Producing vesicle-free cell culture additive for human cells Extracellular Vesicles manufacturing

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Abstract

A new paradigm has emerged recently, which consists in shifting from cell therapy to a more flexible acellular “extracellular vesicle (EV) therapy” approach, thereby opening a new and promising field in nanomedicine.

Important technical limitations have still to be addressed for the large-scale production of clinical-grade EV. Cells are cultured in media supplemented with human platelet lysate (hPL) (xenogenic-free) or GMP-grade fetal calf serum (FCS). However, these additives contain high amounts of EV that cannot be separated from cell-secreted -EV. Therefore, cells are generally maintained in additive-free medium during the EV secretion phase, however this can substantially limit their survival. In the present work, we developed a method to prepare vesicle-free hPL (EV-free hPL) or vesicle-free FCS (EV-free FCS) using tangential flow filtration (TFF). We show a very efficient EV depletion (>98%) for both pure hPL and FCS, with a highly conserved protein content. Culture medium containing our EV-free additives supported the survival of human bone marrow MSC (BM-MSC). MSC could survive at least 216 h, their conditioned medium being collected and changed every 72 h. Both the cell survival and the cumulative EV production were substantially higher than in the starving conditions classically used for EV production. In EV-free hPL containing medium, we show that purified EV kept their morphologic and molecular characteristics throughout the production.

Finally, we tested our additives with 3 other cell types, human primary Endothelial Colony Forming Cells (ECFC) and two non-adherent human cell lines, Jurkat and THP-1. We confirmed that both EV-free hPL and FCS were able to maintain cell survival and EV production for at least 216h.

Our method provides therefore a new option to help producing large amounts of EV from virtually any mammalian cells, particularly those that do not tolerate starvation. This method can apply to any animal serum for research and development purpose. Moreover, EV-free hPL is clinical-grade compatible and allows preparing xenobiotic-free media for massive therapeutic EV production in both 2D (cell plates) and 3D (bioreactor) setting.

Introduction

Cell therapy approaches aim at restoring organ or tissue function. Various cell types are used depending on the indication, including stem or progenitor cells. The efficiency of cell therapy relies on two main processes, deficient cell replacement or indirect trophic effects. Human cells use multiple modes of communication including direct cellular communication through the expression of cell surface molecules or tunnelling nanotubes, secretion of soluble factors or production of extracellular vesicles (EV). This intercellular communication via extracellular cargo is highly conserved across species and EV are likely to be a highly efficient, a robust manner of exchanging information between cells[1].

Several cell types may be of interest for cell therapy, MSC are one of the few cell types to be currently used in clinical practice as a therapeutic agent for immunomodulation or tissue repair[2]. Mesenchymal stromal cells (MSC) are multipotent cells, residing in a large number of adult tissues where they contribute to maintain homeostasis thanks to their immunomodulatory, cyto-protective, pro-angiogenic or anti-fibrotic capacities[3,4]. These effects are mainly mediated by a wide range of soluble molecules and protein-containing EV, mRNAs, miRNAs or mitochondrial fragments[5,6]. Evidence is now accumulating that EV could recapitulate a substantial part of the effects of the cells they originate from and have been proposed as a substitute to cell therapy[7,8]. Switching from cell to EV therapy could improve safety and ease of use with "off the shelf" products immediately available. Moreover, EV are convenient new vehicles for drug delivery, because of their ability to protect their cargo and cross the biological barriers[9].

EV can be isolated from the culture media using various methods including ultra-centrifugation, tangential flow filtration (TFF), immuno-capture, or precipitation[10]. The culture media commonly used for expanding cells require serum (generally Fetal Calf Serum, FCS) or human Platelet Lysate (hPL) in clinical production processes. Both FCS and hPL contain large amounts of EV that cannot be distinguished or separated from the cell-secreted EV. Therefore, the purification of the cell-derived EV needs prior elimination of exogenous EV contained in additives.[11,12,13] This is commonly achieved by expanding the cells in standard conditions, and then incubating them without serum or platelet lysate for a short time to collect the EV. However, starving is stressful and can alter the physiology of the cells. Moreover, cells cannot be maintained for a long time under these conditions, thereby limiting EV production. Another option is to use serum-free or hPL-free complete culture media but these media have important limitations. They contain specific cocktails of growth factors and additives, most often adapted to the expansion of a single cell type. In most cases, these media do not allow cell adhesion, it is necessary to make a coating of the culture surfaces. Few of these media are GMP compliant and finally. They are very expensive, which substantially limits the possibility of producing large volumes of conditioned media. Commercial "exosome/EV-depleted serum and other supplements are available from an increasing number of vendors. Since the method of depletion is usually not indicated, consequences on cell growth and EV release may not be predictable, therefore they should be used with caution[11,12,14].The presence of residual bovine EV has important consequences in EV investigations since it may contaminate the cell-specific EV of human origin produced[12].

This may also preclude the clinical use of the EV since regulatory authorities recommend avoiding animal components in the process of cell expansion. (EMEA/CHMP/410869/2006). Consequently, xenobiotic-free/EV-free culture conditions must be considered for clinical grade EV production. Human platelet lysate offers such a possibility and is a useful substitute to FCS to isolate, amplify and maintain human cells[15]. However, up to now, the production and use of EV-free hPL by TFF has never been reported.

In the present work, our aim was to set-up a reliable method to produce EV-free hPL using scalable, GMP-compatible methods. We used TFF and found optimal conditions to efficiently remove endogenous EV. We showed that our EV-free hPL is a relevant culture additive for human MSC, reducing cell mortality compared to starving conditions and allowing a substantially higher EV production of constant quality over time. We found similar results with EV-free FCS prepared with the same method. We further demonstrated the functionality of our additives on the survival and EV production of very starving-sensitive primary human cells (endothelial colony forming cells) and non-adherent human cell lines (Jurkat and THP-1).

Materials and methods

Preparation of EV-free Human Platelet Lysate by Tangential Flow Filtration (TFF)

Human platelets were provided by the Centre de Transfusion Sanguine des Armées, Clamart, France. A mix of Human Platelet Rich Plasma (hPRP) was obtained by mixing platelets obtained by apheresis (2 donors) and from whole blood buffy coats (5 donors). Subsequently, human Platelet Lysate (hPL) was prepared by a freezing – thawing cycle (-20°C to +37°C) clarification by centrifugation (6000 g for 30 min at +10°C) and filtration of the supernatant on a 0.22 µm filtration unit[16].

Undiluted hPL or FCS samples were subjected to TFF using hollow fiber filters connected to a KrosFlo Research II system under the control of the KF Comm Software Suite. We used dry filters made of Polysulfone (PS) or modified Polyesthersulfone (mPES) (Spectrum Lab) with a nominal cut-off of 50 nm, 500 kDa or 100 kDa (C02-S05U-05-N, C04-E500-10-N, C04-E100-10-N). Filters were first rinsed and hydrated with PBS with at least 2 ml/cm^2 of the filter surface area, as recommended by the manufacturer. Filters were then further conditioned by allowing passage of the hPL or FCS sample into the internal part of the fibers for 10 min at a feed rate allowing a 4000 s^{-1} shear rate. The filtration was then initiated by opening the filtrate port (see figure 1). Pressure sensors allowed monitoring the feeding pressure (P_{feed}), retentate pressure ($P_{retentate}$) and filtrate pressure ($P_{filtrate}$) through the KF Comm acquisition software and the continuous measurement of the trans-membrane pressure (TMP) according to the following equation: $TMP = ((P_{feed}+P_{retentate})/2)-P_{filtrate}$. The EV-Free hPL or FCS samples obtained after TFF were filtered on a 0.22 µm filtration unit.

hPL, the filtrates and the retentates aliquots were first analyzed by Nanoparticule Tracking Analysis (NTA) for their EV content and secondly by size exclusion chromatography (SEC) for protein size distribution analysis as describe below.

Preparation of EV-free hPL and FCS by ultracentrifugation (UC).

Two ml of undiluted hPL or FCS were ultracentrifuged at 120 000 g for 18 h at +4°C or +22 °C (Beckman Coulter TL100 optima max XP equipped with NLS50 rotor, k factor = 158.9,). Only the light-colored top layers of the supernatants (1.5ml) were collected. The same experiment was performed with culture medium consisting of 10% hPL or FCS in αMEM (complete cell culture medium). Following collection, EV contents of UC supernatants were analyzed by NTA. Percentage EV depletion was calculated by comparison of the supernatant EV content after UC (EV_{after}) with EV content of the solutions before UC (EV_{before}): ((EV_{before}-EV_{after})/EV_{before})x100

Bone Marrow Mesenchymal Stromal Cells preparation and culture

Human Bone Marrow MSC (BM-MSC) were obtained after written informed consent from patients undergoing total hip replacement surgery in Percy Hospital (Clamart, France). According to French law a declaration but no ethic comity approval was required for using these surgical residues. As previously reported[17], spongious bone residues were mixed in a solution of phosphate buffered saline (PBS; PAN-Dominique Dutcher) + 1mM EDTA (Prolabo-VWR), + ACD-A (0.32 $g.l^{-1}$ of Citric Acid, 0.88 $g.l^{-1}$ of Sodium Citrate, 0.98 $g.l^{-1}$ of Dextrose) + 0.5% of human serum albumin (HSA; LFB). After 20 min of settling (two times), the supernatant was collected, centrifuged at 460g for 10 min and filtered at 70 µm. The bone marrow mononuclear cells (BM-MNCs) were counted using an automated cell analyzer (Sysmex). BM-MNCs were seeded at 100 000 $cell.cm^{-2}$ in αMEM + 5% hPL + 10 $\mu g.ml^{-1}$ ciprofloxacine (Bayer Pharma) + 2 $U.ml^{-1}$ Heparin (Choay) and cultivated until they reached confluency upon incubation at 37°C in a humidified atmosphere of 5% CO_2. At the end of the first culture step, the population of adherent cells, the BM-MSC, were detached with 0.05% of trypsin-EDTA (Gibco) and frozen in αMEM + 10% human serum albumin (HSA; LFB) + 10% dimethylsulfoxide (DMSO) or seeded again at 2 000 $cells.cm^{-2}$. BM-MSC isolated from 9 donors were amplified and frozen separately or amplified, pooled in equal amounts and then frozen.

For EV's production, BM-MSC from individual donors or a pool of donors were thawed and amplified upon incubation at +37°C in an incubator under a humidified atmosphere of 5% CO_2, cell incubation conditions that were used for all following experiments. When reaching confluency, cells were passaged with trypsin-EDTA and seeded again at a density of 2 000 $cell.cm^{-2}$. Upon reaching 70-80% of confluence, the medium was removed and cells washed 2 times with PBS, placed for one hour in αMEM, then washed one more time in PBS. Then, αMEM supplemented or not with 5% EV-free hPL or 10% EV-free FCS was added for 72h (2.5 ml of medium were added for a 25 cm^2 culture flask and 30 ml for a 300 cm^2 culture flask). The conditioned media were then recovered, centrifuged at 460 g for 5 min to pellet cell in suspension and debris, and the supernatant was kept for EV quantification by NTA. For the analysis of the 3 consecutive 72h incubation periods experiments: the culture medium was recovered at the end of each 72h incubation to quantify and isolate the EV. At each time

and condition, three flasks were used for quantifying cell number and immunophenotyping following cell collection with trypsin-EDTA. Dry pellets of suspended BM-MSC were prepared by pelleting 10^6 cells through centrifugation at 460 g for 5 min and washing the cell pellet two times with PBS before freezing and storing at -80°C until used for protein quantification and western-blot analysis.

