular vesicles display enhanced cell specificity and circulation time. *Journal of Controlled Release*, 224, 77–85. <https://doi.org/10.1016/j.jconrel.2016.01.009>
- Lai, C., Mardini, O., Ericsson, M., Prabhakar, S., Maguire, C., Chen, J., Tannous, B. A., & Brakefield, X. O. (2014). Dynamic biodistribution of extracellular vesicles in vivo using a multimodal imaging reporter. *ACS Nano*, No. 1, 483–494.
- Lázaro-Ibañez, E., Faruqi, F. N., Saleh, A. F., Silva, A. M., Wang, J. T., Rak, J., Al-jamal, K. T., & Dekker, N. (2021). Selection of fluorescent, bioluminescent, and radioactive tracers to accurately reflect extracellular vesicle biodistribution in vivo. *ACS Nano*, 15, 3212–3227. <https://doi.org/10.1021/acsnano.0c09873>
- Leenaars, C. H. C., Kouwenaar, C., Stafleu, F. R., Bleich, A., Ritskes-Hoitinga, M., De Vries, R. B. M., & Meijboom, F. L. B. (2019). Animal to human translation: A systematic scoping review of reported concordance rates. *Journal of Translational Medicine*, 17(1), 1–22. <https://doi.org/10.1186/s12967-019-1976-2>
- Lener, T., Gioma, M., Aigner, L., Börger, V., Buzas, E., Camussi, G., Chaput, N., Chatterjee, D., Court, F. A., delPortillo, H. A., O'Driscoll, L., Fais, S., Falcon-Perez, J. M., Felderhoff-Mueser, U., Fraile, L., Gho, Y. S., Görgens, A., Gupta, R. C., & Hendrix, A. (2015). Applying extracellular vesicles based therapeutics in clinical trials – An ISEV position paper. *Journal of Extracellular Vesicles*, 4(July 2016), 1–31. <https://doi.org/10.3402/jev.v4.30087>
- Luo, W., Dai, Y., Chen, Z., Yue, X., Andrade-powell, K. C., & Chang, J. (2020). Spatial and temporal tracking of cardiac exosomes in mouse using a nano-luciferase-CD63 fusion protein. *Communications Biology*, No. 2020, 1–9. <https://doi.org/10.1038/s42003-020-0830-7>
- McNamara, R. P., Caro-Vegas, C. P., Costantini, L. M., Landis, J. T., Griffith, J. D., Damania, B. A., & Dittmer, D. P. (2018). Large-scale, cross-flow based isolation of highly pure and endocytosis-competent extracellular vesicles. *Journal of Extracellular Vesicles*, 7(1), 1541396. <https://doi.org/10.1080/20013078.2018.1541396>
- Mendt, M., Kamerkar, S., Sugimoto, H., McAndrews, K. M., Wu, C. C., Gagea, M., Yang, S., Blanko, E. V. R., Peng, Q., Ma, X., Marszalek, J. R., Maitra, A., Yee, C., Rezvani, K., Shpall, E., Lebleu, V. S., & Kalluri, R. (2018). Generation and testing of clinical-grade exosomes for pancreatic cancer. *JCI Insight*, 3(8), e99263. <https://doi.org/10.1172/jci.insight.99263>
- Morishita, M., Takahashi, Y., Nishikawa, M., Sano, K., Kato, K., Yamashita, T., Imai, T., Saji, H., & Takakura, Y. (2015). Quantitative analysis of tissue distribution of the B16BL6-derived exosomes using a streptavidin-lactadherin fusion protein and iodine-125-labeled biotin derivative after intravenous injection in mice. *Journal of Pharmaceutical Sciences*, 104(2), 705–713. <https://doi.org/10.1002/jps.24251>
- Németh, K., Varga, Z., Lenzinger, D., Visnovitz, T., Koncz, A., Hegedűs, N., Kittel, Á., Máthé, D., Szigeti, K., Lőrincz, P., O'Neill, C., Dwyer, R., Liu, Z., Buzás, E. I., & Tamási, V. (2021). Extracellular vesicle release and uptake by the liver under normo- and hyperlipidemia. *Cellular and Molecular Life Sciences*, 78, 7589–7604. ; No. 0123456789. <https://doi.org/10.1007/s00018-021-03969-6>
