Cytotherapy 25 (2023) 138–147

Contents lists available at ScienceDirect

CYTOTHERAPY

journal homepage: www.isct-cytotherapy.org

FULL-LENGTH ARTICLE
Basic Research

CD73 activity of mesenchymal stromal cell-derived extracellular vesicle preparations is detergent-resistant and does not correlate with immunomodulatory capabilities

Fabiola Nardi Bauer[1], Tobias Tertel[1], Oumaima Stambouli[1], Chen Wang[2], Robin Dittrich[1], Simon Staubach[1], Verena Börger[1], Dirk M. Hermann[2], Sven Brandau[3], Bernd Giebel[1,*]

[1] *Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany*
[2] *Department of Neurology, University Hospital Essen, University of Duisburg-Essen, Essen, Germany*
[3] *Department of Otorhinolaryngology, University Hospital Essen, University of Duisburg-Essen, Essen, Germany*

ARTICLE INFO

Article History:
Received 7 April 2022
Accepted 23 September 2022

Key Words:
AMP
CD73 activity
ecto-5'-nucleotidase
extracellular vesicles
mesenchymal stromal cells
potency assay

ABSTRACT

Background aims: Extracellular vesicles (EVs) derived from human mesenchymal stromal cells (MSCs) show immunomodulatory activity in different assays both *in vitro* and *in vivo*. In previous work, the authors compared the immunomodulatory potential of independent MSC-EV preparations in a multi-donor mixed lymphocyte reaction (mdMLR) assay and an optimized steroid-refractory acute graft-versus-host disease (aGVHD) mouse model. The authors observed that only a proportion of the MSC-EV preparations showed immunomodulatory capabilities and demonstrated that only MSC-EV preparations with mdMLR immunomodulating activities were able to suppress aGVHD symptoms *in vivo* and vice versa. Since the mdMLR assay is complex and depends on primary human cells of different donors, the authors sought to establish an assay that is much easier to standardize and fulfills the requirements for becoming qualified as a potency assay.
Methods: The bona fide MSC antigen CD73 possesses ecto-5'-nucleotidase activity that cleaves pro-inflammatory extracellular adenosine monophosphate into anti-inflammatory adenosine and free phosphate. To test whether the ecto-5'-nucleotidase activity of the MSC-EV preparations reflected their immunomodulatory potential, the authors adopted an enzymatic assay that monitors the ecto-5'-nucleotidase activity of CD73 in a quantitative manner and compared the activity of well-characterized MSC-EV preparations containing or lacking mdMLR immunomodulatory activity.
Results: The authors showed that the ecto-5'-nucleotidase activity of the MSC-EV preparations did not correlate with their ability to modulate T-cell responses in the mdMLR assay and thus with their potency in improving disease symptomatology in the optimized mouse aGVHD model. Furthermore, the ecto-5'-nucleotidase activity was resistant to EV-destroying detergent treatment.
Conclusions: Ecto-5'-nucleotidase activity neither reflects the potency of the authors' MSC-EV preparations nor provides any information about the integrity of the respective EVs. Thus, ecto-5'-nucleotidase enzyme activity is not indicative for the immunomodulatory potency of the authors' MSC-EV products. The development of appropriate potency assays for MSC-EV products remains challenging.

Introduction

Extracellular vesicles (EVs) harvested from the conditioned medium (CM) of human mesenchymal stromal cells (MSCs) reveal therapeutic activities in many disease models. However, their exact mechanism of action (MoA) has not yet been unraveled [1]. The immunomodulatory capabilities of MSC-EVs have been found in a number of *in vitro* assays and pre-clinical disease models [2]. At the clinical level, the authors successfully treated a steroid-refractory acute graft-versus-host disease (aGVHD) patient with an MSC-EV product harvested from CM of allogeneic bone marrow-derived MSCs. During repeated treatment with escalating doses of the selected MSC-EV product, the pro-inflammatory immune responses of the patient were effectively suppressed [3]. Upon comparing the

* Correspondence: Bernd Giebel, PhD, Institute for Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Virchowstr. 179, Essen 45147, Germany.
E-mail address: bernd.giebel@uk-essen.de (B. Giebel).

https://doi.org/10.1016/j.jcyt.2022.09.006

pro- and anti-inflammatory cytokine content of the applied and other MSC-EV preparations, the authors observed differences in the cytokine content of various MSC-EV preparations; the applied MSC-EV product revealed the highest ratio of anti- to pro-inflammatory cytokines. In recent studies involving an ischemic stroke and a Niemann–Pick type C mouse model, the authors observed functional differences in the therapeutic activity of independent MSC-EV preparations (all produced according to the authors' standardized procedure) for the first time [4,5]. Despite the observed difference in potency in suppressing ischemic stroke and Niemann–Pick type C symptoms, the applied MSC-EV preparations revealed comparable particle numbers and protein content [5].

More recently, the authors compared the ability of independent MSC-EV preparations to modulate allogeneic T-cell responses in a multi-donor mixed lymphocyte reaction (mdMLR) assay and their potency in suppressing aGVHD symptoms in an optimized mouse model. The results showed a correlation between the MSC-EV preparations' *in vitro* immunomodulatory capabilities and their aGVHD-suppressing potency: only some of the MSC-EV preparations revealed immunomodulatory capabilities in the mdMLR assay, and only these MSC-EV preparations were able to efficiently suppress aGVHD symptoms in mice. Similar to the ischemic stroke model, the authors did not find any correlation between any of the EV characteristics, which were evaluated according to Minimal Information for Studies of Extracellular Vesicle 2018 criteria [6], and the MSC-EV preparations' functional properties [7].