Cell culture conditions for amplification of ECFC, Jurkat and THP-1 cells as well as EV production were described in supplementary Material and Methods.

BM-MSC-EV concentration and partial purification by TFF

The BM-MSC conditioned media were centrifuged at 480g for 10 min to discard detached cells and debris. Then, the pellet was discarded and the supernatant filtrated on a 0.22 µm mPES filtration membrane. The BM-MSC-EV content of each harvest was quantified by NTA and stored at -20 °C before proceeding to the isolation of the EV.

After thawing at +4°C overnight, the conditioned media underwent a TFF as described for the preparation of the EV-free media. From our results, the 500 kDa cut-off filter (C04-E500-10-N) was selected. Following a 80% decrease of the conditioned medium volume, an additional PBS diafiltration step was introduced using the same filter by continuously adding 5 volumes of PBS under the control of the KFcom software. Performing this buffer exchange step, that increase TFF time was necessary to allow the wash away of most of the low and medium molecular weight proteins still present in the retentate at the end of the first TFF concentration step. The TFF was further extended until reaching the minimal “retentate" volume leading to the maximal “retentate” concentration (usually up to 50 times). This final “retentate” volume, corresponds to the “dead volume” of the system and therefore does not depend on the volume of the starting conditioned media solution but on the size of the filter and the connections used. Because of the scalability of TFF, it was optimized by adapting hollow fiber filtration surface to the filtration volume subjected to the TFF. This final “retentate” constituted the concentrated and partially purified EV fraction.

Nanoparticle Tracking Analysis

EV size distribution and concentration were determined by nanoparticle tracking analysis (NTA) using a Nanosight NS300 (Malvern-Panalytical, UK) equipped with a 488nm laser. Before measurements, samples were diluted to an appropriate concentration (between 10^8 and 10^9 Particles.ml^{-1} as confirmed by NTA) with sterile PBS (which particle contribution was independently determined by NTA). Samples were injected with a micrometric automatic syringe pump, set at 50 flow rate by the NTA Software, in a +25°C thermostated measuring chamber. Acquisition of data and analysis were performed using the NTA Analytical Software (version 3.4). Optimal focusing was performed on the first “positive” sample of a series. For each sample, 5 movies of 60 s were recorded using a camera level of 14 and analyzed at a detection threshold of 5. Focusing as well as camera level and detection threshold parameters need to be kept constant for comparison of EV number and size distribution in a group of experiments. All others settings were in automatic mode. Particle

concentration ($EV.ml^{-1}$) was calculated following subtraction of PBS NTA background noise and tacking into account the sample dilution factor.

Size Exclusion Chromatography Analysis (SEC)

Analytical Size Exclusion Chromatography. For quality control purpose, hPL samples were analyzed before and after TFF using a Superose 6 increase HR10/300 column (4.10^7 Da exclusion limit) connected either to an AKTA Purifier HPLC or an AKTA-FPLC apparatus from GE-healthcare. 200 µl samples were injected at room temperature, elution was performed at 1 $ml.min^{-1}$ flow-rate with filtrated PBS and 280 nm UV absorbance, as well as conductivity were recorded in-line. Control of the different chromatographic steps, acquisition of data and analysis were performed using the UNICORN software (version 5.31). Exclusion volume was determined using Blue Dextran (2.10^6 Da) and void volume using aprotinin (6500 Da). Low and high molecular weight gel filtration calibration kits (GE-healthcare) were used to test for column efficiency before unknown samples analysis.

BM-MSC-EV polishing step by semi-preparative Size Exclusion Chromatography (SEC). TFF retentates of conditioned media (containing EV) were further purified by SEC using a semi-preparative column (1.6 × 60 cm) containing 120 ml of a matrix of Superose 6 PrepGrade (GE-HealthCare) and connected to an AKTA-FPLC system. Exclusion and void volumes were determined and column calibration performed as described above. TFF retentate, 5 ml samples volume, were injected at room temperature, elution was performed at 1 $ml.min^{-1}$ flow-rate with filtrated PBS and 280 nm UV absorbance was recorded in-line. 5 ml fractions were collected all along the separation run. Each fraction was quantified for EV content, following optimal dilution, using NTA. BM-MSC-EV containing fractions were pooled and concentrated or not using 100 kDa cut-off Amicon ® ultra-15 centrifugal filters (Millipore) exactly as described by the manufacturer.

Cryomicroscopy analysis

BM-MSC-EV samples were processed for cryo-TEM as follows. Briefly, 3 µl of each sample were spotted on 300 mesh glow discharged Lacey grids and cryo-fixed using a Leica EMGP the excess liquid was blotted off with a filter paper, and the grid was then quickly plunged into liquid ethane. EM grids were stored in cryo-boxes under liquid nitrogen until observation.–Samples were imaged using a Tecnai F20 under 200kv, EPU software (Thermofisher) and a Falcon II direct detector (Thermofisher).

Western blotting

Dry pellet of BM-MSC containing 10^6 cells were lysed at 4°C in RIPA buffer (50mM Tris, 150mM NaCl, 0.5% Deoxycholic Acid, 1% Nonidet P-40, 0.1% SDS, pH=8) for 30 min under constant shacking, centrifuged at 15 000 g for 30 min at 4°C and the supernatant was stored at -20°C until use. Protein concentrations of cell lysates as well as

purified BM-MSC-EV were determined with microBCA Protein Assay kit (Thermo scientific) with BSA as standard as described by the manufacturers.

BM-MSC-EV proteins separation by SDS-PAGE was carried out on Novex™ WedgeWell™ 4-12% Tris-Glycine gel (InVitroGen). An amount of 12 µg of proteins per well was denatured by heating at 95 °C for 5 min following addition of 3 volumes of 4X Laemmli Sample Buffer solution in either reducing conditions (5% β-mercaptoethanol final concentration) or not depending on the primary antibody used for blot analysis. After a 2 h migration (in Tris 25 mM / Glycine 192 mM / SDS 0.1% buffer) under a constant electric field of 100 V, separated proteins were transferred onto a 0.45 µm Nitrocellulose membrane (Amersham-Prothan). The non-specific membrane binding sites were blocked with 5% non-fat milk in PBS - Tween 0.1%. Membranes were incubated overnight at 4 °C under constant shaking with the following primary antibodies targeting Alix (cl. OTI1A4, Biorad), Calnexin (Poly cl. Elabscience), CD9 (cl. HI9a, Biolegend), CD63 (cl. TS63, Invitrogen) and CD81 (cl. 5A6, Biolegend), all diluted 1000 times in 1% non-fat milk in PBS -Tween 0.05%.

After 3 washing steps (10 min each) with PBS-Tween 0.05%, the membranes were incubated with a species specific fluorochrome-coupled secondary antibody. The bound fluorescence intensity was observed and quantified using Odyssey infrared scanner (Licor) and the Odyssey v1.2 dedicated software.

EV Proteome Analyses by Liquid Chromatography coupled with Mass Spectrometry

Sample preparation.

Protein concentration in BM-MSC-EV samples were determined using microBCA Protein Assay kit (Thermo scientific) with BSA as standard as described by the manufacturers. BM-MSC-EV proteins obtained after 72, 144 and 216 h were solubilized in 3 volumes of 4X laemmli electrophoresis sample buffer under denaturing (SDS 1% final concentration) and reducing conditions (β-mercaptoethanol 5% final concentration). Duplicate 36 µg of each sample proteins were separated by SDS-PAGE in Novex™ WedgeWell™ 10% Tris-Glycine gel (InVitroGen). After 30 min migration at 100 V, the gels were recovered and each migration track was cut into 10 strips. Each strip was cut into 1 mm^3 cubes and stored in low-binding *Eppendorf* vials. The gel cubes were washed with a (50/50) Acetonitrile (ACN) - 50 mM Ammonium Bicarbonate (AMBIC, Sigma-Aldrich, 09830) solution at room temperature and dehydrated in 100% ACN for 10 min. After ACN removal and gel cube drying on air, the protein disulfide bridges were reduced by adding a 10 mM solution of Dithiothreitol (DTT, Ref: D 0632, Sigma Aldrich) in 50 mM AMBIC for 30 min at +56°C. Reduced cysteines were further alkylated by the addition of a 50 mM Iodoacetamide (IAA, Ref I1149, Sigma Aldrich) in 50 mM AMBIC solution for a 20 min incubation at room temperature in the dark. Then, the gel pieces were washed for 10 min in a 50 mM AMBIC solution and dehydrated in 100% ACN. Finally, the ACN was evaporated and the pieces of gel dried. The alkylated proteins were digested with 10 $ng.\mu l^{-1}$ Trypsin (Sigma-Aldrich, T6567) in AMBIC overnight at +37°C. The trypsin digestion was stopped at room temperature in a 1 mM Formic Acid solution (FA). After centrifugation, the peptide containing supernatants were recovered in new polypropylene tubes. The gel pieces were further washed by adding 100% ACN. The combined supernatants were finally evaporated

under vacuum (Speed-Vac) to remove the solvent and dried samples were stored at -80°C until analysis by liquid chromatography coupled with mass spectrometry (LC-MS-MS).

Liquid Chromatography coupled with Mass Spectrometry.

The dried peptides were reconstituted in 15 µl of loading buffer (0.05% FA) and separated by reverse phase C18 chromatography using the high performance liquid chromatography system (nanoUPLC) nanoACQUITY (Waters, Milford, USA) coupled with a Nanoelectrospray system, hybrid quadrupole ionization, ion mobility mass spectrometry, Time-Of-Flight SYNAPT G2-SI High Definition Mass Spectrometer (NanoESI-Q-IMMS-TOF-HDMSE) from Waters, Manchester, UK controlled by MassLynx software version 4.1.
Once the peptides have been eluted through the HPLC column and injected into the mass spectrometer, the MS data were collected in Data Independent Analysis (DIA) mode and the mass spectrometer was used in positive mode with the analyzer used in the mode resolution 50–4000 m/z.

Data analysis.

Peptides sequences were reconstructed using ProteinLyns Global SERVER 3.0.3 software (PLGS, Waters). These peptides lists were compared to the human SwissProt database (UniProtKB/Swiss-Prot, www.uniprot.org) to identify the source protein using the following parameters: peptide and tolerance of fragment in automatic mode, minimum 1 matching ions per peptide, minimum 1 fragment of matching ions, minimum 3 fragment of ions matching by proteins, minimum 1 single peptide, maximum 1 missed cleavage by trypsin, with the following post modifications translation possibilities: carbamidomethylation of Cysteines and oxidation of Methionine. The maximum false discovery rate (FDR) was set at 1%.

Statistics

Group data were expressed as the mean and SD. Data were analyzed using GraphPad Prism version 7 (GraphPad Software). A Shapiro-Wilk normality test was first performed and statistical significances were determined by one-way anova followed by multiple comparison approach, leading to multiplicity adjusted P values. Calculated two-sided P values were reported on the figures (* for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$, **** for $p < 0.0001$) with p-values < 0.05 considered statistically significant.

