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TECHNICAL NOTE

Inter-laboratory multiplex bead-based surface protein profiling of MSC-derived EV preparations identifies MSC-EV surface marker signatures

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Abstract

Mesenchymal stromal cells (MSCs) are promising regenerative therapeutics that primarily exert their effects through secreted extracellular vesicles (EVs). These EVs – being small and non-living – are easier to handle and possess advantages over cellular products. Consequently, the therapeutic potential of MSC-EVs is increasingly investigated. However, due to variations in MSC-EV manufacturing strategies, MSC-EV products should be considered as highly diverse. Moreover, the diverse array of EV characterisation technologies used for MSC-EV characterisation further complicates reliable interlaboratory comparisons of published data. Consequently, this study aimed to establish a common method that can easily be used by various MSC-EV researchers to characterise MSC-EV preparations to facilitate interlaboratory com-

Bas W. M. van Balkom and André Görgens contributed equally to this study.

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parisons. To this end, we conducted a comprehensive inter-laboratory assessment using a novel multiplex bead-based EV flow cytometry assay panel. This assessment involved 11 different MSC-EV products from five laboratories with varying MSC sources, culture conditions, and EV preparation methods. Through this assay panel covering a range of mostly MSC-related markers, we identified a set of cell surface markers consistently positive (CD44, CD73 and CD105) or negative (CD11b, CD45 and CD197) on EVs of all explored MSC-EV preparations. Hierarchical clustering analysis revealed distinct surface marker profiles associated with specific preparation processes and laboratory conditions. We propose CD73, CD105 and CD44 as robust positive markers for minimally identifying MSC-derived EVs and CD11b, CD14, CD19, CD45 and CD79 as reliable negative markers. Additionally, we highlight the influence of culture medium components, particularly human platelet lysate, on EV surface marker profiles, underscoring the influence of culture conditions on resulting EV products. This standardisable approach for MSC-EV surface marker profiling offers a tool for routine characterisation of manufactured EV products in pre-clinical and clinical research, enhances the quality control of MSC-EV preparations, and hopefully paves the way for higher consistency and reproducibility in the emerging therapeutic MSC-EV field.

KEYWORDS
culture conditions, extracellular vesicles, exosomes, inter-laboratory assessment, mesenchymal stromal cells, MSC, MSC-marker, quality control, regenerative medicine, standardisation, surface markers

1 | INTRODUCTION

Many of our communities are rapidly aging and grappling with the enormous challenge in managing the accelerating occurrence of age-related non-communicable chronic diseases, including diabetes, atherosclerosis and kidney diseases (Luyckx et al., 2018). During the past few decades, novel regenerative therapies addressing such diseases have emerged, including numerous stem cell-based therapies. Remarkably, the therapeutic benefits of mesenchymal stromal cell (MSC) therapy for acute myocardial infarction were found to rely mainly on the action of secreted extracellular vesicles (EVs) (Lai et al., 2010). This finding has sparked considerable interest in using EVs as potential off-the-shelf therapeutics for various ailments (De Jong et al., 2014; Lener et al., 2015; Reiner et al., 2017).

Since then, (pre-)clinical data from multiple studies have demonstrated the regenerative potential of MSC-derived EVs in several human diseases. These EVs carry RNAs and proteins that could mediate regeneration and have anti-inflammatory, immune-modulatory effects. Importantly, similar to MSCs, MSC-EVs appear not to induce adverse immunological reactions, making them suitable for allogeneic use (Pachler et al., 2017). In animal studies, injected MSC-EVs have been demonstrated to facilitate tissue regeneration and immune modulation (Di Trapani et al., 2016; Doeppner et al., 2015; Fan et al., 2020). MSC-EVs promote tissue regeneration by creating a pro-regenerative environment and guiding endogenous cells to repair affected tissues by switching pro-inflammatory to tolerogenic immune responses. Unlike their parent cells, EVs are non-self-replicating and can be sterilised through filtration, allowing for a standardised, off-the-shelf regenerative therapy (Lener et al., 2015). This realisation has led to rapid and extensive expansion of research on the therapeutic potential of EVs, and consequently the amount of registered clinical trials is progressively increasing. Early clinical evidence demonstrated safe and successful application of therapeutic MSC-EVs in a patient with refractory graft-versus-host disease (Kordelas et al., 2014).

MSCs can be sourced from various tissues, such as the umbilical cord, bone marrow and adipose tissue. MSCs obtained from different sources exhibit distinct properties, behaviour and regenerative capabilities, making them suited for different therapeutic applications. This inherent diversity among MSCs from different sources may offer exciting possibilities for tailoring therapies to specific medical conditions. However, this diversity together with variations in isolation, culture, conditioning of MSCs and preparation of MSC-EVs by different research groups, introduces significant variability in MSC-EV properties. This poses a challenge when interpreting the results of studies and comparing data across different laboratories.

To mitigate the heterogeneity among MSCs, the ISCT proposed a minimal defining criterion for MSCs, namely plastic adherence, ability to differentiate towards adipocytes, chondrocytes and osteoblasts, surface expression of CD73, CD90 and CD105, and absence of CD11b, CD14, CD34 and CD45 (Dominici et al., 2006). However, this minimal criterion was not intended to be comprehensive. Therefore, many MSCs fulfilling this criterion are still very heterogenous. In view of the exponentially increasing data about the therapeutic potentials of MSC-EVs, there is a need for an evidence-based and broadly applicable EV characterisa-

tion methodology that is suitable to identify MSC-EVs as being a MSC product, that is, qualify bona fide identity characteristics of an MSC-EV preparation (Witwer et al., 2019). An additional need in the field relates to defining characteristics and establish assays which can be utilised to predict therapeutic efficacy, that is, the potency of MSC-EV preparations (Witwer et al., 2019). In terms of MSC-derived EV characterisation, we recently demonstrated that MSC-EVs, despite originating from MSCs from different sources and isolated in various ways, are characterised by a robust and unique proteomic signature (van Balkom et al., 2019).

In this context, we here hypothesised that the assessment of MSC-EV surface proteins could contribute to increased comparability and normalisation of MSC-EV research data. In this study, we objectively evaluate different surface marker signatures on EVs derived from different MSC sources. Based on a multiplex bead-based EV flow cytometry assay we previously optimised for assessment of EV surface marker signatures (Welsh et al., 2022; Wiklander et al., 2018), we employed a panel specifically designed to evaluate MSC-EV surface phenotypes, ultimately aiming to define a robust MSC-EV consensus surface marker signature.

To investigate the variability of MSC-EV preparations, we assessed 11 different MSC-EV preparations from 5 different labs. The 11 preparations differ in their MSC origin, culture conditions, EV isolation and preparation methods. Additionally, they were analysed using different flow cytometric instrumentation and data collection methods. Flow cytometric analysis was performed and data was collected by each lab, using their equipment and by their protocols of choice. However, using the same novel multiplex bead-based assay MSC panel, we detected a set of surface markers that are either consistently present or absent in all 11 preparations, despite notably different inter-laboratory variation in preparation and analytical procedures. Together, our results revealed that these surface marker components are suitable candidates to minimally define the identity of MSC-EV preparations and could be used to improve consistency and reproducibility in the emerging therapeutic MSC-EV field.