- Noren Hooten, N., Yañez-Mó, M., DeRita, R., Russell, A., Quesenberry, P., Ramratnam, B., Robbins, P. D., Di Vizio, D., Wen, S., Witwer, K. W., & Languino, L. R. (2020). Hitting the bullseye: Are extracellular vesicles on target? *Journal of Extracellular Vesicles*, 10, e12032; No. September. <https://doi.org/10.1002/jev2.12032>

- O'Loughlin, A. J., Mäger, I., de Jong, O. G., Varela, M. A., Schiffelers, R. M., El Andaloussi, S., Wood, M. J. A., & Vader, P. (2017). Functional delivery of lipid-conjugated siRNA by extracellular vesicles. *Molecular Therapy*, 25(7), 1580–1587. <https://doi.org/10.1016/j.ymthe.2017.03.021>
- Ophelders, D., Wolfs, T., Jellema, R., Zwanenburg, A., Andriessen, P., Delhaas, T., Ludwig, A., Radtke, S., Peters, V., Janssen, L., Giebel, B., & Kramer, B. (2016). Mesenchymal stromal cell-derived extracellular vesicles protect the fetal brain after hypoxia-ischemia. *Stem Cells Translational Medicine*, No. 5, 754–763.
- Pascucci, L., Coccè, V., Bonomi, A., Ami, D., Ceccarelli, P., Ciusani, E., Viganò, L., Locatelli, A., Sisto, F., Doglia, S. M., Parati, E., Bernardo, M. E., Muraca, M., Alessandri, G., Bondiolotti, G., & Pessina, A. (2014). Paclitaxel is incorporated by mesenchymal stromal cells and released in exosomes that inhibit in vitro tumor growth: A new approach for drug delivery. *Journal of Controlled Release*, 192, 262–270. <https://doi.org/10.1016/j.jconrel.2014.07.042>
- Pathipati, P., Lecuyer, M., Faustino, J., Strivelli, J., Phinney, D. G., & Vexler, Z. S. (2021). Mesenchymal stem cell (MSC) – Derived extracellular vesicles protect from neonatal stroke by interacting with microglial cells. *Neurotherapeutics*, 18, 1939–1952. <https://doi.org/10.1007/s13311-021-01076-9>
- Pauwels, M. J., Vandendriessche, C., & Vandenbroucke, R. E. (2021). Special DeVeRY: Extracellular vesicles as promising delivery platform to the brain. *Biomedicine*, 9, 1734.
- Pham, T. C., Jayasinghe, M. K., Pham, T. T., Yang, Y., Wei, L., Usman, W. M., Chen, H., Pirisinu, M., Gong, J., Kim, S., Peng, B., Wang, W., Chan, C., Ma, V., Nguyen, N. T. H., Kappei, D., Nguyen, X. H., Cho, W. C., & Shi, J. (2021). Covalent conjugation of extracellular vesicles with peptides and nanobodies for targeted therapeutic delivery. *Journal of Extracellular Vesicles*, 10(4), e12057. <https://doi.org/10.1002/jev2.12057>
- Potz, B. A., Scrimgeour, L. A., Pavlov, V. I., Sodha, N. R., Ruhul Abid, M., & Sellke, F. W. (2018). Extracellular vesicle injection improves myocardial function and increases angiogenesis in a swine model of chronic ischemia. *Journal of the American Heart Association*, 7(12), e008344. <https://doi.org/10.1161/JAHA.117.008344>
- Praetorius, J., & Damkier, H. H. (2017). Transport across the choroid plexus epithelium. *American Journal of Physiology - Cell Physiology*, 312(6), C673–C686. <https://doi.org/10.1152/ajpcell.00041.2017>
- Reiner, A., Witwer, K., van Balkom, B., de Beer, J., Brodie, C., Corteling, R., Gabrielsson, S., Gimona, M., Ibrahim, A., de Kleijn, D., Lai, C., Lötvall, J., Del Portillo, H., Reischl, I., Riazifar, M., Salomon, C., Tahara, H., Toh, W., & Wauben, M. (2017). Concise review: Developing best-practice models for the therapeutic use of extracellular vesicles. *Stem Cells and Development*, 6, 1730–1739.