Results

TFF parameters optimizations

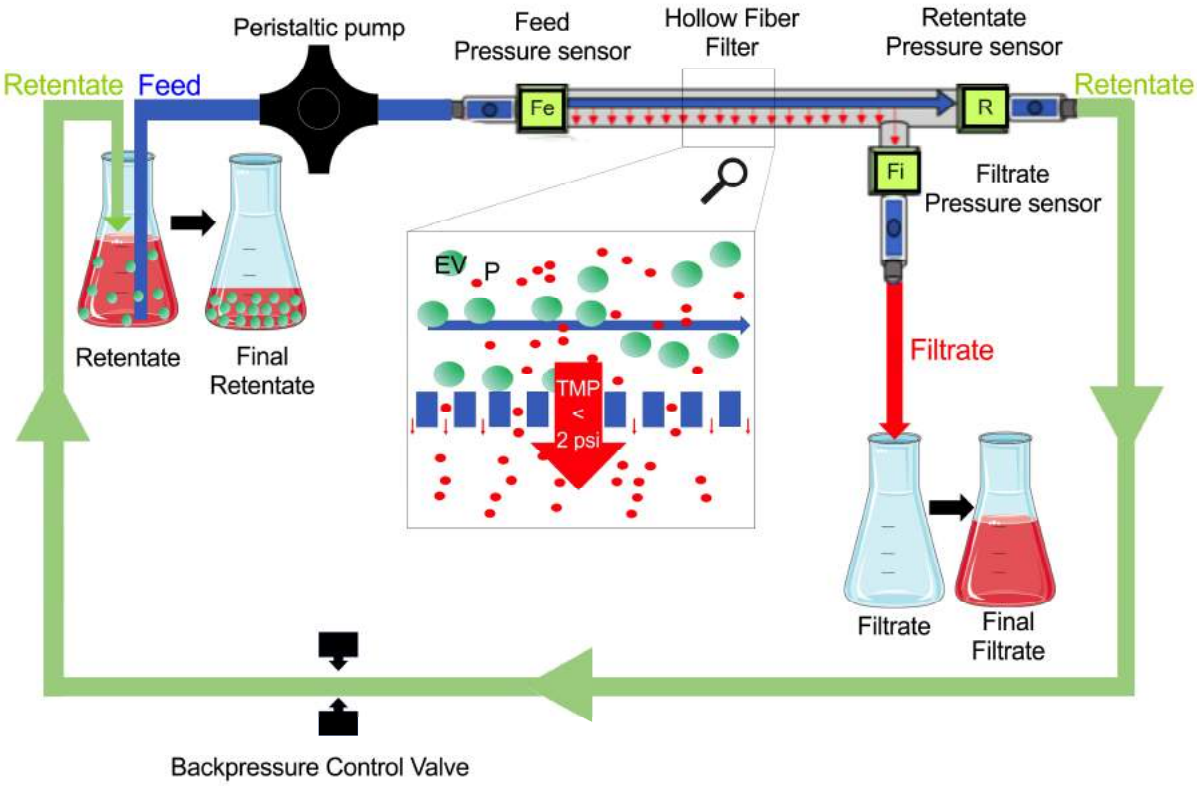

Figure 1. Principle of hPL Tangential Flow Filtration

hPL can be schematize as a solution with small solutes such as proteins (P) (red) and large particle including EVs (green). hPL is placed in a bottle and pumped (peristaltic pump) to bring hPL through a Hollow Fiber Filter. The solution directed to the membrane surface is called the feed and the solution that passes along the filter surface and go back to the bottle is the retentate. The recirculated solution is thus continuously separated in two different compartments: particles smaller than filter cut-off pass through and are collected in the filtrate; particles larger than filter cut-off are retained and go back to the retentate. This cycle is repeated as much as it is necessary to concentrate the larger hPL components in the retentate. Using a filter of the convenient cut-off (see results), EV may be concentrated in the retentate fraction whereas the filtrate provides an EV-free hPL solution. Pressure sensors allow the continuous measurement of pressure in the feed, retentate and filtrate ways and calculation of Trans-Membrane Pressure (TMP). A back pressure control valve that pinches the retentate way allows fixing the TMP to a predefined value through the control software of the TFF system. EV: Extracellular Vesicles, P: soluble proteins

Hundred ml aliquots of hPL were subjected to Tangential Flow Filtrations (TFF). As shown in the TFF diagram of Figure 1**,** a peristaltic pump circulates hPL through a hollow fiber filter of convenient cut-off thus separating hPL in two different fractions: the "filtrate" consisting in EV-free hPL and the EV-enriched "retentate" (Figure 1). The efficiencies of filters with a nominal cut-off of 50 nm, 500 kDa or 100 kDa were compared. The feed stream (hPL or FCS) contains fragile components such as vesicles that may be damaged by high circulation rates. Therefore, the feed rate of the pump was adapted for each filter to obtain a recommended shear rate of 4 000 sec^{-1} (the shear rate is calculated as 8 x Velocity ($m.s^{-1}$)/Fiber Internal Diameter (m) and expressed in sec^{-1}). The resulting flow applied tangentially to the filtration membrane permanently sweeps any un-filtrated material from the membrane surface thus preventing clogging.

Pressure sensors allowed monitoring the feeding pressure (P_{feed}), retentate pressure ($P_{retentate}$) and filtrate pressure ($P_{filtrate}$) (Figure 1) and the continuous measurement of the trans-membrane pressure (TMP) according to the following equation: $TMP = ((P_{feed}+P_{retentate})/2)-P_{filtrate}$. The use of a backpressure valve that pinches the retentate tubing allows TMP to be automatically and permanently adjusted to a preset value. The flow through the filter decreases with time because the fluid concentration increases and the compaction of the macromolecules create a "gel layer resistance". To maintain a sufficient filtration flow rate, TMP between 6.89 and 41.37 kPa (i-e between 1 and 6 psi) were tested on the filtration of hPL through a 500 kDa pore size hollow fiber filter operating at a shear force of 4000 s^{-1} (data not shown). We found that a TMP value of 13.78 kPa (2 psi) was optimal. In any case the filtration process was stopped as soon as the TMP reached 34.47 kPa (5 psi).

Two different fractions were obtained for each filter; the "filtrate" consisting in EV-free hPL and the EV-enriched "retentate".

TFF allowed the production of highly EV-free hPL.

We first compared 3 different TFF filters. The 50 nm and 500 kDa filters allowed us to filter 90% of the starting hPL volume (10 fold retentate concentration) (Figure 2A) with a trans-membrane pressure (TMP) value kept below 5 psi (34.47 kPa) and at an average filtration rate (permeate flux) of 17.3 $l.m^{-2}.h^{-1}$ and 12.1 $l.m^{-2}.h^{-1}$ respectively for the overall process

Conversely, the 100 kDa filtration was stopped because of an undesired and uncontrolled increase in TMP above 5 psi and only 84% volume could be filtrated at a lower average filtration rate of 7.8 $l.m^{-2}.h^{-1}$(Figure 2 B,C).

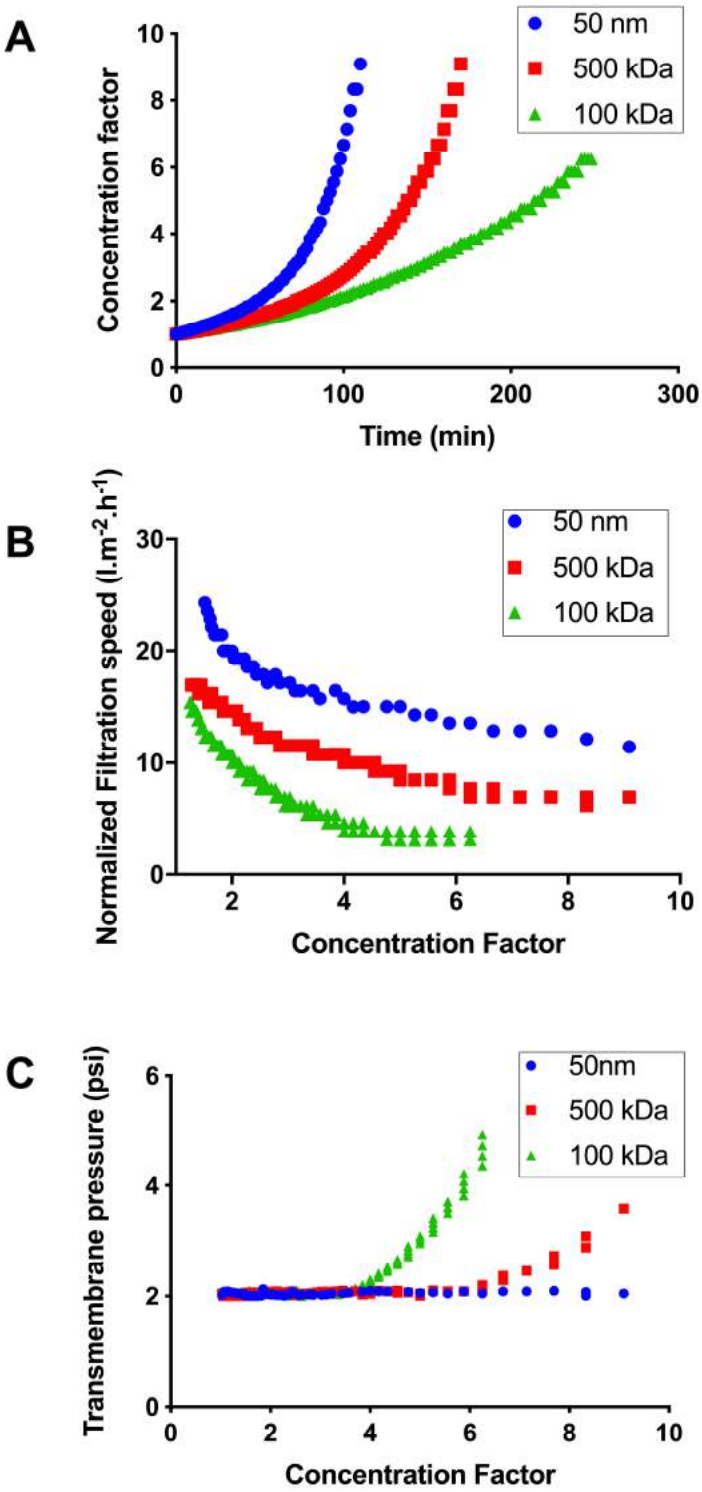

Figure 2. hPL TFF monitoring

Evolution of the Concentration Factor (CF) versus time (**A**), the Trans-Membrane Pressure (TMP) (**B**) and the normalized filtration speed (**C**) versus the CF. Hollow fiber filters of 100 kDa (green), 500 kDa (red) or 50 nm (blue) are registered by the control software of the KR2i TFF system.

The particles concentration and size were measured by Nano Tracking Analysis NTA showed that hPL contained a high concentration of endogenous EV ($2.03x10^{11}$ +/- $9.25x10^{10}$

EV.ml^{-1} from 9 different hPL). More than 90% of those EV had a size range from 50 nm to 200 nm with a median size of 125.5 nm (Figure 3A for a representative experiment). 50 nm, 500 kDa and 100 kDa cut-off filters efficiently removed EV, providing hPL filtrates containing no more than 2.3%, 0.3% and 0.3% of the initial EV content, respectively. As could be expected, the 50 nm filtration was slightly less efficient than the others, especially for EV of hydrodynamic diameters below 100 nm (Figure 3A).