2 | MATERIALS AND METHODS

2.1 | Multiplex bead-based EV flow cytometry assay

The principle of the multiplex bead-based EV flow cytometry assay is based on the use of hard-dyed polystyrene bead (4.8 μm in diameter) populations, with each bead population coupled to different antibodies that recognise potential EV surface antigens and are used to capture antigen-positive EVs, respectively. Internal isotype controls are included to monitor any potential unspecific binding. Capture bead populations are distinguished from each other by their respective hard-dyed fluorescence characteristics detected in FITC versus PE channels, and bead-bound EVs are detected by using APC-conjugated detection antibodies and subsequent quantification of APC fluorescence over controls. A mixture of detection antibodies against the commonly used EV markers CD9, CD63 and CD81 (Pan-tetraspanin detection; in short referred to as PAN) is used for most measurements in this study to obtain higher signal intensities, ensure detection of particular EV markers with lower abundance, and to detect the majority of the (tetraspanin-positive) EVs bound to capture beads within each sample (Figure 1a). Single, APC-conjugated detection antibodies are additionally used for experiments shown in Figure 4b, to investigate potentially differential co-abundance of EV-associated proteins with either CD9, CD63 or CD81. The classical version of the multiplex bead-based EV flow cytometry assay (MACSPlex EV Kit IO, Miltenyi Biotec, order no 130-108-813) was previously described and optimised by us and others for detection and quantification of EV surface marker signatures in EV containing samples derived from cell cultures and biological fluids (Conzelmann et al., 2020; Koliha et al., 2016; Javadi et al., 2021; Welsh et al., 2022; Wiklander et al., 2018). The IO kit includes 39 different capture bead populations mainly directed against a panel of surface markers relevant for immuno-oncology research (Figure 1b). The MSC-EV kit used for most experiments in this study (MACSPlex EV Kit MSC, Miltenyi Biotec, pre-release version) is based on the same principle but contains a total of 58 capture bead populations divided over 2 panels: MSC Panel A (39 populations) and MSC Panel B (19 populations, including 2 internal isotype controls) (Figure 1c,d; full list of markers in Table S1).

Multiplex bead-based EV flow cytometry assays were performed following different protocol variations by respective labs, based on protocols provided by the manufacturer and depending on the individual lab's preferences (Table 1): Briefly, prepared EV samples (assay input dose: Labs 1, 3, 4: 1×10^9 NTA-based particles; Lab 2: 20 μg protein; Lab 5: 4×10^5 cell equivalents) were diluted with MACSPlex buffer to a final volume of 120 μL and incubated either for 1 h (Labs 2, 5) or over-night (Labs 1, 3, 4) with MACSPlex Exosome Capture Beads (MSC Panel A: 2.79 μL/assay; MSC Panel B: 2 μL/assay; IO Panel: 15 μL/assay) on an orbital shaker at room temperature (RT) in the dark. Beads were washed with MACSPlex buffer, and then 5 μL of APC-conjugated CD9, CD63 and CD81 detection antibodies were added to each sample in a volume of 135 μL MACSPlex buffer. Following incubation for 1 h on an orbital shaker at RT, protected from light, samples were washed again and incubated for another 15 min before a final washing step was performed. Incubation and washing steps were performed in 1.5 mL tubes (Labs 1, 2, 5) or 0.22 μm filter plates (Lab 3, 4). The impact of varying respective assay parameters mentioned above on assay results has been extensively evaluated and compared before (Wiklander et al., 2018). Final samples were resuspended in 100–150 μL MACSPlex buffer and analysed by flow cytometry. Samples were acquired on CytoFLEX S, CytoFLEX LX (Beckman Coulter) or MACSQuant Analyzer 10 (Miltenyi Biotec) instruments (see Table S2 for details).

TABLE 1 Overview of MSC, EV and assay characteristics of the participating laboratories.

	MSC				EV		Multiplex bead-based assay			Characterisation	
Lab	Source	Primary/immor	Culture	Harvest	Harvest period	Preparation method	Assay input dose	Assay protocol	Flow cytometer	Cells	EVs
1	BM	Primary	10% FBS	0% FBS	24 h	UC	10^9 particles	O/N	CytoFLEX LX	(Nguyen et al., 2022)	(Nguyen et al., 2022; van Rhijn-Brouwer et al., 2019)
2	ESC	Immortalised	10% FBS	0% FBS	72 h	TFF-UF	20 μg protein	'short'	MACSQuant Analyzer 10	(Lian et al., 2007, Chen et al., 2011)	(Arslan et al., 2013; Lai et al., 2010; Lai et al., 2012)
3	WJ/CB	Immortalised	10% FBS	0% FBS	48 h	TFF-UF	10^9 particles	O/N	MACSQuant Analyzer 10	(Hagey et al., 2023)	(Cavallaro et al., 2021; Dar et al., 2021; Hagey et al., 2023; Zheng et al., 2023)
4	AT	Immortalised	10% FBS	0% FBS	24 h	TFF-SEC	10^9 particles	O/N	MACSQuant Analyzer 10[#]	(ASC52telo)	(van de Wakker et al., 2024)
5	BM	Primary	10% hPL	hPL	48 h	PEG-UC	4×10^5 cell equivalents	'short'	CytoFLEX S	(Kordelas et al., 2014)	(Börger et al., 2020; Kordelas et al., 2014)

Abbreviations: AT, adipose tissue; BM, bone marrow; CB, umbilical cord blood; ESC, embryonic stem cells; FBS, fetal bovine serum; hPL, human platelet lysate; PEG, polyethylene glycol; SEC, size exclusion chromatography; TFF, tangential flow filtration; UC, ultracentrifugation; WJ, Wharton Jelly.

[#]Multiplex bead-based EV flow cytometry assays for MSC-EVs from Lab 4 were performed by Lab 3, all other samples were measured by the lab that prepared the EVs. Further details are provided in Section 2, in Table 2, and in Table S2.