- Saleh, A. F., Lázaro-Ibáñez, E., Forsgard, M. A. M., Shatnyeva, O., Osteikoetxea, X., Karlsson, F., Heath, N., Ingelsten, M., Rose, J., Harris, J., Mairesse, M., Bates, S. M., Clausen, M., Etal, D., Leonard, E., Fellows, M. D., Dekker, N., & Edmunds, N. (2019). Extracellular vesicles induce minimal hepatotoxicity and immunogenicity. *Nanoscale*, 11(14), 6990–7001. <https://doi.org/10.1039/c8nr08720b>
- Schwartz, M. W., Bergman, R. N., Kahn, S. E., Taborsky, G. J., Fisher, L. D., Sipols, A. J., Woods, S. C., Steil, G. M., & Porte, D. (1991). Evidence for entry of plasma insulin into cerebrospinal fluid through an intermediate compartment in dogs. Quantitative aspects and implications for transport. *Journal of Clinical Investigation*, 88(4), 1272–1281. <https://doi.org/10.1172/JCI115431>
- Somiya, M., Yoshioka, Y., & Ochiya, T. (2018). Biocompatibility of highly purified bovine milk-derived extracellular vesicles. *Journal of Extracellular Vesicles*, 7(1), 1440132. <https://doi.org/10.1080/20013078.2018.1440132>
- Southam, D. S., Dolovich, M., O'Byrne, P. M., & Inman, M. D. (2002). Distribution of intranasal instillations in mice: Effects of volume, time, body position, and anesthesia. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, 282(4 26-4), 833–839. <https://doi.org/10.1152/ajplung.00173.2001>
- Su, Y., Walker, J. R., Park, Y., Smith, T. P., Liu, L. X., Hall, M. P., Labanieh, L., Hurst, R., Wang, D. C., Encell, L. P., Kim, N., Zhang, F., Kay, M. A., Casey, K. M., Majzner, R. G., Cochran, J. R., Mackall, C. L., Kirkland, T. A., & Lin, M. Z. (2020). Novel NanoLuc substrates enable bright two-population bioluminescence imaging in animals. *Nature Methods*, 17(8), 852–860. <https://doi.org/10.1038/s41592-020-0889-6>
- Théry, C., Witwer, K. W., Aikawa, E., Alcaraz, M. J., Anderson, J. D., Andriantsitohaina, R., Antoniou, A., Arab, T., Archer, F., Atkin-Smith, G. K., Ayre, D. C., Bach, J.-M., Bachurski, D., Baharvand, H., Balaj, L., Baldacchino, S., Bauer, N. N., Baxter, A. A., Bebawy, M., & Zuba-Surma, E. K. (2018). Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018): A position statement of the international society for extracellular vesicles and update of the MISEV2014 guidelines. *Journal of Extracellular Vesicles*, 8(1), 1535750. <https://doi.org/10.1080/20013078.2018.1535750>
- Thomi, G., Joergger-Messlerli, M., Haesler, V., Muri, L., Surbek, D., & Schoeberlein, A. (2019). Intranasally administered exosomes from umbilical cord stem cells have preventive neuroprotective effects and contribute to functional recovery after perinatal brain injury. *Cells*, 8(8), 855. <https://doi.org/10.3390/cells8080855>
- Upadhyay, R., Madhu, L. N., Attaluri, S., Gitaí, D. L. G., Pinson, M. R., Kodali, M., Shetty, G., Zanirati, G., Kumar, S., Shuai, B., Weintraub, S. T., & Shetty, A. K. (2020). Extracellular vesicles from human IPSC-derived neural stem cells: MiRNA and protein signatures, and anti-inflammatory and neurogenic properties. *Journal of Extracellular Vesicles*, 9(1), 1809064. <https://doi.org/10.1080/20013078.2020.1809064>