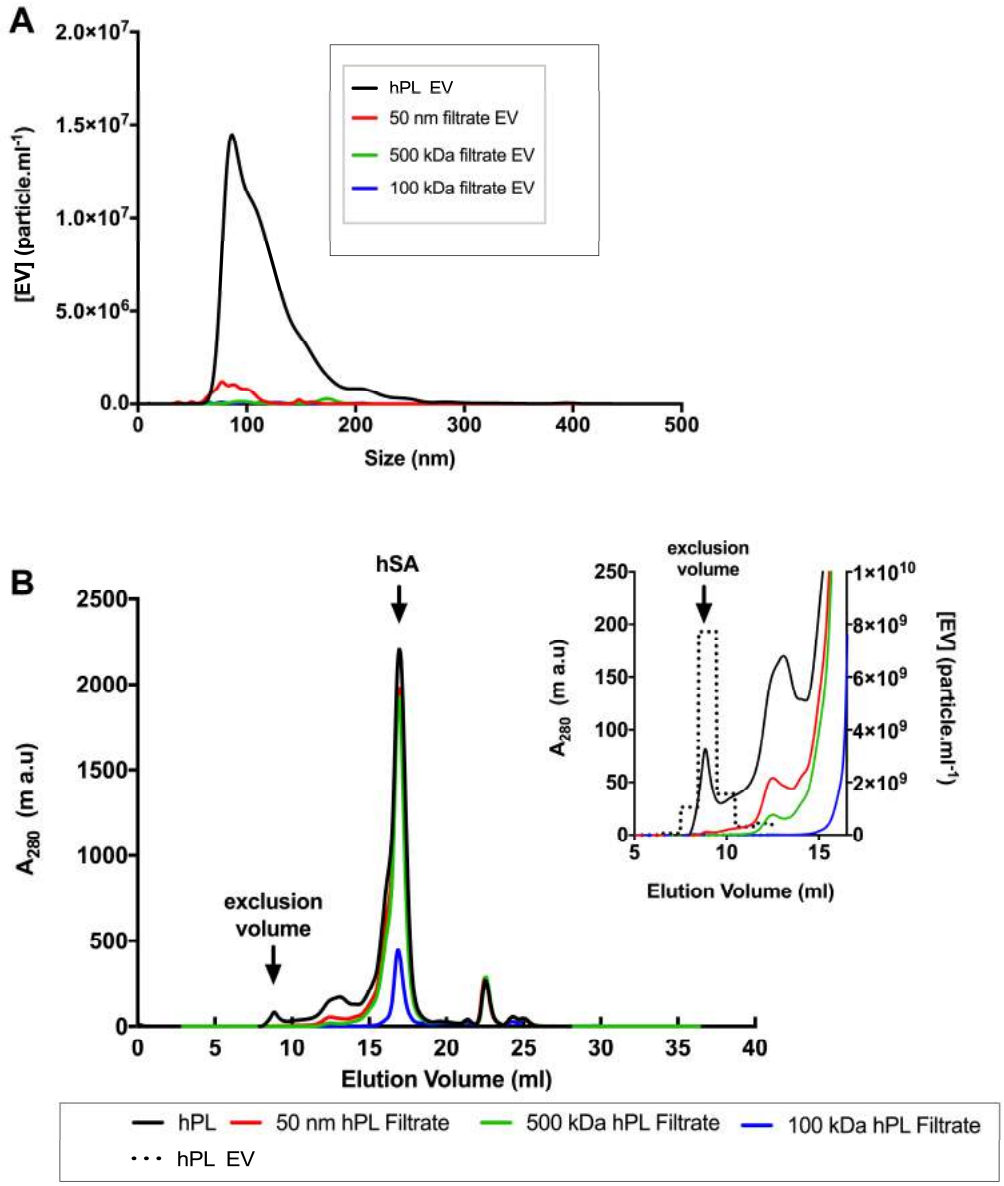

Figure 3. hPL EV depletion by Tangential Flow Filtration

A) Nanoparticle Tracking Analysis (NTA) of hPL and TFF hPL filtrates. EV hydrodynamic diameter distribution in hPL (black) and in 50 nm (red), 500 kDa (green) and 100 kDa (blue) filtrates.

B) Analytical Size Exclusion Chromatograph (SEC) of hPL and TFF hPL filtrates. Proteins content of hPL (black), 50 nm (red), 500 kDa (green) and 100 kDa (blue) filtrates are analyzed by chromatography on superose 6 increase column, elutions are monitored following 280 nm absorbance. **B Insert)** One ml fractions from hPL and of 100 kDa (green), 500 kDa (red) or 50 nm (blue) filtrates eluted from the SEC chromatography are collected and analysed by NTA. EV content of hPL exclusion volume fractions are reported. EV content of the of 100 kDa, 500 kDa or 50 nm filtrates exclusion volume fractions are not visible at this scale.

hPL and 500 kDa hPL filtrate had similar protein profiles.

For quality control purpose, hPL filtrates were further analyzed by size exclusion chromatography (SEC). The protein content in the effluent was continuously monitored by measuring the optic deviation (OD) at 280 nm. The protein elution profile of 500 kDa and 50 nm filtrates were very similar to that of the hPL, (Figure 3B) except for the higher molecular weight proteins (or protein complexes) eluted before 15 ml (Figure 3B insert). The 100 kDa filtrate (blue curve, Figure 3B) contained less low molecular weight proteins such as human serum albumin (hSA) eluting at 17 ml that were more retained in the 100 kDa retentate (Figure 4C) than the others (Figure 4 A, B)

As expected, NTA showed that hPL EV were eluted in the "exclusion volume" of the column (around 9 ml) as shown with the black dotted curves (Figure 3B Insert). In the 100 kDa, 500 kDa and 50 nm filtrates SEC fractions, the exclusion volume contains negligible (50 nm filter) or undetectable (100 & 500 kDa filters) amounts of EV (supplementary Figure S1). Indeed, NTA showed that EV were retained in the TFF retentates and appeared in the exclusion volumes following SEC, as shown on Figure 4.

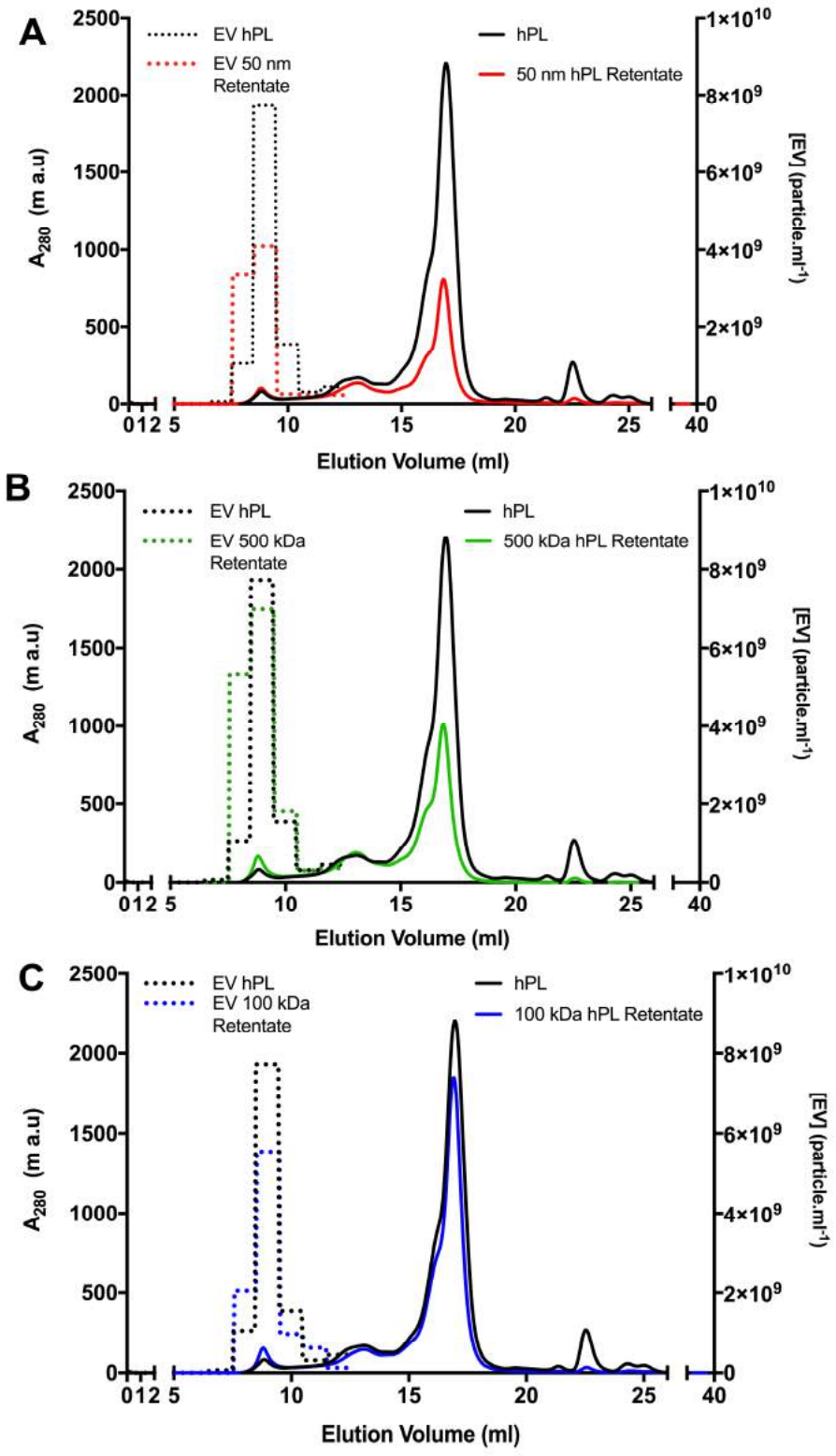

Figure 4. hPL EV concentration in the Tangential Flow Filtration retentates

Analytical Size Exclusion Chromatograph of hPL and TFF hPL retentates. Proteins content (solid lines) of hPL (black), 50 nm (red **A**), 500 kDa (green **B**) and 100k Da (blue **C**) retentates are analysed by chromatography, elutions are monitored following 290 nm absorbance (left scale). One ml eluted fractions from hPL and each retentate is collected and analyzed by NTA (dotted lines). EV content of exclusion volume fractions is reported (right scale).

Considering the excellent EV depletion (>99%), the similarity between hPL and 500 kDa-filtrated hPL 280 nm OD profiles and the ability to maintain optimal filtration settings, the 500k Da cut-off filter has been chosen for the following experiments to produce an hPL filtrate that will be called "EV-free hPL" in all following experiments.

TFF is more efficient than ultracentrifugation for hPL and FCS EV depletion.

As stated in Théry et al.[14], EV depletion from FCS is usually performed by an ultra-centrifugation step (UC) of the culture medium (usually a 10% FCS in culture medium, or following at least 1:4 dilution) for at least 18 h at 100 000g or shorter period at higher speed, without recommending a specific temperature. Here, a 120 000 g UC for 18 h at +4°C or +22°C was applied to hPL or FCS. The efficiency of UC depletion was compared to TFF using a 500 kDa cut-off filter. TFF was highly efficient at depleting EV from undiluted hPL or FCS, with depletion percentages of 99.5 +/- 0.5 % (n=8) for hPL and 97.89 +/- 1.9 % (n=12) for FCS. Conversely, 120 000 g UC for 18h, either at 4°C or 22°C allowed only limited EV depletion of see results on table 1. Dilution of hPL or FCS to 10% in culture media do not improve UC depletion at any time and temperature (data not shown).