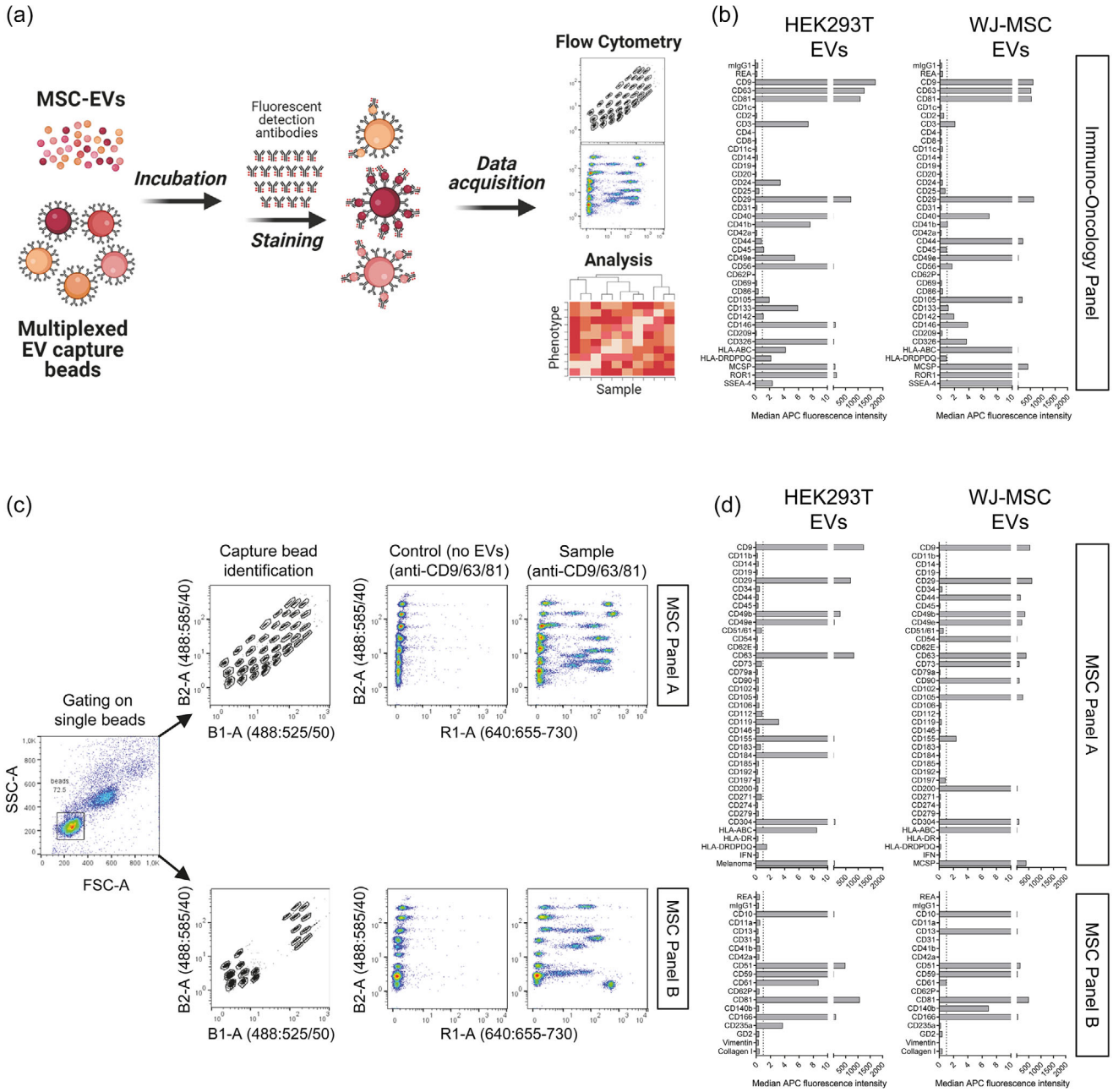


FIGURE 1 Multiplex bead-based EV flow cytometry assay principle and example data output. (a) Principle and workflow of multiplex bead-based EV flow cytometry assay platform. (b) Example surface marker profiles for HEK293T- and MSC-derived EVs for the previously established immuno-oncology panel. (c) Flow cytometric gating strategy for the MSC assay measured in two separate panels. Single capture beads were gated based on FSC-A versus SSC-A parameters, and bead subpopulations were identified by green (525/50 nm filter) versus orange light (585/40 nm filter) emitted after excitation with a blue laser (488 nm). Signals derived from APC-conjugated detection antibodies were quantified in the R1-A channel (655–730 nm filter) after excitation with a red laser (640 nm). (d) Example dataset showing differential surface marker profiles for HEK293T- and MSC-derived EVs.

2.2 | MSC culture and characterisation

MSC lines used in this study were cultured and characterised by each lab, respectively, as follows (numbering of Labs and MSC lines refers to numbers used in Table 1, Figures 3 and 4): MSC01 and MSC02 used by Lab 1 were derived from bone marrow and obtained from two separate production batches of donors who had given written consent. The UMC Utrecht Gene and Cell Therapy facility provided the cells, which were cultured in MEM-α media (ThermoFisher Scientific). The culture medium was supplemented with 10% fetal bovine serum (FBS; Biowest), 100 U/mL penicillin, 100 µg/mL streptomycin, 0.1 µg/mL primocin, 200 µM L-ascorbic acid (Sigma), and 1 ng/mL basic Fibroblast Growth Factor (ThermoFisher Scientific). MSC03, MSC04 and

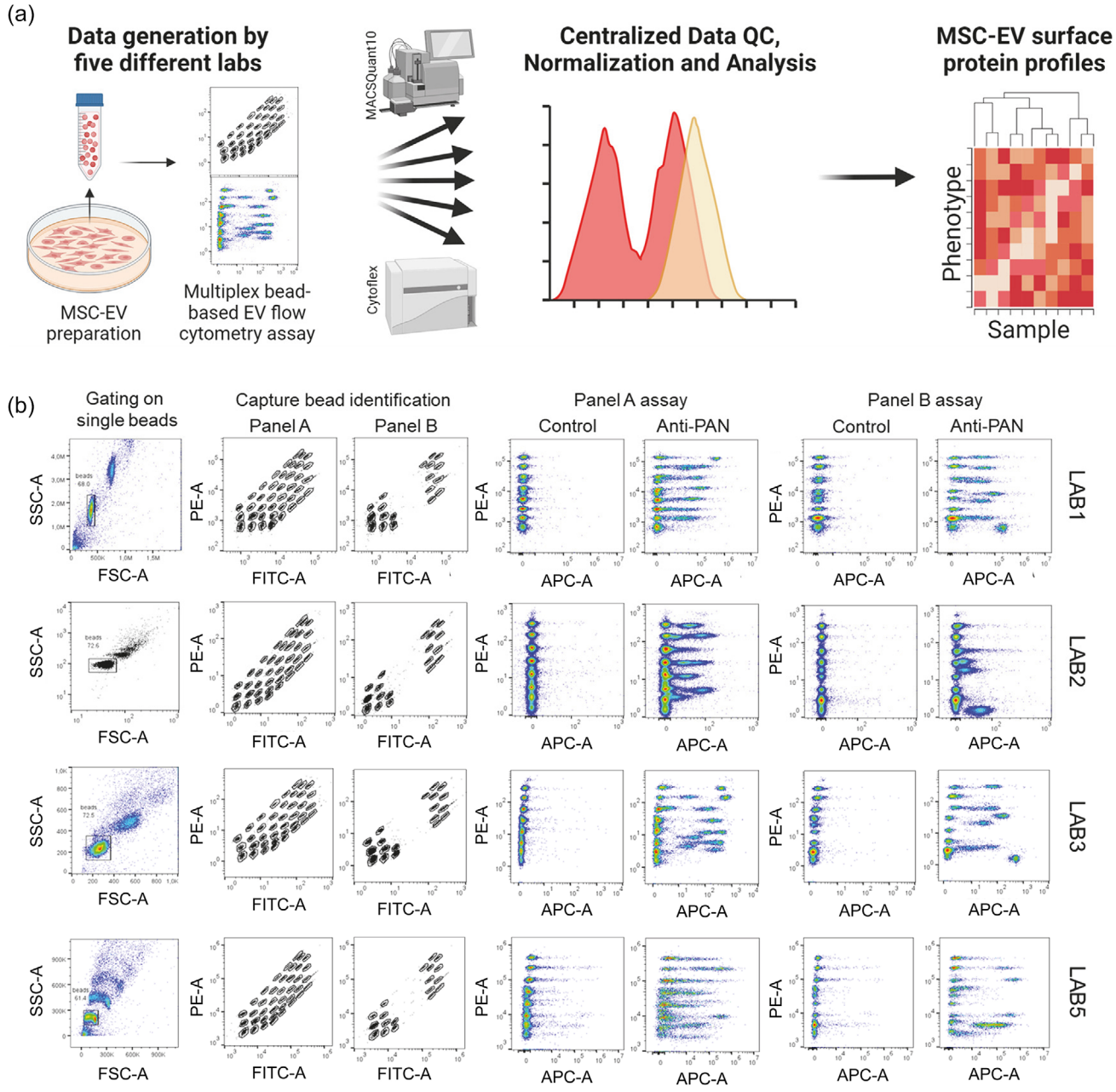


FIGURE 2 Inter-laboratory assay assessment. (a) Schematics summarising the study. MSC-EVs were prepared and measured by multiplex-bead based EV flow cytometry assays in participating labs before data was normalised and analysed centrally. (b) Example data showing assay analysis including gating strategy and capture bead identification steps for all lab participants (note that EVs provided by Lab 4 were measured in Lab 3). See Section 2, Table 1 and Table S1 for instrument and assay details.