- Urzi, O., Raimondo, S., & Alessandro, R. (2021). Extracellular vesicles from plants: Current knowledge and open questions. *International Journal of Molecular Sciences*, 22(10), 5366. <https://doi.org/10.3390/ijms22105366>
- Usman, W. M., Pham, T. C., Kwok, Y. Y., Vu, L. T., Ma, V., Peng, B., Chan, Y. S., Wei, L., Chin, S. M., Azad, A., He, A. B. L., Leung, A. Y. H., Yang, M., Shyh-Chang, N., Cho, W. C., Shi, J., & Le, M. T. N. (2018). Efficient RNA drug delivery using red blood cell extracellular vesicles. *Nature Communication*, 9(1), 2359. <https://doi.org/10.1038/s41467-018-04791-8>
- Van Deun, J., Mestdag, P., Agostinis, P., Akay, Ö., Anand, S., Anckaert, J., Martinez, Z. A., Baetens, T., Beghein, E., Bertier, L., Berx, G., Boere, J., Boukouris, S., Bremer, M., Buschmann, D., Byrd, J. B., Casert, C., Cheng, L., & Cmoach, A. (2017). EV-TRACK: Transparent reporting and centralizing knowledge in extracellular vesicle research. *Nature Methods*, 14(3), 228–232. <https://doi.org/10.1038/nmeth.4185>
- Verweij, F. J., Revenu, C., Arras, G., Dingli, F., Loew, D., Pegtel, D. M., Follain, G., Allio, G., Goetz, J. G., Zimmermann, P., Herbolme, P., Del Bene, F., Raposo, G., & van Niel, G. (2019). Live tracking of inter-organ communication by endogenous exosomes in vivo. *Developmental Cell*, 48(4), 573–589.e4. <https://doi.org/10.1016/j.devcel.2019.01.004>
- Watson, D. C., Bayik, D., Srivatsan, A., Bergamaschi, C., Valentin, A., Niu, G., Bear, J., Monninger, M., Sun, M., Morales-Kastresana, A., Jones, J. C., Felber, B. K., Chen, X., Gursel, I., & Pavlakis, G. N. (2016). Efficient production and enhanced tumor delivery of engineered extracellular vesicles. *Biomaterials*, 105, 195–205. <https://doi.org/10.1016/j.biomaterials.2016.07.003>
- Welsh, J. A., van der Pol, E., Arksteijn, G. J. A., Bremer, M., Brisson, A., Coumans, F., Dignat-George, F., Duggan, E., Ghiran, I., Giebel, B., Görgens, A., Hendrix, A., Lacroix, R., Lannigan, J., Libregts, S. F. W. M., Lozano-Andrés, E., Morales-Kastresana, A., Robert, S., & de Rond, L. (2019). MIFlowCyt-EV: A framework for standardized reporting of extracellular vesicle flow cytometry experiments. *Journal of Extracellular Vesicles*, 9(01), 1713526. <https://doi.org/10.1080/20013078.2020.1713526>
- Wiklander, O. P., Bostancioglu, R., Welsh, J., Zickler, A., Murke, F., Corso, G., Felldin, U., Hagey, D., Evertsson, B., Liang, X., Gustafsson, M., Mohammad, D., Wiek, C., Hanenberg, H., Bremer, M., Gupta, D., Björnstedt, M., Giebel, B., Nordin, J. Z., & Görgens, A. (2018). Systematic methodological evaluation of a multiplex bead-based flow cytometry assay for detection of extracellular vesicle surface signatures. *Frontiers in Immunology*, 9(June), 1326. <https://doi.org/10.3389/fimmu.2018.01326>
- Wiklander, O. P. B., Nordin, J. Z., O'Loughlin, A., Gustafsson, Y., Corso, G., Mäger, I., Vader, P., Lee, Y., Sork, H., Seow, Y., Heldring, N., Alvarez-Erviti, L., Edvard Smith, C. I., Le Blanc, K., Macchiarini, P., Jungebluth, P., Wood, M. J. A., & El Andaloussi, S. (2015). Extracellular vesicle in vivo biodistribution.