Table 1. Comparison of EV depletion by UltraCentrifugation and 500 kDa Tangential Flow Filtration

Comparative analysis of particles/EV content in ultracentrifugation supernatants (120 000g for 18h at +4°C or +22°C) and 500 kDa TFF (+22°C) filtrates were performed by NTA. EV depletion was expressed as a percentage of particles concentration remaining compared to initial values. Results are expressed as mean +/- SE of n different experiments as reported on the table.

		EV Depletion Percentage after UC 18h at 120 000 g		EV Depletion Percentage after TFF 500 kDa	
Temperature	Sample	Mean +/- SE	n	Mean +/- SE	n
+ 4°C	Undiluted FCS	47.12 % +/- 18.9	3	NA	NA
	Undiluted hPL	16.46 % +/- 2.5	3	NA	NA
+ 22°C	Undiluted FCS	41.10 % +/- 5.79	3	97.89 % +/- 1.9	12
	Undiluted hPL	10.27 % +/- 7.95	5	99.54 % +/- 0.5	8

EV-free hPL supplemented media supported BM-MSC EV production

EV production by BM-MSC prepared from 9 different donors. We evaluated the EV production by BM-MSC from 9 donors after 72h of incubation in αMEM supplemented with 5% EV-free hPL. Important amounts of EV were produced (Figure 5) with some variability between donors (from 1 to 2.5 10^9 EV.ml^{-1} with a mean production value of $1.42x10^9$ +/- $3.5x10^8$ EV.ml^{-1}). When a pool containing equal amounts of BM-MSC from each donor was prepared, NTA showed that the EV secretion ($1.34x10^9$ +/- $2.4x10^8$ EV.ml^{-1}) was similar to the calculated mean production of the 9 donors. Thus, in order to overcome any inter-donor variability, the pool of BM-MSC donors was used for all following experiments. The NTA analyzes carried out on the non-depleted αMEM + 5% hPL indicate the presence of a very large quantity of particles per mL around $1.56x10^{10}$ EV.mL^{-1} +/- $3.84x10^9$ (Figure 5). Therefore, a depletion of at least 99% of the hPL NTA signal was necessary to obtain EV from BM-MSC with limited "contamination" by remaining hPL EV. Indeed, 5% hPL depleted at 99% will contribute to $1x10^8$ EV.ml^{-1} in the conditioned media (Figure 5, T0 condition), a 90% depletion will lead to a ten folds higher contribution of 10^9 EV.ml^{-1}. This may have a very important impact on the proportion of the EV signal that could be attributed to cells. Indeed, if we apply these values to the quantification of EV production by our BM-MSC pool, 92.6 +/- 1.63 % (n=9) could be attributed to BM-MSC following a 99% depletion whereas, hPL EV contamination and BM-MSC EV production become comparable under a limited 90 % depletion.

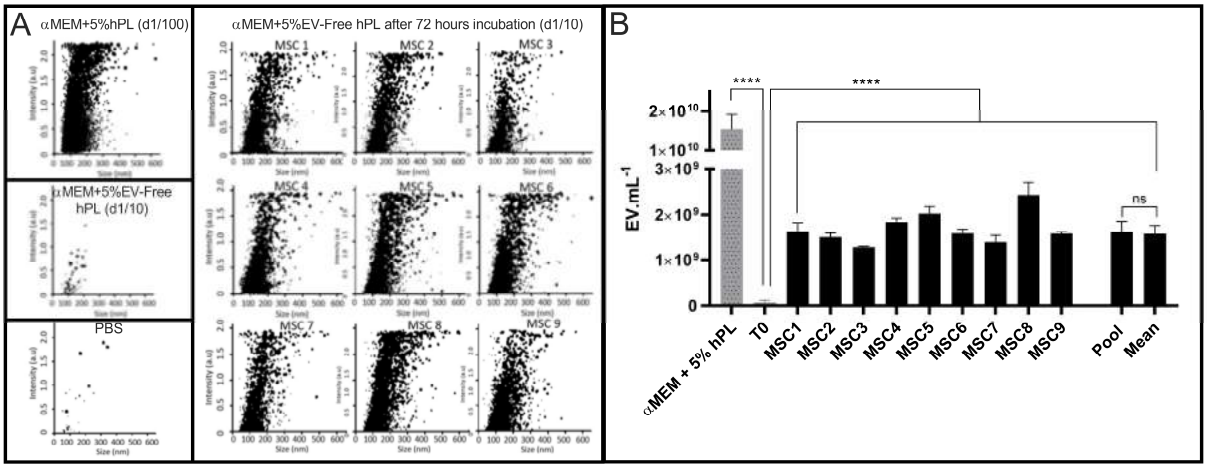

Figure 5. BM-MSC EV production in αMEM supplemented with EV-Free hPL

A) Representation of NTA analyses from PBS, αMEM supplemented with 5% hPL or EV-Free hPL as well as BM-MSC-EV productions (72 h) from 9 individual donors. B) Quantitative analysis by NTA of the BM-MSC-EV productions from 9 donors and from of a pool of the 9 donors (72 h), the Mean condition corresponds to the average calculation of the EV.ml-1 obtained for each MSC donor. Results were expressed as mean +/- SE from 3 independent incubations of each donor and the pool of all donors. The calculated mean EV content of the 9 different individual BM-MSC conditioned media was also indicated (Mean). Statistical significance of change in EV concentration between conditions was evaluated using multiple comparison analysis following one-way ANOVA. Multiplicity adjusted P values for difference in EV content in the presence of either 5% hPL or 5% EV-free hPL (T0) as

well as between 72h production of EV by BM-MSCs in 5% EV-free hPL compared to T0 are reported on the figure . NS: Not Significant *: P<0.05; **: P<0.01; ***: P<0.001; ****: P<0.0001.

EV-free hPL supplemented media supported prolonged BM-MSC cell survival and EV production. EV productions are usually performed in starving condition, often limiting the production duration to 48 h or 72 h. Therefore, the ability of αMEM containing EV-free hPL to sustain BM-MSC survival and EV production was compared to starving condition. Cells were first grown in amplification medium with 5% full hPL until near confluency, then extensively washed and incubated for 3 successive periods of 72 h (72 h, 144 h and 216 h) in αMEM (without antibiotics and heparin) supplemented or not with 5% EV-free hPL or 10% EV-free FCS. We observed that whereas the addition of heparin in the incubation medium is absolutely necessary to prevent clot formation in the presence of full hPL, αMEM containing EV-free hPL do not clot (data not shown) and heparin was thus omitted. The morphology of the cells was preserved in all conditions regardless of the incubation time (data not shown). Cell count confirmed the decrease in cell number in starving condition and the ability of EV-free hPL and EV-free FCS to maintain cell survival (Figure 6A). As shown on Figure 5B, improved cell survival of MSC in EV-free hPL or FCS supplemented media correlates with a time-dependent increased EV production without any changes in the EV hydrodynamic diameter distribution as quantified by NTA (Figure 6C). Following the three consecutive 72h periods, the cumulative productions of EV were increased by 2.5 in the presence of 5% EV-free hPL and by 3.5 in the presence of 10% EV-free FCS compared to αMEM alone.

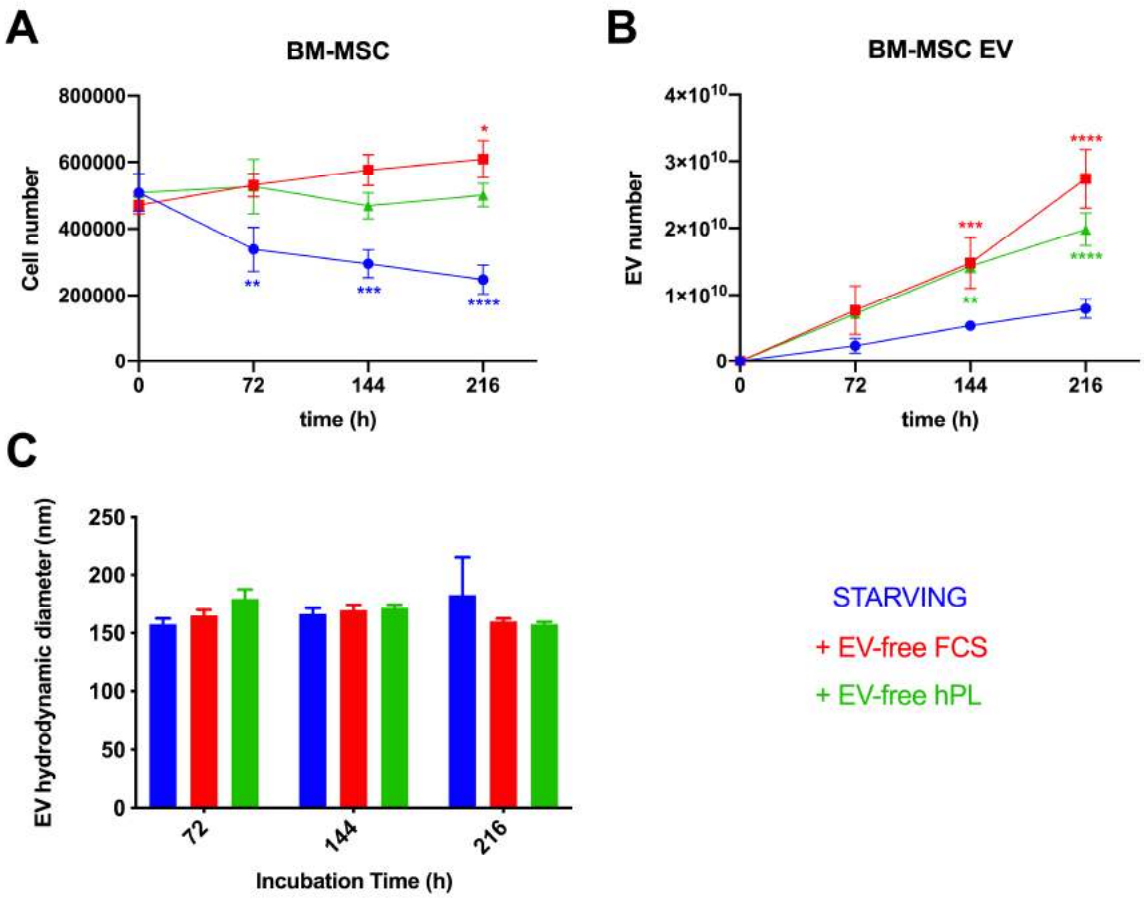

Figure 6. BM-MSC survival and EV production during secretion phase in αMEM media supplemented or not with 5% EV-Free hPL or 10% EV-Free FCS

A) Quantification of BM-MSC number following 3 consecutive 72 h periods (72 h, 144 h and 216 h) in αMEM alone (STARVING, blue) or supplemented with 5% EV-Free hPL (green) or 10% EV-Free FCS (red) with medium change every 72 h. Results are expressed as mean +/- SE from 3 independent incubations for each time and condition. Statistical significance of change in cell number compared to zero time (T0) was evaluated using multiple comparison analysis following one-way ANOVA. *: P<0.05; **: P<0.01; ***: P<0.001. **B)** Conditioned Media of MSC for 3 consecutive 72 h periods in αMEM alone (STARVING, blue) or supplemented with 5% EV-Free hPL (green) or 10% EV-Free FCS (red) were analysed by NTA for their EV content. Cumulative productions are reported (mean +/- SE) from 3 independent incubations for each time and condition. Multiple comparison analysis was performed following one-way ANOVA. Only multiplicity adjusted P values for difference in EV content in the presence of either 5% EV-free hPL or 10% EV-free FCS compared to αMEM alone at the same time are reported on the figure. *: P<0.05; **: P<0.01; ***: P<0.001; ****: P<0.0001. **C)** Median EV hydrodynamic diameter is measured by NTA for αMEM alone (STARVING, blue) or supplemented with 5% EV-Free hPL (green) or 10% EV-Free FCS (red) at 72 h, 144 h and 216 h collection times (mean +/- SE) from 5 independent determinations. No significant statistical difference between any conditions was observed using multiple comparison following one-way ANOVA.