Differences in the ability to suppress aGVHD symptoms have also been reported for MSC-based cellular therapeutics. For example, in a phase 3 clinical trial on adult aGVHD patients, a commercial MSC product failed to show efficacy [8,9], whereas in a later one-armed phase 3 clinical trial on pediatric aGVHD patients, the commercial product remestemcel-L demonstrated clinical recovery-promoting effects [10,11]. Despite the latter favorable results, the United States Food and Drug Administration rejected approval for remestemcel-L in the treatment of pediatric aGVHD. Within their statement, the Food and Drug Administration argued that the product is derived from the bone marrow of varying donors and that potency is not appropriately tested and, consequently, product heterogeneities are not sufficiently controlled (www.fda.gov/media/140988/download).

Thus, setting up appropriate potency assays is an essential need for the manufacture of MSC and MSC-EV products. However, as the authors recently comprehensively discussed specifically with regard to MSC-EV products, setting up appropriate potency assays is itself a challenge [1]. Ideally, potency assays should be robust, fast, reproducible and quantifiable in order to assess how accurately a therapeutic product would exert the desired function. Furthermore, potency assays have to fulfill specific regulatory requirements (https://database.ich.org/sites/default/files/Q6B_Guideline.pdf) and ideally address the product's MoA (www.fda.gov/media/79856/download).

Although the authors' mdMLR assay results turned out to correlate with the results of the murine GVHD model, the assay is complex and time-consuming [7]. Furthermore, to set up the assay, mononuclear cells (MNCs) of 12 different healthy peripheral blood donors were pooled, aliquoted and stored at −80°C until use. Although mdMLR results for each of the given pooled MNC batches are reproducible, because of the use of primary materials, batch-to-batch variabilities cannot be avoided.

Consequently, the authors aim to establish accurate functional assays that can be performed in a much easier and reproducible manner and qualified as potency assays that fulfill regulatory requirements. To this end, assays monitoring the enzymatic activity of MSC-EV-associated contents provide several advantages and have already been used for the functional testing of MSC-EV products [12–14].

CD73 is a bona fide cell surface antigen of MSCs and their EVs [15,16]. It possesses ecto-5'-nucleotidase activity and participates in the purinergic signaling pathway, which, upon activation by adenosine phosphates, triggers pro-inflammatory immune responses [17,18]. Although extracellular adenosine triphosphate and adenosine diphosphate are cleaved by the ectoenzyme nucleoside triphosphate diphosphohydrolase 1/CD39 into adenosine monophosphate (AMP) and free phosphate, the enzymatic activity of CD73 converts AMP into anti-inflammatory adenosine and free phosphate [27,28,29,30]. Since deficiency of CD73 enhances the severity of clinical aGVHD symptoms in mice [19], the authors decided to explore the utility of ecto-5'-nucleotidase activity in distinguishing MSC-EV preparations revealing or lacking immunomodulatory activity in the mdMLR assay. Accordingly, in this study, the authors adopted an enzymatic ecto-5'-nucleotidase activity assay quantifying free phosphate concentration and determined this activity in independent MSC-EV preparations.

Methods

Cultivation of MSCs

Human MSCs were cultivated from the bone marrow of healthy donors after obtaining informed consent according to the Declaration of Helsinki as described previously [3,20]. Briefly, MSCs were expanded in low-glucose Dulbecco's Modified Eagle's Medium (Lonza, Basel, Switzerland) supplemented with 10% human platelet lysate (hPL), 100 U/mL penicillin–streptomycin–L-glutamine (Thermo Fisher Scientific, Waltham, MA, USA) and 5 IU/mL heparin (Ratiopharm, Ulm, Germany). Medium exchange was performed when MSCs reached a confluency of 50%, after which media were collected every 48 h and centrifuged at 2000 × g for 15 min to remove cellular debris until MSC culture reached a confluency of about 80%. CMs were stored at −20°C until use. All processed CMs tested negative for mycoplasma. Cultivated MSCs were characterized by flow cytometry, and their osteogenic and adipogenic differentiation potential was confirmed *in vitro* in full accordance with the MSC identity criteria recently recommended by MSC-EV experts [15].

MSC-EV preparation with polyethylene glycol from MSC-CM

EVs were prepared from the CM of MSCs by applying a polyethylene glycol (PEG) ultracentrifugation-based purification strategy as described previously [21,22]. Briefly, MSC-CMs were thawed and centrifuged at 6800 × g for 45 min at 4°C in an Avanti J-26 XP centrifuge using a JS-5.3 swing-out rotor with a k-factor of 7728 (Beckman Coulter, Krefeld, Germany). This was followed by a 0.22-μm filtration step using a Nalgene bottle-top filter (Thermo Fisher Scientific). PEG 6000 (Sigma-Aldrich, Deisenhofen, Germany) and sodium chloride (NaCl) were added to clarified MSC-CM to a final concentration of 10% PEG 6000 (v/v) and 75 mM NaCl. Following incubation overnight at 4°C, EVs were pelleted in a JS-5.3 swing-out rotor in an Avanti J-26 XP centrifuge at 1500 × g for 30 min at 4°C (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 reprecipitated at 110 000 × g for 130 min at 4°C in an XPN-80 ultracentrifuge using a Ti45 tight-angle rotor with a k-factor of 133 (Beckman Coulter). Obtained pellets were resuspended in 0.9% NaCl and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES;Thermo Fisher Scientific) at 4 × 10^7 cell equivalents/mL, aliquoted into micro-centrifugation low-retention tubes (Kisker Biotech GmbH & Co KG, Steinfurt, Germany) and stored at −80°C until use.