MSC05 (AC111, AC114, AC117) by Lab 2 were produced by E1MYC 16.3, an immortalised monoclonal MSC line generated using MSCs derived from the differentiation of human embryonic stem cells, cultured in DMEM (ThermoFisher Scientific) supplemented with 10% FBS (Thermofisher Scientific) as described previously (Chen et al., 2011; Lian et al., 2007). Lab 3 used cord blood-derived MSCs (CB-MSC; MSC06) and Wharton Jelly-derived MSCs (WJ-MSC; MSC07): CB-MSC (ATCC, PCS-500-010, Umbilical Cord-Derived Mesenchymal Stem Cells, Normal, Human) were cultured in MEM-α media (ThermoFisher Scientific) supplemented with basic fibroblast growth factor (5 ng/mL; Sigma Aldrich), 10% FBS (Invitrogen) and 1% Antibiotic-Antimycotic (anti-anti; ThermoFisher Scientific); WJ-MSCs were cultured in DMEM (1 g/L glucose; containing GlutaMAX-I and sodium pyruvate, Invitrogen) supplemented with 1% anti-anti (ThermoFisher Scientific) (Hagey et al., 2023). Adipose tissue-MSCs (AT-MSC; MSC08) by Lab 4 (ATCC #SCRC-4000, ASC52telo hTERT immortalised adipose derived mesenchymal stem cells) were cultured in MEM-α media (Gibco, without ribonucleotides, without deoxyribonucleotides) supplemented with GlutaMAX-I, 1% penicillin/streptomycin and 10% FBS (Gibco). MSC09, MSC10 and MSC11 used by Lab 5 were raised from bone marrow of

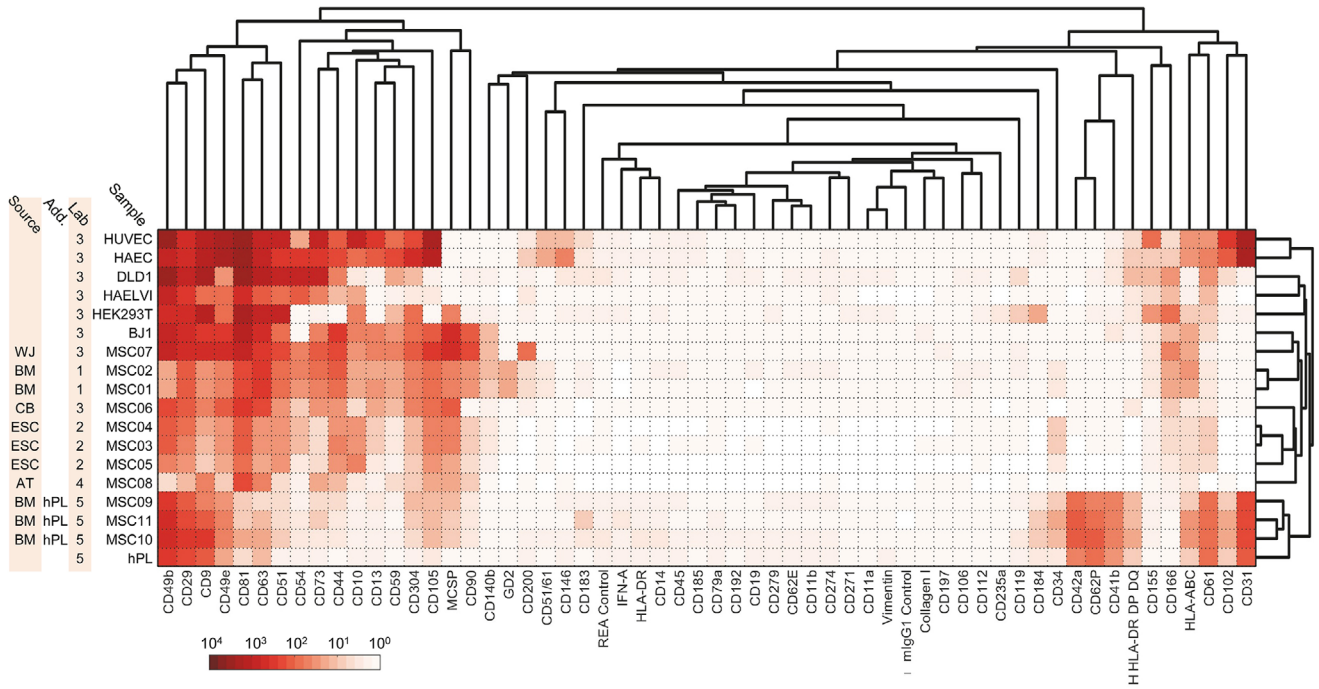


FIGURE 3 Normalised protein surface marker data from all labs. Clustering heatmap combining the normalised signals of all samples for all markers. Add., media additive; AT, adipose tissue; BM, bone marrow; CB, umbilical cord blood; ESC, embryonic stem cell; hPL, human platelet lysate; WJ, Wharton Jelly.

healthy donors after informed consent according to the declaration of Helsinki, as described previously (Kordelas et al., 2014; Radtke et al., 2016). Briefly, MSCs were expanded in DMEM (1 g/L glucose; Lonza) supplemented with 10% human platelet lysate (hPL), 100 U/mL penicillin-streptomycin-L-glutamine (ThermoFisher Scientific) and 5 IU/mL Heparin (Ratiopharm). Further details and references of the MSCs used in this study, including characterisation, are summarised in Tables 1 and 2.

2.3 | Cell culture of other human cell lines

HEK293T cells were cultured in DMEM (containing Glutamax-I and sodium pyruvate; 4.5 g/L glucose; Invitrogen) supplemented with 10% FBS (Invitrogen), 1% anti-anti (ThermoFisher Scientific). BJ-5ta fibroblast cells (BJ1; ATCC CRL-4001) were cultured with a 4:1 mixture of DMEM and Medium 199 (4 parts DMEM with Glutamax-I, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, and 1 part Medium 199 supplemented with 0.01 mg/mL hygromycin B and 10% FBS; all ThermoFisher Scientific). DLD1 (DLD1; ATCC CCL-221) epithelial colon adenocarcinoma cells were cultured in RPMI1640 medium supplemented with GlutaMAX-I, 1% penicillin/streptomycin and 10% FBS (all ThermoFisher Scientific). Human alveolar epithelial cells (HAELVI; CI-hAELVi, Inscreenex #INS-CI-1015 (Kuehn et al., 2016)) were cultured in huAEC medium with supplements (Inscreenex #INS-ME-1013). Primary human aortic endothelial cells (HAEC; ATCC #PCS-100-011) and immortalised human umbilical vein endothelial cells (HUVEC; HUVEC/TERT2; ATCC #CRL-4053) were cultured in Vascular Cell Basal Medium (ATCC #PCS-100-030) supplemented with endothelial cell growth Kit-VEGF (ATCC #PCS-110-041). All cell lines were grown at 37°C, 5% CO_2 in a humidified atmosphere and regularly subjected to mycoplasma testing.

2.4 | Preparation and characterisation of EVs

EVs were isolated from conditioned media (CM) of MSCs by participating labs as follows: Lab 1 used a previously described differential ultracentrifugation method (van Balkom et al., 2013; van Rhijn-Brouwer et al., 2019). In brief, MSCs were cultured until 80% confluence. The culture medium was replaced with EV-collection media (MSC culture media without FBS and Primocin). After 24 h, the CM was collected and subjected to centrifugation for 15 min at 1500 × *g* to remove cellular debris. Subsequently, larger particles were eliminated by centrifugation for 30 min at 10,000 × *g* using a Beckman XE90 ultracentrifuge with a SW32 Ti rotor (Beckman Instruments). EVs were finally subjected to ultracentrifugation for 60 min at 100,000 × *g*, followed by two washes with PBS and ultracentrifugation for 60 min at 100,000 × *g*, all performed using a Beckman XE90 ultracentrifuge with a SW32 Ti rotor. The final EV pellet was collected by centrifugation at 100,000 × *g* using a Beckman XE90 centrifuge with a SW60 Ti rotor.

TABLE 2 Summary of EV characterisation as performed by each laboratory.

Lab	Diameter	NTA	EM	SG	Protein	Function	References
1	149 nm (modal)	X		X	Flotillin-1, Lamin A/C, TOM20, ATP5A, GAPDH	Regeneration, angiogenesis, cellular uptake	(van Balkom et al., 2013; van Rhijn-Brouwer et al., 2019)
2	122.7 nm (modal)	X			CD81, ALIX, TSG101, CD73	CD73/NT5E activity	(Shi et al., 2023)
3	109.7–134.7 nm (mean)	X	X		CD9, CD63, CD81, Syntenin A1, CD29, CD44, CD49e, CD105, ROR1	Cellular uptake, cellular transcriptional response	(Görgens et al., 2022; Hagey et al., 2023; van de Wakker et al., 2022)
4	50–110 nm (modal)	X			CD9, CD63, CD81, Syntenin A1, TSG101, HSP90, CD151, ITGB1, HLA-ABC, CD73	AKT stimulation	(van de Wakker et al., 2022, 2024;
5	103–132.2 nm (mean)	X			CD9, CD63, CD81, Syntenin A1, Calnexin	in vivo immune modulation	(Börger et al., 2020; Madel et al., 2023)

Abbreviations: EM, electron microscopy; NTA, nanoparticle tracking analysis; SG, sucrose gradient.