- bution is determined by cell source, route of administration and targeting. *Journal of Extracellular Vesicles*, 4(2015), 1–13. <https://doi.org/10.3402/jev.v4.26316>
- Witwer, K. W., & Wolfram, J. (2021). Extracellular vesicles versus synthetic nanoparticles for drug delivery. *Nature Reviews Materials*, 6(2), 103–106. <https://doi.org/10.1038/s41578-020-00277-6>
- Wu, A. Y. T., Sung, Y. C., Chen, Y. J., Chou, S. T. Y., Guo, V., Chien, J. C. Y., Ko, J. J. S., Yang, A. L., Huang, H. C., Chuang, J. C., Wu, S., Ho, M. R., Ericsson, M., Lin, W. W., Cheung, C. H. Y., Juan, H. F., Ueda, K., Chen, Y., & Lai, C. P. K. (2020). Multiresolution imaging using bioluminescence resonance energy transfer identifies distinct biodistribution profiles of extracellular vesicles and exomeres with redirected tropism. *Advancement of Science*, 2001467, 1–17. <https://doi.org/10.1002/adv.202001467>
- Yao, X., Lyu, P., Yoo, K., Yadav, M. K., Singh, R., Atala, A., & Lu, B. (2021). Engineered extracellular vesicles as versatile ribonucleoprotein delivery vehicles for efficient and safe CRISPR genome editing. *Journal of Extracellular Vesicles*, 10(5), e12076. <https://doi.org/10.1002/jev.12076>
- Zhu, X., Badawi, M., Pomeroy, S., Sutaria, D. S., Xie, Z., Baek, A., Jiang, J., Elgamal, O. A., Mo, X., Perle, K. L., Chalmers, J., Schmittgen, T. D., & Phelps, M. A. (2017). Comprehensive toxicity and immunogenicity studies reveal minimal effects in mice following sustained dosing of extracellular vesicles derived from HEK293T cells. *Journal of Extracellular Vesicles*, 6(1), 1324730. <https://doi.org/10.1080/20013078.2017.1324730>
- Zhuang, X., Xiang, X., Grizzle, W., Sun, D., Zhang, S., Axtell, R. C., Ju, S., Mu, J., Zhang, L., Steinman, L., Miller, D., & Zhang, H. G. (2011). Treatment of brain inflammatory diseases by delivering exosome encapsulated anti-inflammatory drugs from the nasal region to the brain. *Molecular Therapy*, 19(10), 1769–1779. <https://doi.org/10.1038/mt.2011.164>

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Driedonks, T., Jiang, L., Carlson, B., Han, Z., Liu, G., Queen, S. E., Shirk, E. N., Gololobova, O., Liao, Z., Nyberg, L. H., Lima, G., Paniushkina, L., Garcia-Contreras, M., Schonvisky, K., Castell, N., Stover, M., Guerrero-Martin, S., Richardson, R., Smith, B., ... Witwer, K. W. (2022). Pharmacokinetics and biodistribution of extracellular vesicles administered intravenously and intranasally to *Macaca nemestrina*. *Journal of Extracellular Biology*, 1, e59. <https://doi.org/10.1002/jex2.59>