Nanoparticle tracking analysis

MSC-EV preparations were characterized according to the updated Minimal Information for Studies of Extracellular Vesicle guidelines [6]. Nanoparticle tracking analyses were performed on a

ZetaView (Particle Metrix GmbH, Meerbusch, Germany) for particle quantification and average size estimation. Polystyrene beads measuring 100 nm (Thermo Fisher Scientific) were used for calibration. Videos were recorded at all 11 positions with five repetitions. Positions two and 10 were excluded for the final result. The machine was set up as follows: sensitivity 75, shutter 75, minimum brightness 20, minimum size 5 and maximum size 200 (see supplementary Table 1).

Protein content of MSC-EV preparations

The protein content of the MSC-EV preparations was determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions (see supplementary Table 1).

Western blot

Western blot was performed as described previously [21]. Briefly, MSC-EV preparations (25 μg) were solubilized and denatured using Laemmli sample buffer (4×) containing dithiothreitol (AppliChem GmbH, Darmstadt, Germany) and separated by 12% one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were plotted on polyvinylidene fluoride membranes (MilliporeSigma, Darmstadt, Germany), and membranes were blocked with Tris-buffered saline and 0.1% Tween 20 containing 5% (w/v) skim milk powder (Sigma-Aldrich). For the detection of exosomal marker proteins, the following antibodies were used: anti-Syntenin (clone EPR8102; Abcam, Cambridge, UK), anti-CD81 (clone JS-81; BD Biosciences, San Jose, CA, USA), anti-CD9 (clone VJ1/20.3.1; kindly provided by Francisco Sánchez, Madrid, Spain) and anti-CD63 (clone H5C6; BioLegend, San Diego, CA, USA). Anti-calnexin (clone ab10286; Abcam) antibodies were used for the detection of contaminants. Bound antibodies were counter labeled with horseradish peroxidase-conjugated secondary antibodies (Dianova, Hamburg, Germany). SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Darmstadt, Germany) was used for signal detection. Protein bands were visualized using a Fusion FX7 chemiluminescence detection system (Vilber Lourmat Deutschland GmbH, Eberhardzell, Germany). EM-MENU 4.09.83 image acquisition software was used to take 16-bit images. Image post-processing was carried out using ImageJ 1.52b software (National Institutes of Health, Bethesda, MD, USA).

Imaging flow cytometry

Unless otherwise mentioned, staining of MSC-EV preparations was performed as described previously [23,24]. Briefly, 5 μL PEG-prepared MSC-EV samples were labeled with 0.5 μL allophycocyanin (APC)-conjugated mouse anti-human CD63 (clone MEM-259; EXBIO Praha, a.s., Vestec, Czech Republic) or mouse IgG1 isotype (clone MOPC-21; BioLegend) for the detergent experiments. Additionally, for further characterization, 5 μL PEG-prepared MSC-EV samples were labeled with phycoerythrin (PE)-conjugated mouse anti-human CD9 (clone MEM-61; EXBIO Praha, a.s.) or fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD81 (clone JS-84; Beckman Coulter). Unstained samples and buffer controls without MSC-EVs but with antibodies were used as controls. All samples were incubated for 1 h in the dark at room temperature and diluted 100-fold for anti-CD9 and 40-fold for anti-CD63 or anti-CD81 with pH 7.4 phosphate-buffered saline (PBS, Thermo Fisher Scientific) before analysis.

Next, without further washing, samples were analyzed with an Amnis ImageStream^{X}Mk II instrument (Luminex Corporation, Seattle, WA, USA). Different concentrations of non-ionic detergents Tween 20 and NP-40 (0.1%, 0.25%, 0.5% and 1%) were used for evaluation of the EV characteristics of recorded objects. All samples, which were applied in U-bottom 96-well Falcon plates (Corning GmbH, Kaiserslautern, Germany), were analyzed in triplicate with 5-min acquisition time per well. All data were acquired at 60× magnification at low flow rate and with the removed beads option deactivated. More details are provided in supplementary Tables 2 and 3. Data analysis was performed exactly as described before using IDEAS 6.2 software (Luminex Corporation) [23,24]. All fluorescent events were plotted against the side scatter. A combined mask feature (MC and NMC) was used to improve the detection of fluorescent images. Images were analyzed for coincidences (swarm detection) using the spot counting feature; any event with multiple spots was excluded. All remaining events with low side scatter (<500) and a fluorescent intensity higher than 300 were used for calculation of concentrations. Data were visualized with Prism 8.4.2 software (GraphPad Software, San Diego, CA, USA).

Multi-donor mixed lymphocyte reaction

To evaluate *in vitro* the immunomodulatory activities of prepared MSC-EV samples, the authors performed mdMLR assays as described previously [7]. Briefly, peripheral blood MNCs (PBMCs) from 12 different donors were isolated by conventional Ficoll density gradient centrifugation and pooled using equivalent cell numbers from each donor and aliquoted. The aliquots of PBMC pools were stored in the vapor phase of liquid nitrogen until use. Upon thawing, PBMCs were cultured in Roswell Park Memorial Institute 1640 medium (Thermo Fisher Scientific) supplemented with 10% human AB serum (produced in-house), 100 U/mL penicillin and 100 μg/mL streptomycin (Thermo Fisher Scientific). A total of 6×10^5 cells in a final volume of 200 μL per well were cultured at 37°C and 5% carbon dioxide in the presence and absence of MSC-EV preparations in 96-well U-bottom Falcon plates. PBMCs cultured in the absence of MSC-EVs served as the stimulated control. For functional testing, 25 μg of given MSC-EV preparations was applied to the respective wells. After 5 days of culture, cells were harvested and stained with a cocktail of fluorescently labeled antibodies: anti-CD4-BV785 (clone RPA-T4, 300554; BioLegend), anti-CD25-PE (clone BC-96, 12-0259-42; Thermo Fischer Scientific) and anti-CD54-AF700 (clone 1H4, A7-429-T100; EXBIO Praha, a.s.). The authors used 7-aminoactinomycin D for the identification of dead cells. Data acquisition was performed on a CytoFLEX flow cytometer with CytExpert 2.3 software (Beckman Coulter). Obtained data were analyzed with Kaluza Analysis 2.1 software (Beckman Coulter). Flow cytometry data are shown as total cell counts.