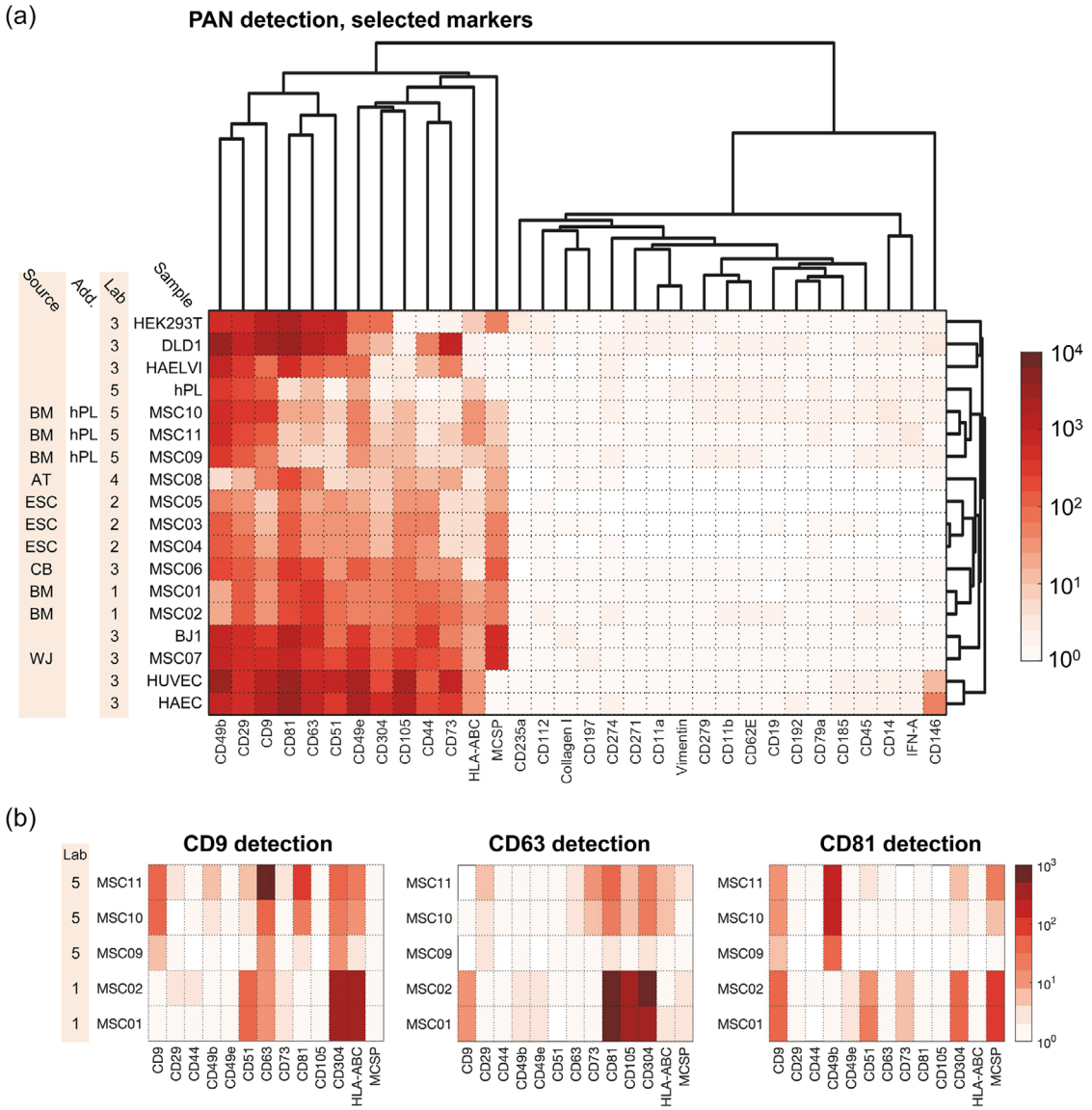


FIGURE 4 Candidate MSC-EV marker selection and characterisation. (a) Summary of selected MSC-MBFCM surface markers classified as either consistently positive or negative on all MSC-EV samples included in this study. (b) Characterisation of MSC-EV markers classified as positive on all MSC-EV samples by using single anti-tetraspanin antibodies for detection. Add., media additive; AT, adipose tissue; BM, bone marrow; CB, umbilical cord blood; ESC, embryonic stem cell; hPL, human platelet lysate; WJ, Wharton Jelly.

EVs were transferred into 1.5 mL tubes and stored at −80°C until measurement. Lab 2 prepared EVs as previously described (Shi et al., 2023). In brief, cells were grown to a confluency of 80% in chemically defined media composed of DMEM (ThermoFisher Scientific) supplemented with 1% non-essential amino acids, 1% glutamine, 1% insulin-transferrin-selenium-X, 1 mM sodium pyruvate, 0.05 mM βmercaptoethanol, 5 ng/mL fibroblast growth factor (FGF-2; ThermoFisher Scientific) and 5 ng/mL platelet-derived growth factor (PDGF-AG; Cytolab Ltd) for 3 days. The conditioned media was size fractionated by tangential flow filtration (TFF) and concentrated 50× using a membrane with a molecular weight cut-off of 100 kDa (Sartorius). EV samples were 0.22 µm filtered and stored at −20°C until use. EVs from MSCs, HEK293T, BJ1, HUVEC, HAEC, DLD1 and HAELVI cells by Lab 3 were prepared as described recently (Hagey et al., 2023). In brief, media was changed to OptiMem (Invitrogen) 48 h before harvest of CM as described before (Corso et al., 2017). All CM samples were directly subjected to a low-speed centrifugation step at 500 × g for 5 min followed by a 2000 × g spin for 10 min to remove larger particles and cell debris. Precleared cell culture supernatant was subsequently filtered through 0.22 µm vacuum filters (Corning, cellulose acetate, low protein binding) to remove larger particles and concentrated via TFF by using the KR2i TFF system (Spectrum Labs) equipped with modified polyethersulfone hollow fibre filters with 300 kDa membrane pore size (MidiKros, 370 cm^2 surface area, Spectrum Labs) at a flow rate of 100 mL/min (transmembrane pressure at 3.0 psi and shear rate at 3700 s^{-1}) as described previously (Corso et al., 2017). Amicon Ultra-0.5 10-kDa MWCO spin-filters (Millipore) were used to concentrate samples to a final volume of 100 µL,