Purification and characterization of BM-MSC EV

EV Concentration / pre-purification by TFF. TFF is an efficient way to concentrate EV from cell-conditioned media[18]. With this method, EV stay in the retentate (Figure 1). TFF fully recovered EV in the retentate fraction of BM-MSC conditioned medium with 5% EV-free hPL or 10% EV-free FCS (data not shown). TFF was performed to

concentrate/enrich 500 ml of BM-MSC-conditioned media collected following three consecutive 72 h incubation periods. NTA quantification after 72 h, 144 h and 216 h were $1.07x10^9$; $1.39x10^9$ and $1.58x10^9$ EV.ml^{-1} respectively. Each production underwent a 500 kDa cut-off TFF with a final PBS diafiltration step. This allowed a first EV concentration / pre-purification resulting in 'retentates' fractions containing $4.06x10^{10}$; $1.33x10^{11}$ and $1.44x10^{11}$ EV.ml^{-1}, after 72 h, 144 h and 216 h respectively, the volumes of which are indicated in Table 2

Table 2. Summarized data of EV content during the purification process
Conditioned media from BM-MSC incubated for 72 h, 144 h and 216 h in the presence of αMEM supplemented with 5% EV-Free hPL were prepared by TFF and SEC. The table summarizes the volumes and EV concentrations at the different steps of the EV purification process.

		72H	144H	216H
Conditioned Media	Volume (ml)	533	511	479
	[EV] (EV.ml^{-1})	$1.07 x10^9$	$1.39 x10^9$	$1.58 x10^9$
Retentat 500kDa TFF	Volume (ml)	11.5	12	15
	[EV] (EV.ml^{-1})	$4.06 x10^{10}$	$1.33 x10^{11}$	$1.44 x10^{11}$
After SEC	Volume (ml)	30	30	45
	[EV] (EV.ml^{-1})	$7.80 x10^9$	$1.55 x10^{10}$	$1.89 x10^{10}$
After 100kDa concentration	Volume (ml)	2.78	3.86	4.76
	[EV] (EV.ml^{-1})	$1.50 x10^{11}$	$1.50 x10^{11}$	$1.50 x10^{11}$

Final purification by SEC. TFF retentates from each incubation period of 72 h were subjected to an additional SEC step. As expected, EV were eluted in the exclusion volume of the column which was clearly separated from the main protein peaks (Figure 7A, B, C). Thus, the addition of the SEC step was very efficient to separate EV from most of the other components of the conditioned media including medium molecular weight (MMW) and low molecular weight (LMW) proteins that are incompletely eliminated by TFF despite the diafiltration step.

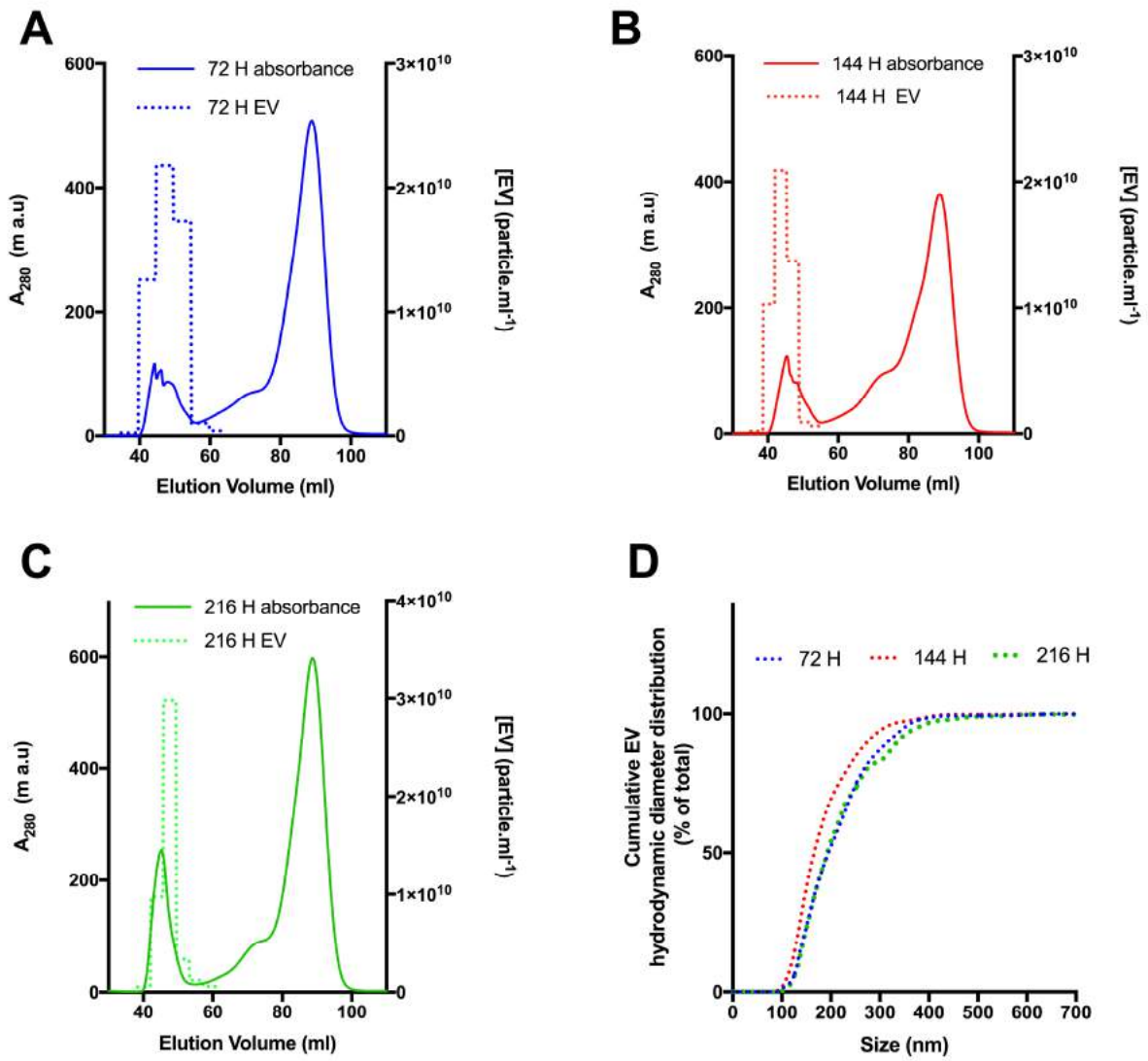

Figure 7. EV purification by Size Exclusion Chromatography following Tangential Flow Filtration

Conditioned media from BM-MSC incubated for 3 consecutive 72 h time periods (72 h (A), 144 h (**B**) and 216 h (**C**)) in the presence of αMEM supplemented with 5% EV-Free hPL were first concentrated by 500 kDa TFF and the retentate further analysed and purified by SEC. Proteins content was analysed following 280 nm absorbance of the eluted fractions (solid lines). Five ml fractions were collected and analyzed by NTA for their EV content (dotted lines). Normalised cumulative EV hydrodynamic diameter distributions were further determined by NTA for each time point and reported in (**D**).

After SEC, the EV containing fractions of the exclusion volume (usually fraction 4 to 6 or 7) were pooled providing samples containing $7.80x10^{9}$; $1.55x10^{10}$; $1.89x10^{10}$ EV.ml^{-1} for 72 h, 144 h and 216 h respectively (Table 2). NTA showed that EV size distribution was very similar for all three 72 h incubation periods (Fig 7D). The samples were further concentrated by ultrafiltration on 100 kDa Amicon ultra centrifugal filter unit in order to readjust their concentrations to an identical value of $1.5x10^{11}$ EV.ml^{-1}. The 72 h, 144 h and 216 h EV-enriched samples were further used for cryo-TEM and proteomic analysis.

EV characterization

Cryomicroscopy analysis of the EV sample purified from all three consecutive 72 h

incubation period showed round-shape membrane delimited vesicular structures (Figure 8A) in a size range similar to that observed by NTA.

Protein composition analyzis. Samples were further analyzed by Western blot for the presence of EV markers. As shown on Figure 8B, the membrane-associated proteins CD9, CD63, CD81 and the soluble protein Alix, suggested as EV positive controls[14], were detected in similar amounts in all three (72 h, 144 h and 216 h) EV samples. These proteins were also found in BM-MSC lysates. Conversely, the reticulum marker calnexin, suggested as an EV negative control[14] was undetectable in EV samples but detected in cell lysates. The presence of similar amount of each membrane marker (CD9, CD63 and CD81) as well as the soluble marker Alix in all three EV-enriched samples further confirms the presence and production of EV that are qualitatively comparable all along the 3x72h incubation periods of BM-MSC.

This result was further confirmed by the mass-spectrometry analysis of the protein content of these EV-enriched samples. When retaining proteins identified with at least 95% confidence degree (green tag) and discarding potentially contaminating keratin, LC-MS-MS identified 762, 999 and 822 proteins after respectively 72 h, 144 h and 216 h incubation periods, resulting in a total of 1109 different proteins from all three samples taken together (Supplementary Table S1). Finally, 484 proteins were common to all three EV samples (Figure 8C, middle). All samples contained MSC markers CD73, CD90 and CD29 that have been identified at the surface of BM-MSC by FACS analysis (Supplementary Table S2). Conversely, CD105 and CD44 BM-MSC markers were not detected suggesting the possibility of a different sorting of these membrane proteins in this cell type. We then searched for the presence of platelet markers (CD41, CD42, P-selectin) or other circulating cell (CD11b, CD31, CD34, CD45, Glycophorin A) that may indicate the presence of contaminating EV originating from hPL. None of them was detected in any sample