CD73 assay

A Malachite Green colorimetric assay kit (Sigma-Aldrich) was applied to quantify the production of inorganic phosphate from 5' AMP substrate according to the manufacturer's instructions. Briefly, MSC-EV preparations (25 μg) diluted in NaCl and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid were added to 5′ AMP (50 μM) diluted in the same buffer. Following incubation for 1 h at 37°C, the colorimetric reagent was added. The absorbance was measured after 30 min at 620 nm on a PowerWave XS microplate reader (BioTek Instruments, Winooski, VT, USA). Processed AMP amounts were calculated by comparing the absorbance values with a pre-recorded standard curve. As a control, the CD73 inhibitor α,β-methyleneadenosine 5′-diphosphate (APCP) (Sigma-Aldrich) was added to selected samples to a final concentration of 10 μM. To study the requirements for EV integrity, the authors treated selected samples with different concentrations of non-ionic detergents Tween 20 and NP-40 (0.1%, 0.25%, 0.5% and 1%).

Statistical analysis

Data are presented as mean ± standard deviation. After testing for Gaussian distribution, continuous variables were compared using an

unpaired *t*-test. Statistical significance was defined as $P \leq 0.05$. Statistical analyses were performed using Prism 8.4.2 (GraphPad Software).

Results

Immunomodulatory properties differ among independent MSC-EV preparations

With the aim of exploring the utility of enzymatic CD73 assays for the prediction of functional properties of independent MSC-EV preparations, the authors selected a collection of eight independent MSC-EV preparations that showed or lacked immunomodulatory activities in the mdMLR assay in the past. Since in addition to inter-donor variability the authors also observed, to various degrees, batch-to-batch activity variations for MSC-EV products produced from aliquots of the same donor MSC stocks, the authors selected different MSC-EV batches of the two most frequently used donor MSC stocks (i.e., MSC16.3 and MSC41.5). Notably, although most MSC16.3-EV preparations lack immunomodulatory *in vitro* activities, most MSC41.5-EVs contain them.

The authors selected the two atypical MSC16.3-EV preparations that appeared active in mdMLR (16.3-EVa$_1$, 16.3-EVa$_2$) and the two atypical MSC41.5-EV preparations that appeared inactive (41.5-EVi$_1$, 41.5-EVi$_2$). A representative inactive MSC16.3-EV preparation (16.3-EVi) and a representative active MSC41.5-EV preparation (41.5-EVa) were also selected in addition to an active and inactive MSC-EV preparation from other donors (70.5-EVa, 84-EVi).

To exclude the possibility that functional properties had changed during storage of the MSC-EV samples and to have appropriate references, the authors at first retested the immunomodulatory activity of all eight samples in the mdMLR assay. Compared with the stimulated MNCs that were cultured in the absence of MSC-EVs (control), four of the eight MSC-EV preparations did not modulate the content of activated CD4 T cells (i.e., $CD54^+CD25^+CD4^+$ cells), whereas the other four MSC-EV preparations significantly reduced $CD54^+CD25^+$T-cell numbers. Confirming previous evaluations, the authors qualified the MSC-EVs 16.3-EVa$_1$, 16.3-EVa$_2$, 41.5-EVa and 70.5-EVa as MSC-EV preparations with mdMLR immunomodulatory activities (MSC-EVa) and the MSC-EV 16.3-EVi, 41.5-EVi$_1$, 41.5-EVi$_2$ and 84-EVi as MSC-EV preparations without mdMLR immunomodulatory activities (MSC-EVi) (Figure 1).

Immunomodulatory activities of MSC-EV preparations are not reflected by metric EV data

To compare the mdMLR data with metric features of the given MSC-EV preparations accurately, the authors again performed western blot and imaging flow cytometry analyses of all MSC-EV samples used in this study in parallel. 25-μg of given MSC-EV samples and as a control of the respective hPL-EV samples that had been prepared from fresh MSC media (10% pooled hPL-supplemented Dulbecco's Modified Eagle's Medium), the authors observed variabilities in the band intensities of all EV markers tested (CD9, CD63, CD81 and Syntenin) (Figure 2A). Notably, as before, the authors detected lower CD81 and higher CD9 levels in the hPL-EV control than the MSC-EV samples [7]. Calnexin, which was used as an impurity marker, was not detected in any of the EV samples but was detected in a control sample containing material of lysed MSCs (see supplementary Figure 1). For analysis, the authors quantified the intensity of the western blot bands and compared their intensities with the immunomodulating activities of MSC-EVs. However, the authors did not find any correlation (Figure 2B). Next, the authors analyzed the composition of $CD9^+$, $CD63^+$ and $CD81^+$ objects in each of the given MSC-EV samples with imaging flow cytometry (Figure 2C; also see supplementary Figure 2). Consistent with the results of western blot, the abundance of $CD9^+$, $CD63^+$ and $CD81^+$ object populations did not correlate with the mdMLR modulatory properties of the investigated MSC-EV preparations (Figure 2D). Thus, as in the authors' previous study [7], obtained metric EV data did not correlate with the explored immunomodulatory activity of the given MSC-EV preparations.