and final EV samples were stored at −80°C in PBS-HAT [PBS supplemented with HEPES, human serum albumin and D-(+)-Trehalose dihydrate] buffer (Görgens et al., 2022). AT-MSC derived EV samples by Lab 4 were isolated, as recently described (van de Wakker et al., 2024), by washing the cells replacing the media with Optimem (Gibco). CM was removed after 24 h and spun down at 2000 × *g* for 15 min. Supernatant was filtered through a 0.45 µm aPES bottle top filter (Nalgene). Filtrate was concentrated to a volume of approximately 3 mL by TFF using a Minimate TFF capsule (Pall) with a membrane cutoff of 300 kDa. During TFF, a buffer exchange was performed to PBS. Residue was loaded on a HiScreen Capto Core 700 multimodal flowthrough chromatography (MFC) column (Cytiva) connected to an ÄKTA start system (Cytiva). EV-containing fractions were pooled and concentrated using 10 kDa Amicon Ultra-15 spinfilters (Merck). EVs were stored in PBS-HAT buffer in 1.5 mL tubes (Görgens et al., 2022; van de Wakker et al., 2022). MSC-EVs by Lab 5 were prepared from CM of MSCs applying a polyethylene glycol (PEG)-ultracentrifugation based purification strategy as described previously (Börger et al., 2020). Briefly, MSC-CM were centrifuged at 6800 × *g* for 45 min at 4°C in an Avanti J-26 XP centrifuge using a swing-out rotor JS-5.3 (Beckman Coulter; k-factor: 7728), followed by a 0.22 µm filtration step using a bottle-top filter (Nalgene, Thermo Scientific). PEG 6000 (Sigma-Aldrich) and NaCl were added to clarified MSC-CM to a final concentration of 10% PEG 6000 (v/v) and 75 mM NaCl, respectively. Following incubation overnight at 4°C, EVs were pelleted in a swing-out rotor JS-5.3 in an Avanti J-26 XP centrifuge at 1500 × *g* for 30 min at 4°C (Beckman Coulter; k-factor: 7728). Subsequently, pellets were resuspended, washed in 65 mL 0.9% NaCl and transferred into 70 mL polycarbonate centrifuge tubes (Beckman Coulter). EVs were re-precipitated at 110,000 × *g* for 130 min at 4°C in an XPN-80 ultracentrifuge using the tight angle rotor Ti45 (Beckman Coulter; k-factor 133). Obtained pellets were resuspended in 0.9% NaCl/10 mM HEPES (Gibco) (4×10^7 cell equivalents/mL), aliquoted into 1.5 mL low retention tubes (Kisker) and stored at −80°C until usage. In addition to multiplex bead-based EV flow cytometry, EVs prepared by participating labs as described above were characterised by nanoparticle tracking analysis (particle size and concentration) and Western blot (Lab 1–5), single EV Imaging Flow Cytometry (Lab 3, 4, 5), and CD73/ecto-5′-nucleotidase (NT5E) activity assay (Lab 2) as described previously (Tables 1 and 2) (Börger et al., 2020; Cavallaro et al., 2021; Chen et al., 2011; Corso et al., 2017; Dar et al., 2021; Görgens et al., 2019, 2022; Hagey et al., 2023; Kordelas et al., 2014; Lian et al., 2007; Radtke et al., 2016; Shi et al., 2023; Tertel, Bremer, et al., 2020; Tertel, Görgens, et al., 2020; van Balkom et al., 2013; van Rhijn-Brouwer et al., 2019; van de Wakker et al., 2024; van de Wakker et al., 2022; Zheng et al., 2023).

2.5 | Data analysis and statistics

Flow cytometric data from all participating labs was centrally analysed in FlowJo Version 10.9.0. Bar graphs were created with Graphpad Prism 8. MBFCM data shown in Figure 1 was background subtracted (i.e., median fluorescence signal from capture bead population plus EVs plus CD9/CD63/CD81-APC antibody mix minus median fluorescence signal from the same sample processed without EVs) as described before (Wiklander et al., 2018). For all other data, MPA_{PASS} software version 1.10 was used for database construction and dataset normalisation (i.e., expressed as $\log_{10}$-transformed fold change values over respective controls). Further analyses, including heatmaps and hierarchical clustering were performed with custom scripts in MATLAB 2023a (Mathworks Inc.), available upon requestion. Hierarchical clustering analyses used Euclidean distance and average linkage method (Welsh et al., 2022; Wiklander et al., 2018). Schematic figure panels were created with BioRender.

3 | RESULTS

3.1 | Multiplex bead-based EV flow cytometry assay principle

The multiplex bead-based EV flow cytometry (MBFCM) assay platform used in this study was originally introduced by Koliha et al. (2016), and the first 39-plex panel mostly containing immuno-oncology (IO) related markers was commercially released as 'MACSPlex Exosome kit, human' (Miltenyi Biotec; now named 'MACSPlex EV Kit IO'). We and others subsequently utilised and further optimised this MBFCM platform with the IO panel for defined detection of EV surface markers in a variety of cell culture and biological fluid-derived EV containing samples (Gençer et al., 2024; Li et al., 2022; Welsh et al., 2022; Wiklander et al., 2018; Wolf et al., 2022). Figure 1a illustrates the core principle of the MBFCM assay, employing the use of specialised capture beads coated with antibodies targeting specific surface proteins of interest on the EVs. First, EV samples are incubated with the capture beads to selectively bind to the EVs presenting the targeted surface proteins. Then, bead-captured EVs are stained with included fluorescence-conjugated detection antibodies against CD9, CD63 and/or CD81, or custom fluorescence-conjugated detection antibodies, allowing identification of capture beads by flow cytometry and quantification of detection antibody fluorescence derived from antibodies bound to antigens present on EVs (Figure 1a).

With the IO panel (or IO-MBFCM), this yields relative quantification of signals for a total of 39 capture bead populations. Example data for the IO panel is provided for HEK293T and WJ-MSC EVs in Figure 1b. In this study, we employed a modified version of the MBFCM assay, which incorporates a new panel of select multiplexed capture beads targeting surface proteins potentially relevant to EVs derived from MSCs (referred to as MBFCM-MSC or MSC panel). Currently, at the

pre-commercialisation stage, the MSC-MBFCM assay has been designed with two panels of multiplexed capture bead populations measured separately, here referred to as MSC panel (a) and MSC panel (b) (Figure 1c,d and Table S1). Each panel is comprised of specialised capture beads coated with different sets of antibodies, targeting specific surface proteins that have been previously implicated or hypothesised to be present on MSC-derived EVs (Figure 1c,d).

The comparison of surface protein profiles between MSC-EVs and EVs derived from other sources, such as HEK293T and WJ-MSC, using both MSC Panel A and B, revealed (Figure 1d) that the assay successfully detected several commonly used MSC-typical surface markers (e.g., CD90, CD44, CD73, CD105, CD200) exclusively present on MSC-EVs and not on EVs derived from HEK293T cells. On the other hand, other markers were either detected on EVs from both sources or none, among those the commonly used tetraspanins CD9, CD63, CD81 and also integrins such as CD29 and CD49b/CD49e (positive on both) and leukocyte-specific markers such as CD11a, CD11b, CD19 and CD45 (Figure 1d). Comparable positive and negative results obtained for capture bead populations against markers included in both the IO-MBFCM and the MSC-MBFCM panels (e.g., CD14, CD19, CD31, CD41b, CD45, CD49e, CD105) in this first example dataset further validate the overall assay specificity, and specific detection of candidate markers for MSC-EVs demonstrates the ability of the MSC-MBFCM assay panel to identify MSC-derived EVs based on their surface protein signatures (Figure 1b,d).

3.2 | Inter-laboratory assay assessment

To assess the robustness of the assay, an inter-laboratory assessment and comparison of MSC-EV surface marker profiling was performed: EVs were prepared in five different laboratories (referred to as Lab 1–5) according to their preferred MSC type (11 MSC-EV preparations in total, plus EVs derived from the standard cell line HEK293T, from epithelial DLD1 and HAELVI cells, from endothelial HUVEC and HAEC cells, and BJ1 fibroblasts as controls), cell culture method, and EV isolation workflow. Importantly, all cells, procedures, EV isolation, and analysis methods, including cell- and EV-characterisations, have been published (Tables 1 and 2), and all MSC-EV characterisations meet the MISEV criteria (Théry et al., 2018; Welsh et al., 2024) (Table 2). Then, the MBFCM assays, including flow cytometric measurements, were performed in four laboratories, and acquired data were collected by the coordinating laboratory responsible for comprehensive individual and pooled data analyses (Figure 2a). Since standard workflows and protocols as well as other parameters such as MSC source and status (primary vs. immortalised), and instrumentation varied between all laboratories, we first established that the data generated by each group is compatible with the analysis workflow of the coordinating laboratory. For this, analyses were performed using both bead panels, using a combined detection antibody mix (CD9, CD63 and CD81), and data format and quality were compared at the coordinating laboratory. As depicted in Figure 2b, all samples acquired by different labs enable the identification of all capture bead populations for both MSC-MBFCM panels.