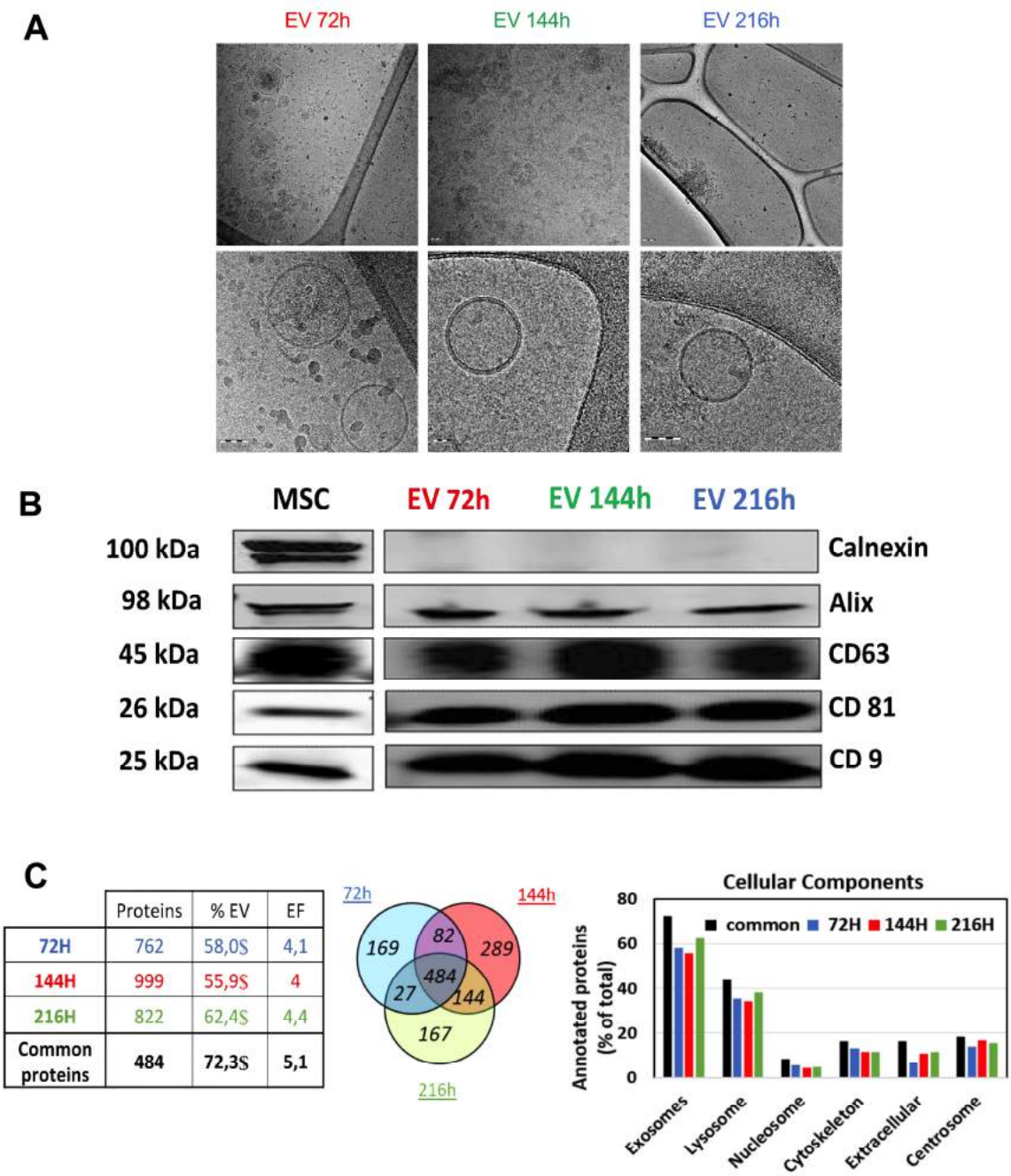

Figure 8. Morphologic and proteomic characterization of BM-MSC EV produced in αMEM supplemented with 5% EV-Free hPL

BM-MSC were incubated for 3 consecutive 72 h time periods (72 h, 144 h and 216 h) in the presence of αMEM supplemented with 5% EV-Free hPL. EV were isolated by TFF and SEC. **A**) Cryo-Electron Microscopy photography of EV produced during the 72h, 144h and 216h incubation times. Scale bars are reported on each photography. **B**) Western blot analysis of the endoplasmic reticulum Calnexin and classical EV markers (*Alix*, CD63, CD81, CD9). Left legend refers to antigen size (kDa) and right legend to antigen name. **C**) LC-MS-MS analysis of BM-MSC EV proteins. Venn diagram (middle) summarizes the distribution of the 1109 proteins identified within the 3 groups 72 h, 144 h and 216 h. Table (left) reports the percentages of EV-annotated proteins in each sample as well as common proteins (determined using Human proteome Funrich Database). Enrichment Factor (EF) was calculated by comparing the percentage of annotated EV-proteins in each sample to the whole human proteome (Funrich Database). On the right, Funrich was also used to provide the percentage of annotated gene/protein for the 6 most represented cellular components for each sample and the common protein lists.

Gene ontology enrichment analysis using the Funrich v3.1.3 software tool (http://www.funrich.org)[18], was carried out considering the protein list of each individual sample and proteins common to all three samples. Only 629, 843, 715 and 433 Uniprot identifiers respectively for the 72 h, 144 h, 216 h samples or common to all three samples where available in the Cellular component database and retained for analysis by the Funrich software (supplementary Table S3). Analysis was carried out in comparison to whole human proteome for cellular component. The top 6 most relevant annotations for 72 h (lowest p value and highest % of annotated proteins) were plotted in comparison to the other groups (144 h, 216 h and common), and very similar distribution were observed. The exosomal compartment was enriched in all data sets (Figure 8C, right) with 58% of the proteins for 72 h, 56% for 144 h and 62% for 216 h, it increased to 72% for the common proteins. Moreover, the exosomal annotations for 72 h, 144 h and 216 h EV proteins displayed similar fold enrichment of 4.1, 4 and 4.4 respectively. This value further increased to 5.1 for the common proteins (Fig 8C, left). A total of 74 proteins for 72 h, 79 for 144 h and 76 for 216 h, were among the "top 100" proteins most often identified in exosomes from Exocarta database (http://www.exocarta.org). This strongly indicates that the protein composition of EV-enriched fraction of BM-MSC did not vary significantly during the 3 consecutive incubation periods in EV-free hPL.

EV-free hPL and FCS sustained survival and EV production of other cell types

MSC-EV are promising for treating several diseases, but other cell types are of interest, including cells that are more difficult to maintain in culture. Therefore, we studied the ability of our EV-free additives to sustain the culture of endothelial cells such as ECFC. As expected, starvation (EBM2+MV without serum) drastically limited the ECFC survival, thereby preventing EV secretion. The supplementation of EBM2+MV with our EV-free hPL or FCS sustained ECFC survival for at least three consecutive 72 h periods (Figure 9A), therefore allowing unrestricted ECFC-EV secretion (Figure 9B)**.**

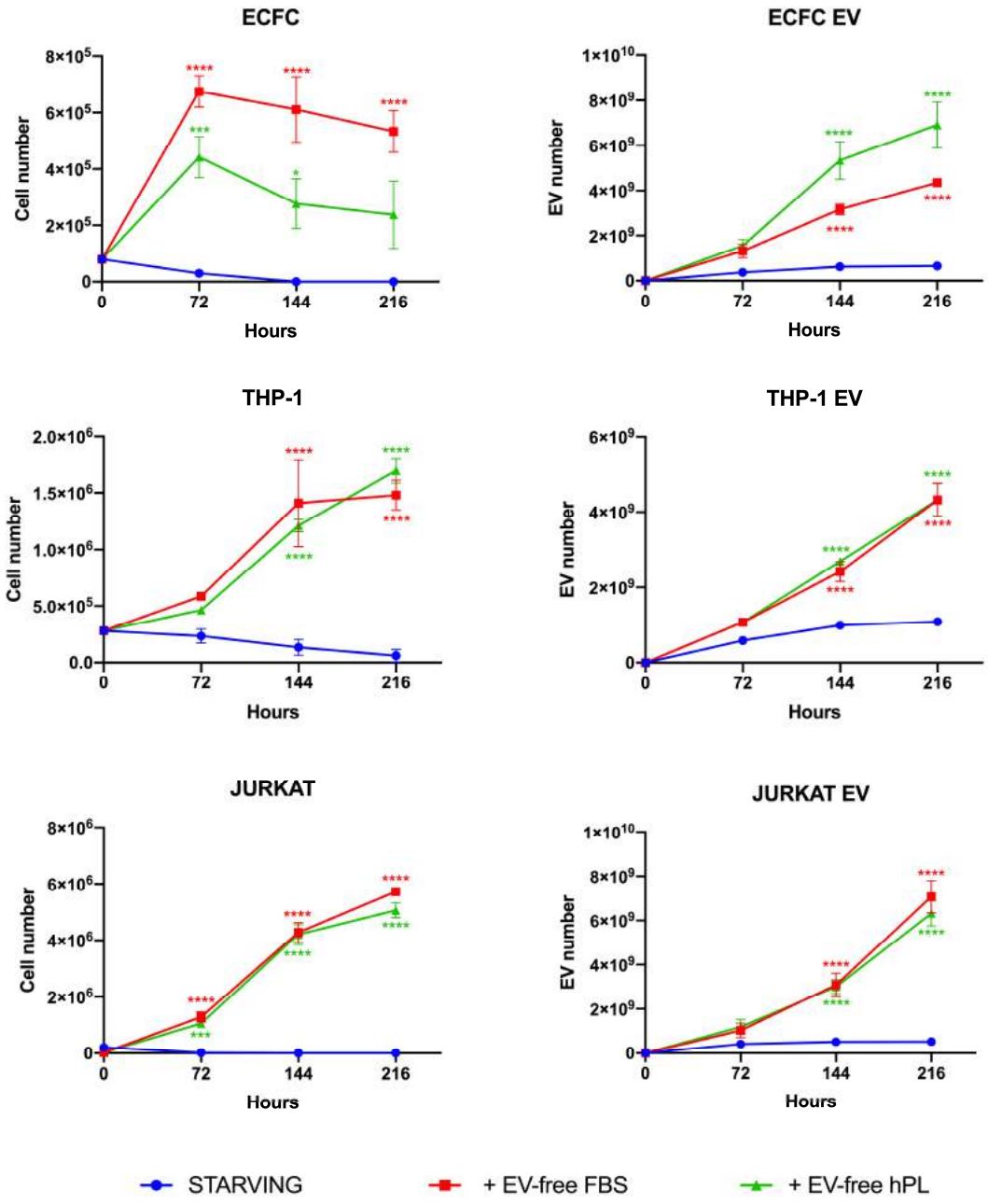

Figure 9. Incubation of ECFC, Jurkat and THP-1 in culture media alone (starving) or supplemented with EV-Free hPL or EV-Free FCS

Cells were cultured for 3 consecutive 72 h periods (72 h, 144 h and 216 h), at each time point the cells were counted in mirrored plates and EV number in the conditioned media were measured by NTA. **A)** ECFC number in EBM2-MV alone (blue) or supplemented with 5% EV-Free hPL (green) or 5% EV-Free FCS (red). **B)** EV produced by ECFC in the same conditions as in A). **C)** THP-1 number in RPMI (blue) or supplemented with 10% EV-Free hPL (green) or 10% EV-Free FCS (red). **D)** EV produced by THP-1 in the same conditions as in C). **E)** Jurkat number in RPMI (blue) or supplemented with 10% EV-Free hPL (green) or 10% EV-Free FCS (red). **F)** EV produced by Jurkat in the same conditions as in E). Cell numbers and cumulative EV productions are expressed as mean +/- SE from 3 independent incubations for each time and condition. Statistical significance of change in cell and EV number compared to zero time (T0) was evaluated using multiple comparison analysis following one-way ANOVA. Only multiplicity adjusted P values for difference in EV content in the presence of either 5% EV-free hPL or 10% EV-free FCS compared to αMEM alone at the same time are reported on the figure . *: P<0.05; **: P<0.01; ***: P<0.001; ****: P<0.0001.

To further test the functionality of our additives, we tested them with 2 non-adherent human cell lines namely THP-1 and Jurkat. Again, following amplification in complete medium such as RPMI supplemented either in FCS or hPL, starvation radically impaired cell survival especially for Jurkat with 100% cell death after 72 h and limited EV production. The supplementation with EV-free hPL or FCS allowed survival and even proliferation of both THP-1 and Jurkat (Figure 9C and E) thus allowing EV secretion (Figure 9D and F), demonstrating that our method allows preparing EV-free additives that could be used with virtually any cell type.