MSC-EV preparations possess ecto-5'-nucleotidase activity

The authors performed dose-finding experiments to explore ecto-5'-nucleotidase concentrations in mdMLR immunomodulatory and non-immunomodulatory MSC-EV preparations. To this end, the authors applied the MSC-EV preparations 16.3-EVa$_1$, 16.3-EVi and 41.5-EVi$_2$ in different concentrations to monitor CD73 enzyme activity. For simplicity, doses were defined according to the protein content of the MSC-EV preparations. In total, six different doses, ranging from 5 μg to 100 μg per sample, were applied in duplicate in three independent experiments. To monitor the impact of the buffer, 5′ AMP and the CD73 inhibitor APCP were applied as controls separately or together with the respective MSC-EV preparations. Both active and inactive MSC preparations showed a dose-dependent increase in free phosphate production that correlated with the amount of MSC-EV preparation that was applied (Figure 3A). As expected, in the presence of APCP, EV samples did not show any ecto-5'-nucleotidase activity.

Since the MSC-EV preparations contained human platelet-derived EVs [24–26], the authors also tested the ecto-5'-nucleotidase activity of EV preparations obtained from hPL-supplemented fresh MSC medium. However, no ecto-5'-nucleotidase activity was detected in any of the three independently prepared hPL-EV samples (hPLa, hPLb and hPLc) (Figure 3B). Thus, the results demonstrated that ecto-5'-nucleotidase activity is of MSC origin.

Ecto-5'-nucleotidase activity of MSC-EV preparations is donor-related and does not correlate with recorded mdMLR immunomodulatory activity

To evaluate whether ecto-5'-nucleotidase concentration was comparable among independent MSC-EV preparations, the authors analyzed the free phosphate production of all selected MSC-EV preparations in duplicates in parallel, as before, the authors again applied 25 μg of each MSC-EV preparation. Remarkably, independent of their immunomodulatory activities within the mdMLR assay, all 16.3 and all 41.5 MSC-EV samples displayed comparable ecto-5'-nucleotidase activities. Of note, the ecto-5'-nucleotidase activities within the 41.5 MSC-EV samples were significantly higher than those in the 16.3 MSC-EV samples ($P = 0.001$) (Figure 4A,B). Notably, there was no correlation between the mdMLR immunomodulatory activity and the recorded ecto-5'-nucleotidase activity ($P = 0.696$) (Figure 4C). Thus, the authors concluded that ecto-5'-nucleotidase activity was independent of the MSC-EV preparation immunomodulatory activity measured in the mdMLR assay.

EV integrity is not required for ecto-5'-nucleotidase activity in MSC-EV preparations

Next, the authors investigated whether ecto-5'-nucleotidase activity depended on the integrity of the EVs within the MSC-EV preparation samples. Since EVs are detergent-sensitive, 25 μg aliquots of the MSC-EV preparation 16.3i were treated with different concentrations of the non-ionic detergent Tween 20 or NP-40 (0.1%, 0.25%, 0.5% and 1%). The impact of the detergent on the EV samples was documented via anti-CD63 antibody labeling by imaging flow cytometry. Compared with untreated control samples, fewer $CD63^+$ objects were recorded after treatment with 0.1% Tween 20 or 0.1% NP-40. After applying NP-40 at a final concentration of 0.25% or greater, almost no $CD63^+$ objects were detected; however, a huge proportion of $CD63^+$ objects were Tween 20-resistant (Figure 5A).