We have previously demonstrated that MBFCM assays, through their specific usage of human-specific antibodies both on capture beads and as detection antibodies, facilitate sensitive, specific and robust detection of human EV surface marker profiles regardless of sample purity, also directly in conditioned medium and in the presence of FBS in conditioned medium or in EV preparations of different purity (Wiklander et al., 2018). However, as one laboratory (Lab 5) used human platelet lysate (hPL) in its standard MSC culturing and EV-isolation procedures, a hPL-EV sample was included as control, next to the six non-MSC-derived EV samples. Thus, in total the surface protein profiles of 11 MSC-EV preparations, six non-MSC EV preparations (HEK293T cells, two epithelial and two endothelial cell sources, and BJ1 fibroblasts), and hPL were assessed and normalised (Welsh et al., 2022) centrally (Figure 3). Despite overall signal variabilities between laboratories and instruments, and evident overlap of hPL surface marker profiles with MSC-EVs from Lab 5 particularly for platelet related markers (CD41b, CD42a), we obtained clear and comparable data for several other candidate MSC-EV markers (Figure 3). Clearly, commonly used MSC-typical surface markers (e.g., CD90, CD44, CD73, CD105) are detected on MSC-EVs, showing a considerable overlap with fibroblast-derived EVs, whose surface marker signature could only be distinguished from some other MSC-derived EVs based on intensity differences, like (in general) lower abundance of CD34, CD54 and HLA-DR DP DQ. Clearer differences can be observed when comparing marker profiles of endothelial, epithelial and HEK293T derived EVs. Here, HEK293T EVs lack the MSC-typical markers, whereas subsets can be observed in endothelial (CD73, CD44, CD105, CD146) or epithelial (CD73, CD44) EVs.

Accordingly, hierarchical clustering analysis identified a total of five separate clusters: Particularly endothelial EVs, epithelial EVs, and HEK293T-EVs appeared distinct from all other samples, and MSC-EV samples and hPL EVs from Lab 5 clustered differently than other MSC-EV samples. Generally, overall clustering of MSC-EV samples was clearly associated with experimental conditions performed in different laboratories, respectively (Figure 3).

3.3 | Identification of MSC-EV-typical surface marker profiles

Based on the obtained data, we selected markers that were either consistently positive (including CD44, CD73 and CD105) or negative (including CD11b, CD45 and CD197) on MSCs from all sources (Figures 3 and 4a) and performed a hierarchical

clustering analysis on these markers as well (Figure 4a). Considering that MSC-EVs from different sources and culture conditions would differ regarding some markers, this led to a robust set of markers that appeared suitable to define MSC-EVs based on both marker positivity and negativity, similarly as it has been performed for cells before. Next, to further characterise markers identified as robustly positive on all MSC-preparations in this study, we analysed the respective co-detection of markers classified as positive by using single tetraspanin detection antibodies (CD9, CD63 or CD81) for a total of five different MSC-preparations from two labs (Figure 4b). This provided further information about potentially differential surface marker co-detection or EV subsets included in respective preparations: for example, CD105 and CD304 appeared to be more co-detected with CD63 compared to CD9 or CD81, MCSP was detected highest on CD81 positive EVs, and for HLA-ABC we obtained the highest signal with CD9 detection antibodies (Figure 4b).

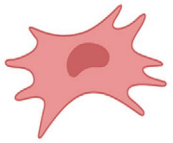
In summary, the MSC-MBFCM results of this study qualify CD73, CD105 and CD44 as most robust positive marker candidates identifying MSC-derived EVs and confirm CD11b, CD14, CD19, CD45 and CD79 as robust negative surface markers consistent with the ISCT minimal criteria for MSCs before (Figure 5) (Dominici et al., 2006). CD90 as positive cellular MSC marker and CD34 and HLA-DR as negative cellular MSC markers appeared to be less robust as MSC-EV markers for the samples tested here. Furthermore, several new consistently negative MSC markers were qualified (Figure 4). Several other surface markers were consistently detected on all MSC-EV preparations in this study and on non-MSC-derived EVs, and can be considered as more general EV markers (Figures 4 and 5).

4 | DISCUSSION

In this study, we assessed the robustness of a novel bead-based flow cytometry assay panel for the semiquantitative and qualitative assessment of potential MSC-EV and non-MSC-EV surface markers (MSC-MBFCM). We evaluated the sensitivity and selectivity of this MSC-MBFCM assay for the identification of MSC-typical or consensus MSC surface protein signatures on extracellular vesicles. Despite a plethora of included parameters varying between labs, such as different MSC-sources (bone marrow, cord blood, adipose tissue and Wharton jelly), MSC culture conditions, EV isolation procedures and MSC-MBFCM assay protocols, we identified a set of reliable surface markers that were consistently detected as either positive or negative on all MSC-EV preparations (summarised in Figure 5). We propose that these markers will be useful to enhance the quality control of MSC-EV preparations regardless of laboratory-specific parameters.

With current developments in MSC-EV-centred research and therapeutic development, a growing number of laboratories and companies is culturing MSCs from different sources, under different conditions, after which a variety of protocols is employed for the isolation and characterisation of EVs. This results in an enormous heterogeneity in EV preparations (Börger et al., 2017, Börger et al., 2016), making comparisons between results or consensus definitions of MSC-EV criteria challenging. Nevertheless, our recent analysis of published proteomics data revealed that despite this apparent heterogeneity, a common protein signature could be identified between various MSC-EV preparations (van Balkom et al., 2019). The identified protein signature was indeed confirmed in later publications (Fang et al., 2020; Hu et al., 2020; Kim et al., 2021), suggesting that protein analysis of MSC-EVs could provide a tool allowing comparison and normalisation of experimental data between research groups. In this study, we focused on surface markers that can be utilised without investigating the whole EV proteome and that can easily and specifically be measured by immuno-detection methods. It should be noted that our earlier proteomics study (van Balkom et al., 2019) did not only identify surface proteins as common MSC-EV proteins, but also soluble extracellular and intracellular proteins, of which vimentin and collagen I were included in our current analysis. Vimentin is an intracellular protein, making it not surprising that it is not detected in our surface profiling assay. Collagen I is an extracellular soluble protein which may bind to the exterior of EVs, hence making it consistently detectable in proteomics analyses. In contrast, it was consistently not detected in our current study, suggesting it may have been too low abundant or washed/stripped during EV preparation.

In line with our previous findings (Wiklander et al., 2018), this novel MBFCM assay panel could accurately distinguish HEK293T-EVs as well as epithelial and endothelial EVs from MSC-derived EVs. Although EVs from different cell types show overlapping patterns, the combination of markers allows differentiation of MSC-derived EVs and could identify potential contamination by EVs from other cell types. Notably, MSC-EVs are positive for the 'gold standard' EV surface: the tetraspanins CD63, CD9 and CD81, be it at different levels, together with MSC surface markers CD44, CD105, CD73 and CD90 which could allow for quality control in terms of sample identity and batch-to-batch comparability, and assessment of contamination by EVs not derived from MSCs (Racchetti & Meldolesi, 2021; van Balkom et al., 2019). Importantly, the identity of an MSC-EV preparation evaluated here differs from its potency, which we did not address in this study. Hence, the detection of surface antigens' presence or absence as established in this study will provide the basis for developing quantitative assays, however this is just the first step towards the goal towards defining clear criteria for MSC-EVs. Ultimately, it is the concentration or ratio of various surface antigens that is crucial in defining the identity of different MSC EV preparations (or any other EV preparations) (Witwer et al., 2019).In terms of general limitations of MBFCM-type assay results, we would like to highlight that markers not being clearly detected as positive cannot ultimately considered as negative, similarly as for basically all other immuno-detection based EV assays including Western blot. As we demonstrated before, particularly low-abundant (meaning either few proteins per EV or