Discussion

EV therapy is an evolution of cell therapy and, because of its efficiency and flexibility, it a very promising new field of nanomedicine. Yet, EV production is a laborious and time-consuming work. Both the production and isolation steps limit the EV production yields. Culture media are supplemented with serum or hPL that may interfere with specific EV production, by introducing exogenous EV that cannot be discarded or separated from specific EV. Here, the aim was to set-up a method allowing an efficient EV depletion to prepare specific additives including a clinical-grade compatible EV-free-hPL. In this work, we used tangential flow filtration to produce EV-free additives. We first showed that these EV-free medium allowed lengthening the EV production duration from MSC, traditionally limited by starvation conditions. We showed that we could thereby increase the amount of produced EV. This was confirmed with three other cell type known to be much more sensitive to starvation.

The tangential flow filtration is a highly relevant method; it is possible to manage large volumes of solution without clogging issue that appears in classical dead-end filtration. In order to optimize TFF, hollow fiber filters of different cut-off were evaluated, with the objective of eliminating a maximum of EV but keeping the composition and efficiency of EV-free hPL as close as possible to hPL. Even if the 50 nm filtration was the fastest compared to 500kDa and 100kDa, it was the least efficient, with only 97.1% depletion. Our choice was then guided by the need to obtain an EV-free hPL sufficiently rich to support cell survival. Since the EV depletion capacity was similar for the 100 kDa and 500 kDa filters, we chose the 500 kDa filter for all our preparations, in order to keep the maximum amount of soluble medium and low molecular weight proteins. The unexpected observation that EV-free-hPL do not clot when diluted up to 10% in α-MEM medium is of great practical interest since there is no longer any need to use heparin as an additive in the EV production phase. Since human fibrinogen is a 45 nm-long plasma glycoprotein with a molecular weight of 340 KDa, it may be suggested that 500 kDa TFF results in depletion of fibrinogen in the filtrate, sufficient to impair clot formation (data not shown). Furthermore, we noted the importance of monitoring the transmembrane pressure to limit membrane fouling and provide an overall higher filtration rate. An uncontrolled and too high TMP may also result in a decrease in size exclusion efficiency by EV deformation, which could thus pass through the fiber pores.

There are many methods for separating EV, traditional ones using size or buoyant density (ultracentrifugation, microfiltration or gel filtration), to those using precipitation (for example with polyethylene glycol), specific interactions with EV membrane molecules, or by microfluidic technics[20]. Each technic has a number of drawbacks. Ultracentrifugation is often

used to remove EV from FCS. However, the results are not optimal with various levels of depletion achieved, which depend on centrifugation conditions and duration[11,12,13,21-23]. It is also reported that a higher depletion is obtained for large vesicles than for small size vesicles such as exosomes[11]. We were able to confirm the inability of ultracentrifugation to sufficiently deplete FCS from its endogenous EV component and further extended this conclusion to hPL. Consistently, we found that TFF was far more effective to remove EV from hPL and FCS. Moreover, TFF allowed carrying out EV removal without prior dilution of hPL and serum, despite their very high viscosity. These EV-free products can then be added to any culture medium or frozen for further utilization. The large volumes of EV-free hPL that can be produced, as well as the convenience of its storage, make it a good cell culture supplement for the production of clinical grade EV. Several companies have developed xenogenic serum free media enabling optimized and reproducible cell productions[24–28]. However, these products are expensive and still difficult to use in the context of large EV productions. Our method provides a cost-effective alternative to such products.

Ultrafiltration (TFF or dead-end) is now used to concentrate/purify EV from conditioned medium[18,29,30]. However, its use to deplete EV from additives such as FCS and hPL is limited. One group suggested that ultrafiltration through Amicon ultra-15 100 kDa cut-off centrifugal filters was more efficient than ultracentrifugation for FCS EV depletion[21]. However, while this study clearly demonstrated the efficiency of EV-depleted FCS to sustain growth and metabolism of different cell types, it is clearly not adapted for large volumes of FCS and do not document other additives such as hPL.

Thus, by controlling key filtration parameters such as shear rate and TMP as well as filter membrane cut-off, we show the ability of TFF to address the EV depletion of viscous solutions such as hPL or FCS. Viscosity, a consequence of the high protein content, is certainly the most important factor that impaired EV depletion of pure hPL or FCS using ultracentrifugation. Since TFF is a highly scalable method, it could manage the isolation of EV from industrial large-scale productions that would be carried out in bioreactors. Thus knowing the filtration rate (expressed as $l.m^{-2}.h^{-1}$) of a particular medium on a known cut-off filter, only the adaptation of the filter surface is necessary to allow estimating the filtration duration required for the volume of the solution to be treated. Using the KrosFlo Research II system from Spectrum, we were able to produce 500 ml of EV-free hPL in about 4 hours using a 155 cm^2 filter (D04-E500-10-N) and up to 10 L of EV-free FCS in about 12 hours using a 1600 cm^2 filter (S06-E500-10-N).

Several studies have shown that using EV-free sera can modify cell behavior, for example with reduced viability[31,32], slowed growth[11,33] or reduced migration[13] as compared to FCS. Whereas growth and migration was not in the scope of this study, our results clearly indicates that cell survival was favored compared to starving conditions in several cell types. To the best of our knowledge, no data are available regarding the use of EV-free hPL and our purpose was to use this additive only during the secretion phase. We did not compare EV-free hPL and native hPL since the latter contains too much particles and will therefore never be suitable for EV production. Yet, our results show that EV-free hPL supplemented media allowed maintaining BM-MSC amount and viability throughout at least 3 secretion periods (from 72h and up to 216h), without modification of their phenotype or protein profile. This was not the case in starving conditions where the cell number decreased significantly and

rapidly. Thus, the amount of-EV obtained with the EV-free hPL was greater than under the classical cell starving condition. This process, which allows carrying out several EV production cycles from a single production of cells, could significantly reduce the costs of clinical-grade EV batches.

By using BM-MSC-EV production as a biological readout, we also show that hPL needs to be at least 99% EV-depleted in order to assign at least 90% of the EV content to the producing cells. This clearly questions the use and characterization of EV produced in condition media containing "EV-depleted" FCS commercially available or obtained by ultracentrifugation. As discussed in previous articles[12,34], knowing the amount of contaminating EV in hPL, FCS or other additives is essential as it determines the ability to correctly assess the contribution of the cultured cell. This is even more important for therapeutic use. When using a media supplemented with hPL, BM-MSC-EV were concentrated in the TFF retentate together with hPL proteins that did not or less efficiently cross the filter. Such proteins were only partially eliminated through the diafiltration step and in a clinical setting, could be considered impurities (molecules from a raw material that have not been fully eliminated from the final product). A size exclusion chromatography step can be added to the TFF isolation process in order to purify the BM-MSC-EV. In this study, we chose to use a column with an exclusion limit of 4.10^7 Da, corresponding to a 40 nm diameter sphere, a size close to the smallest EV[35]. This step successfully discarded the remaining medium and low molecular weight EV-free hPL proteins (such as hSA), that were not completely filtered by TFF. However, it could not eliminate some high molecular weight proteins secreted by BM-MSC co-enriched with EV in the TFF step.

The mass-spectrometry analysis confirms the complete or near complete depletion of EV and mainly platelet EV in the hPL. Taking into account the high sensitivity of LC-MS-MS the absence of platelets membrane marker such as CD41, CD42 and P-selectin confirms the high efficiency of our TFF protocol. The LC-MS-MS also showed consistency in protein composition throughout the 3 consecutive harvests, EV-associated proteins being the most represented.

We showed that our EV-free hPL as well as our EV-free FCS are able to sustain other primary cells or cell lines, including cells known to be very sensitive to their culture conditions such as ECFC, Jurkat or THP-1. This demonstrates that our EV-free additives can be suitable for virtually any cell type; therefore, our method can be useful far beyond the MSC we present here.

Finally, important regulatory issues are now well identified for the production and control of EV-based biological medicinal product, as reviewed recently[36]. For the clinical use, the safety is the first concern. Since virus depletion in hPL and FCS is of major importance, it could be predicted that using 500 kDa cut-off filters will provide secured EV-free hPL and FCS products through depletion of the smallest animal virus known today[37] (i-e Parvoviridae (18-26 nm diameter) and Picornaviridae (20-30 nm diameter) family virus). Human platelet lysate is a well-known blood-derived product, widely used in the world including for MSC production. Its safety is proven and because TFF in closed system is also widely used in GMP-grade production for proteins or vaccine, our method allows fulfilling a currently unmet need for massive GMP-grade EV production.

Conclusion

There is a growing interest in using EV as medicinal products in numerous applications. Some technical limitations are still to address, including the large-scale EV production and manufacturing. We describe here a process that allows producing large amounts of EV-free human platelet lysate, a clinical-grade compatible additive that can be used to support cell culture for prolonged EV production. The novelty mainly concerns hPL, a highly viscous product, but the proof of concept carried out in this article concerning the use of TFF for the depletion of EV can be extended to any type of complete culture medium or directly on the additives, such as FCS or any other animal serum, before mixing in the culture medium. Importantly, these EV-free additives give a new option to produce EV from cells, which do not survive in starving condition. This work provides a new method that can be very useful for both EV research and large-scale EV manufacturing.

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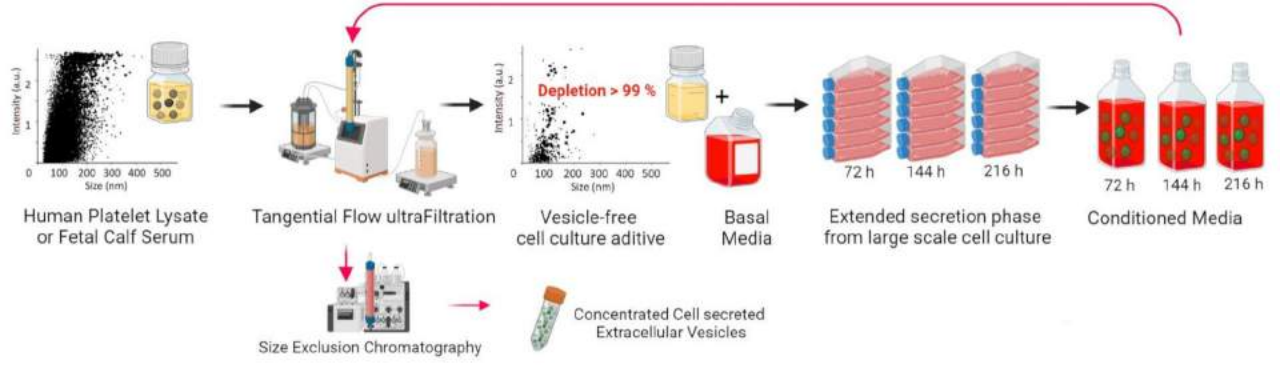
Method to prepare vesicle-free media using tangential flow filtration allowing prolonged cell survival, enhanced EV secretion and manufacturing.


Intensity (a.u.)
Size (nm)
Human Platelet Lysate
or Fetal Calf Serum
Tangential Flow ultraFiltration
Depletion > 99 %
Vesicle-free
cell culture aditive
Basal
Media
72 h
144 h
216 h
Extended secretion phase
from large scale cell culture
72 h
144 h
216 h
Conditioned Media
Size Exclusion Chromatography
Concentrated Cell secreted
Extracellular Vesicles