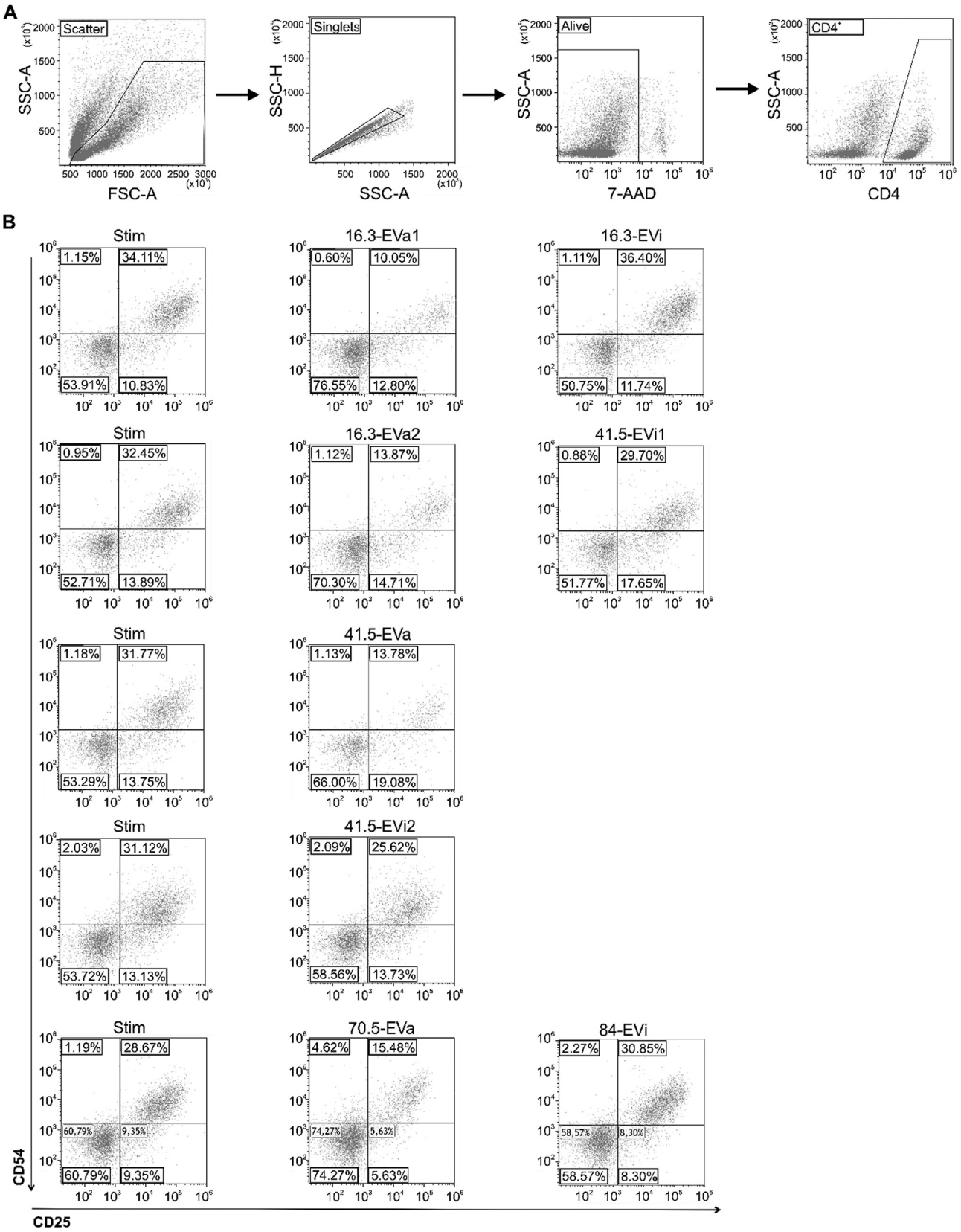

Fig. 1. MSC-EV preparations differ regarding their immunomodulatory activity. The immunomodulatory activity of different MSC-EV preparations was evaluated in the mdMLR assay. Within a culture period of 5 days, in comparison with control cultures, MSC-EV preparations considered to be immunomodulatory active reduced the frequency of activated $CD4^+$ T cells that were identified as $CD25^+CD54^+$ cells. By contrast, MSC-EV preparations judged as immunomodulatory inactive did not considerably influence the content of activated $CD4^+$ T cells in comparison with the respective control cultures. (A) MNCs were gated according to their light scatter features. Among single, non-7-AAD-labeled cells, CD4 T cells were identified as $CD4^+$ cells. (B) Frequencies of living activated CD4 T cells ($CD25^+CD54^+$) cultured for 5 days in the absence (Stim) or presence of MSC-EV preparations derived from different MSC cell stocks (16.3-EVa_1, 16.3-EVa_2, 16.3-EVi, 41.5-Eva, 41.5-EVi_1, 41.5-EVi_2, 70.5-EVa and 84-EVi). Each row represents mdMLR assays that were performed in parallel. FSC-A, forward scatter area; 7-AAD, 7-aminoactinomycin D; SSC-A, side scatter area; Stim, stimulated.