Marker	MSC markers	MSC-EV markers		Robust MSC EV marker candidate
	ISCT minimal criteria*	EV proteomics#	EV MSC-MBFCM	
CD73	pos	pos (7/10)	pos (11/11)	**yes**
CD90	pos	pos (8/10)	pos (10/11)	(yes)
CD105	pos	pos (7/10)	pos (11/11)	**yes**
CD11b	neg		neg (11/11)	
CD14	neg		neg (11/11)	
CD19	neg		neg (11/11)	
CD34	neg		neg (4/11)**	
CD45	neg		neg (11/11)	
CD79	neg		neg (11/11)	
HLA-DR	neg		neg (8/11)***	
CD13	pos (optional)	pos (8/10)	pos (7/11)	no
CD44	pos (optional)		pos (11/11)	**yes**
Vimentin		pos (8/10)	neg (0/11)	no
Collagen I		pos (8/10)	neg (0/11)	no
CD9			pos (11/11)	general EV marker
CD63			pos (11/11)	general EV marker
CD81			pos (11/11)	general EV marker
CD29	pos (optional)		pos (11/11)	general EV marker
CD49b			pos (11/11)	general EV marker
CD49e			pos (11/11)	general EV marker
MCSP			pos (11/11)	general EV marker
CD304			pos (11/11)	general EV marker
CD51			pos (11/11)	general EV marker

FIGURE 5 Comparison of MSC and MSC-EV surface markers. *Surface markers in ISCT minimal criteria for defining MSC (Dominici et al., 2006). #MSC-EV surface marker detected by proteomics (van Balkom et al., 2019). **Detected as positive on MSC-EVs from MSCs cultured in hPL and 5 other MSC EV preparations. ***Detected as positive on MSC-EVs from MSCs cultured in hPL only. Note: 'general EV marker' refers to more common presence also on EVs from other sources, not necessarily from all sources.

relatively few positive EVs per sample) surface markers on EVs can appear negative even though they might be present, which can be tested by increasing assay signals, for example, through increasing EV assay input amounts or more sensitive complementary methods (Wiklander et al., 2018). For example, CD90 was detected positive for all MSC-EV samples except MSC06 (10 out of 11 samples tested), which could mean that MSC06-derived EVs were negative for CD90, or simply too low to detect, for instance because it is generally or for this specific MSC source not loaded as efficiently into released EVs as other, more consistently positively detected markers. For this example, we would conclude that CD90 as classical cellular surface marker for MSCs is still a valid and specific marker for MSC-EVs if detected as positive in such an MSC-MBFCM assay, however for EVs, it here appeared

less robust than other classical cellular MSC markers such as CD44, CD73 or CD105, which were detected in all 11 MSC-EV samples. Similarly, CD13 was detected as positive in 7 out of 11 MSC-EV samples, and its detection levels were relatively low and close to the background, making it a less robust MSC-EV marker here. In addition to this limitation for low-abundant markers in terms of assay limit of sensitivity, it should generally be noted that negative signals also could mean that EVs in respective samples are not positive for both the capture bead-targeted marker and the detection antibody target (in this study CD9, CD63 or CD81), or are truly negative for both markers. Without further testing, this remains unknown.

The inclusion of MSCs from five different sources in this study acknowledges the inherent variability in MSC sources. Donor factors such as age, sex, genetics and overall health can influence the properties and behaviour of MSCs. In addition, immortalisation can affect MSC differentiation capacity (Lai et al., 2010), and culture conditions and passage number, are variables that might impact aspects such as proliferation rate, differentiation potential and EV secretion (Miclau et al., 2023). MSCs can be derived from various tissues, each with its distinct microenvironment and physiological cues. For instance, bone marrow-derived MSCs might exhibit different properties compared to those sourced from adipose tissue, umbilical cord or placenta (Costela-Ruiz et al., 2022). These differences could manifest in terms of surface marker expression, cytokine secretion and EV cargo. The diversity in isolation methods across the five MSC-EV samples adds an additional layer of complexity. Various protocols exist for MSC-EV isolation, including (gradient) centrifugation, tangential flow filtration and polyethylene glycol precipitation. Potentially, these methods can influence the yield, purity and functional characteristics of the isolated MSC-EV (sub)populations (Reiner et al., 2017). However, it is important to note that MSC-EVs consistently present a distinct set of surface markers, irrespective of their origin or preparation method, which sets them apart from EVs generated by other cell types.

The dynamic relationship between culture medium components and EV production remains a focal point in EV research, shaping its course toward clinical translation (Shekari et al., 2023). Notably, the culture medium is enriched with essential supplements such as fetal bovine serum, human serum and human platelet lysate, which serve as both critical growth factors and prominent sources of EVs and EV-like particles. In this intricate milieu, hPL emerges as a promising alternative to FBS. Its production processes are characterised by greater control and consistent quality, making it a superior choice for minimising variability and maintaining a standardised environment for cell culture (Schallmoser et al., 2007). However, even though hPL enables xeno-free cultivation of MSCs, it is important to recognise that hPL contains analogous quantities of exogenous serum-derived EVs and various nanoparticulate entities, encompassing growth factors and protein aggregates (Forteza-Genestra et al., 2020; Gardiner et al., 2016). Studies have indicated that hPL can influence EV surface markers, size, concentration and biological activity. For instance, hPL boosts CD63, CD81 and CD9 in ASC-EVs (Fuzeta et al., 2020), increases BM-MSC-EV size and concentration (Becherucci et al., 2018), and improves immunomodulation and angiogenic potential of UC-MSC-EVs (Palombella et al., 2022). Currently, the influence of these hPL-derived vesicles on the therapeutic properties of MSC-EV preparations obtained from hPL-containing culture medium remains an open question. This necessitates a comprehensive understanding of whether exogenous serum EVs and EV-like particles work synergistically or contrarily to the specific therapeutic effects of MSC-EVs. In this study, one laboratory opted to employ hPL as an additional component for MSCs culture medium. Intriguingly, hPL containing medium sample was integrated into the experimental design as a control counterpart, thereby enhancing the comparability and normalisation of protein abundance and detection between samples. Notably, the immunophenotype of MSC-EVs demonstrated a considerable overlap with that of hPL samples. This strategic aspect serves to provide a more comprehensive understanding of the intricate interplay between hPL and MSC-EVs and emphasises the importance of considering the culture medium's impact in the context of EV research and potential clinical applications.

The modified MBFCM assay was specifically designed to include markers specifically positive or negative for MSC-EVs. Our results indicate that indeed various MSC-EVs appear to have a similar surface protein profile, which is clearly distinct from HEK293T cell-derived EVs as well as epithelial and endothelial EVs. As additional control cell type, fibroblasts (at least the cell line included in this study, and for the surface markers included in these specific MSC-EV panels), appear to have a very much overlapping, though slightly distinct protein profile. This finding is not unexpected given the great physical similarities between these two cell types (Soundararajan & Kannan, 2018; Ugurlu & Karaoz, 2020), and poses a challenge towards further refinement of any assay aiming to specifically distinguish MSC-EVs from fibroblast EVs. Also, despite the general overlapping results, minute variations in detected MSC-EV surface protein profiles exist between labs, like the GD2 positivity, only observed by Lab 1. Our current study protocol does not allow to determine whether such variations find their origin in the used cells or protocols, which can only be determined in future, more intensive, inter-laboratory studies in which also cells and vesicle preparations could be exchanged between labs.

The modified MSC-MBFCM assay allows scientists to examine a broader range of surface proteins, increasing the potential to identify novel markers specific to MSC-EVs. Since the assay is robust, and does not require much input material or purified EV samples, the MSC-MBFCM assay is expected to be useful to monitor MSC-EV production processes at different steps and contribute to validation and quality control of final MSC-EV batches produced for pre-clinical and ultimately therapeutic approaches. Further refinement and optimisation of the assay with additional capture bead populations and antibody combinations could enhance its capacity to distinguish between EVs from diverse cellular sources. This may contribute to a more comprehensive understanding of EV biology and their potential diagnostic and therapeutic applications. As the assay progresses

towards commercialisation, these insights will play a crucial role in its potential utilisation as a valuable tool in EV research and clinical applications.

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CONFLICTS OF INTEREST STATEMENT

BG is a scientific advisory board member of Innovex Therapeutics SL, Mursla Ltd, PL BioScience GmbH and ReNeuron, a consultant of Fujifilm and a founding director of Exosla Ltd; AG and SEA consult for and have financial stakes in Evox Therapeutics Ltd; SKL is a founding director of Paracrine Therapeutics; all other authors report no conflicts of interest.

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SUPPORTING INFORMATION

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

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