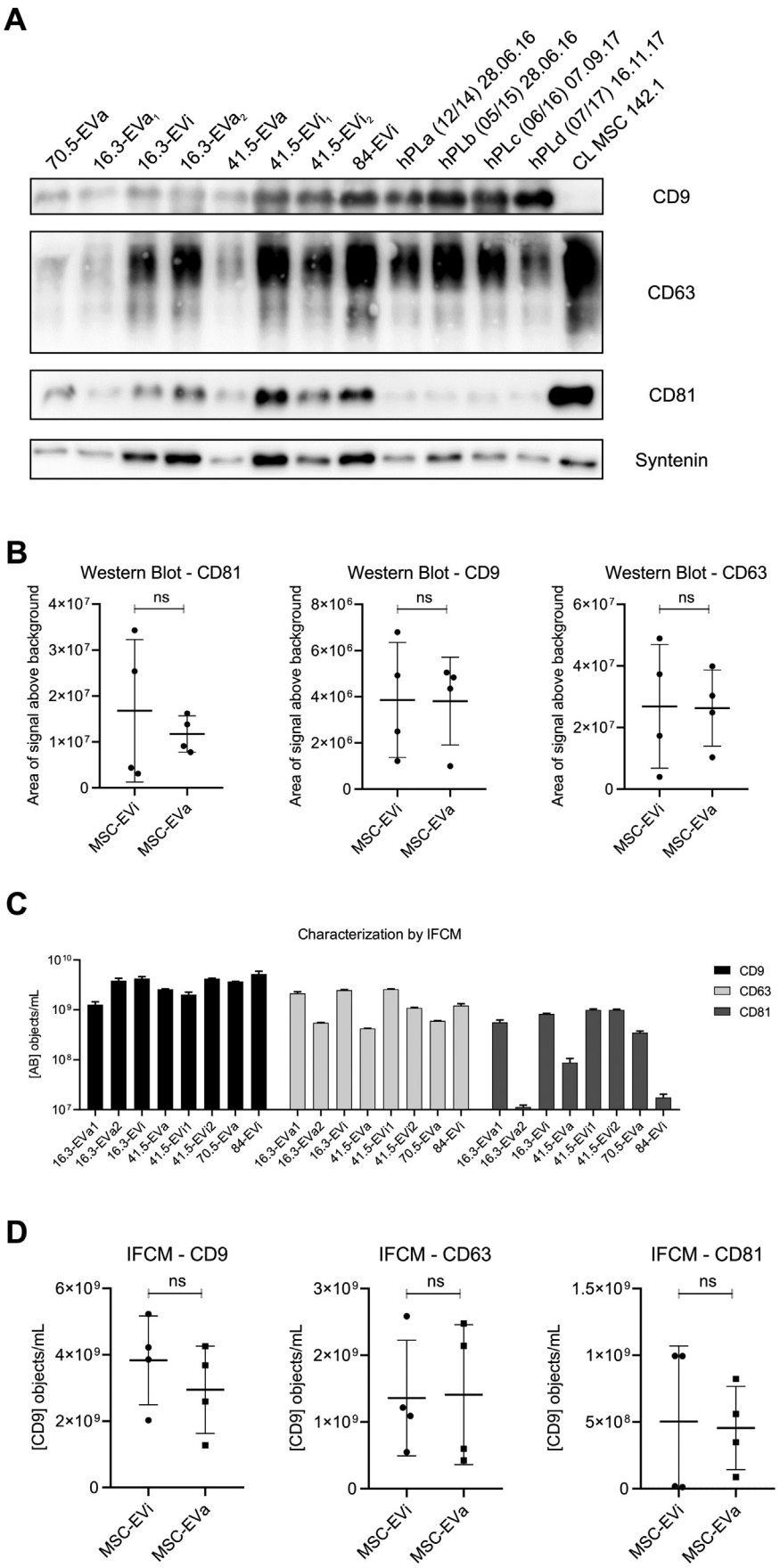

Fig. 2. Western blot and IFCM analyses reveal no correlation of EV marker abundancies with mdMLR results. Western blot and IFCM analyses of all samples were performed in parallel. For western blot, EV samples prepared from fresh hPL-supplemented cell culture media (hPL-EVs) and lysed MSCs were used as controls. (A) Western blot bands following sequential anti-CD81, anti-CD9 and anti-CD63 staining as well as anti-Syntenin staining (images of whole blots are presented in supplementary Figure 1). (B) Correlation analysis

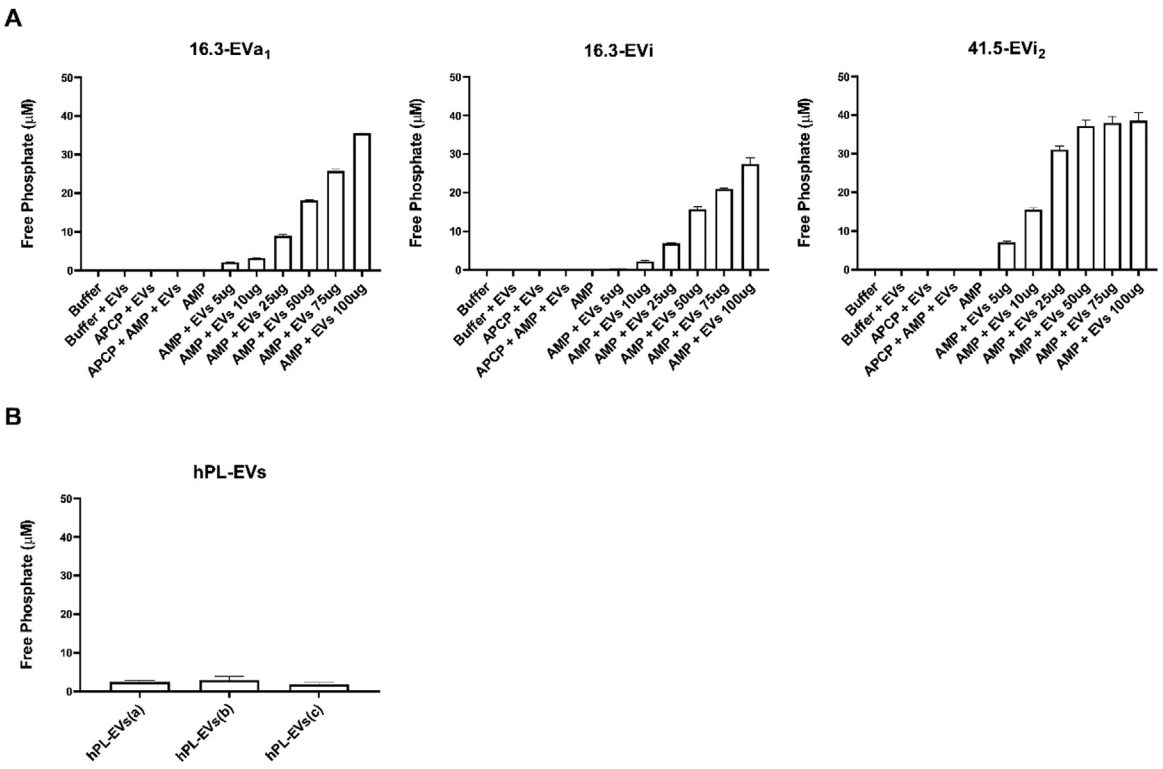

Fig. 3. Ecto-5'-nucleotidase activity is of MSC origin and can be analyzed quantitatively. For quantification of ecto-5'-nucleotidase activity within MSC-EV preparations, a Malachite Green colorimetric assay was used. This assay is based on the quantification of inorganic phosphate that is cleaved by the ecto-5'-nucleotidase activity of provided samples from a given AMP substrate. (A) MSC-EV preparation samples were applied according to their protein content (5–100 μg). A couple of controls were provided, containing different combinations of sample buffer (Buffer), 25 μg of given MSC-EV preparations, the ecto-5'-nucleotidase inhibitor APCP and/or the AMP substrate. (B) As additional controls, 25 μg of each of three independent hPL-EV preparations was tested for ecto-5'-nucleotidase activity. Data were obtained from three independent experiments performed in duplicates. Results are presented as mean ± standard deviation of duplicates.

These results demonstrated that CD63$^+$ EVs in the authors' MSC-EV preparations were NP-40-sensitive. In addition, the authors determined the ecto-5'-nucleotidase activity of detergent-treated samples. Independent of the detergent concentration, no significant reduction in recorded ecto-5'-nucleotidase activity was observed among detergent-treated and untreated MSC-EV samples (Figure 5B). Overall, in the authors' MSC-EV preparations, the results demonstrated that EV integrity was not required for ecto-5'-nucleotidase activity.

Discussion

Here the authors explored whether the ecto-5'-nucleotidase activity of given MSC-EV preparations reflected their immunomodulatory ability to suppress T-cell activation in an allogeneic setting, especially in the mdMLR assay. Independent of recorded immunomodulatory activity, the authors demonstrated that independent MSC-EV batches produced from CMs of MSCs cultivated from the same donor cell stocks contain comparable ecto-5'-nucleotidase activity and that this activity differs between MSC-EV preparations of different donors. Furthermore, the authors demonstrated that EV-destroying detergent treatment does not reduce the ecto-5'-nucleotidase activity of MSC-EV samples. Thus, ecto-5'-nucleotidase activity provides no information about the immunomodulatory activity of given MSC-EV samples, as measured by the mdMLR assay, or the quality of EVs, especially with regard to their integrity. Consequently, the authors conclude that although this assay is very precise and reproducible, it does not allow functional discrimination of MSC-EV samples containing or lacking immunomodulatory activity.

Since the predictions obtained from the mdMLR assay correlate well with the ability of MSC-EV preparations to suppress aGVHD symptoms in aGVHD mice, the authors conclude that the CD73 activity assay is also not appropriate for discriminating MSC-EV samples containing or lacking the ability to suppress aGVHD symptoms.

The fact that MSC-EV samples lacking the ability to suppress aGVHD symptoms contain ecto-5'-nucleotidase demonstrates that this activity is not sufficient for improving aGVHD. As discussed more recently, the authors hypothesize that MSC-EV preparations act in multifaceted ways and that, depending on disease-specific MoA combinations, attributes need to act together to suppress a certain disease [1]. Thus, even if the authors' results clearly demonstrate that ecto-5'-nucleotidase itself is not sufficient to suppress aGVHD, it remains an open question as to whether CD73 activity contributes to MSC-EV anti-GVHD activity. The observation that CD73 deficiency enhances the severity of aGVHD symptoms in mice [19] argues for its involvement in controlling aGVHD. To

performed with unpaired *t*-test for western blot band intensities as calculated by ImageJ and immunomodulatory properties of active (MSC-EVa) and inactive (MSC-EVi) MSC-EV samples. (C) Abundance of CD9$^+$, CD63$^+$ and CD81$^+$ objects in the different MSC-EV samples as quantified by IFCM. The applied gating strategy and examples for the analyses are provided in supplementary Figure 2. The concentration of recorded objects is provided as object/mL ± standard deviation. (D) Correlation analysis performed with unpaired *t*-test for obtained IFCM data and immunomodulatory properties of active (MSC-EVa) and inactive (MSC-EVi) MSC-EV samples. IFCM, imaging flow cytometry.

The p values for the western blots are: CD81 p=0.551; CD9 p=0.976; CD63 p=0.964.

The p values for the IFCM analyses in D are: CD9 p=0.383; CD63 p=0.944; CD81 P=0.884.

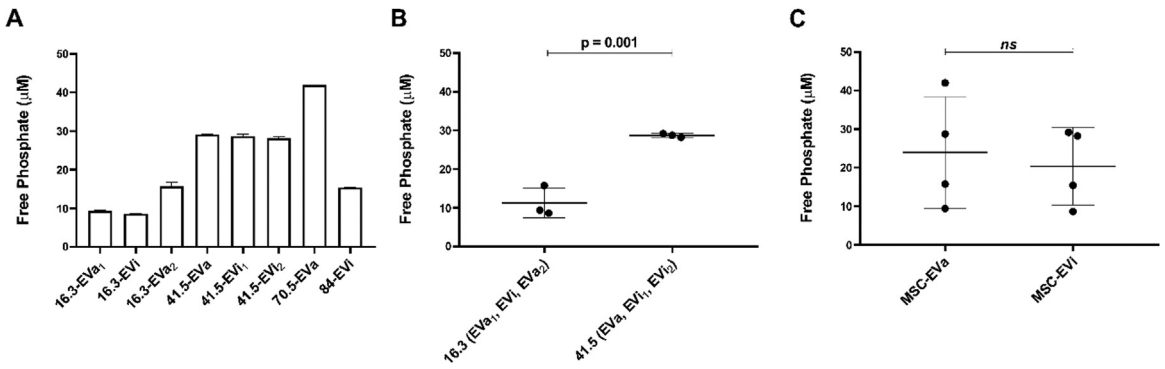

Fig. 4. Ecto-5'-nucleotidase activity is related to the donor material rather than the MSC-EV preparation's ability to modulate allogeneic immune reactions within the mdMLR assay. (A) For the ecto-5'-nucleotidase assay, 25 μg of each of the eight different MSC-EV preparations was applied in duplicate. The recorded ecto-5'-nucleotidase activity is depicted in relation to the (A) different samples, (B) MSC donor and (C) MSC-EV preparation's ability to modulate allogeneic immune responses within the mdMLR assay. (B,C) Each data point represents the mean of the duplicate. Data are presented as mean ± standard deviation. Unpaired *t*-test was used for statistical analysis. ns, not significant ($P = 0.696$).

understand whether CD73 contributes to the pre-clinical effect, CD73 activity needs to be specifically blocked or depleted in MSC-EVs. This could be achieved by, for example, CRISPR-Cas9-mediated knockout strategies as long as the loss of CD73 gene function does not significantly affect other biological properties of engineered MSCs. However, even though it will be interesting to learn about the role of CD73 activity in MSC-EV MoA, the authors' MSC-EV preparations cannot be functionally discriminated by this assay. Therefore, there must be another component that determines whether the authors' MSC-EV preparations can modulate the allogeneic immune reaction *in vitro* or *in vivo*. Although the authors compared the particle and EV abundance of selected EV markers in this study, it was impossible to identify any surrogate that allowed for discrimination of immunomodulatory active and inactive MSC-EV preparations. Notably, in the context of their aGVHD study [7], the authors compared potent and non-potent MSC-EV preparations at the proteomic and microRNA level and failed to identify any concrete surrogate marker. Thus, at the molecular level, immunomodulatory active and inactive MSC-EV samples are very similar, especially as presented by means of

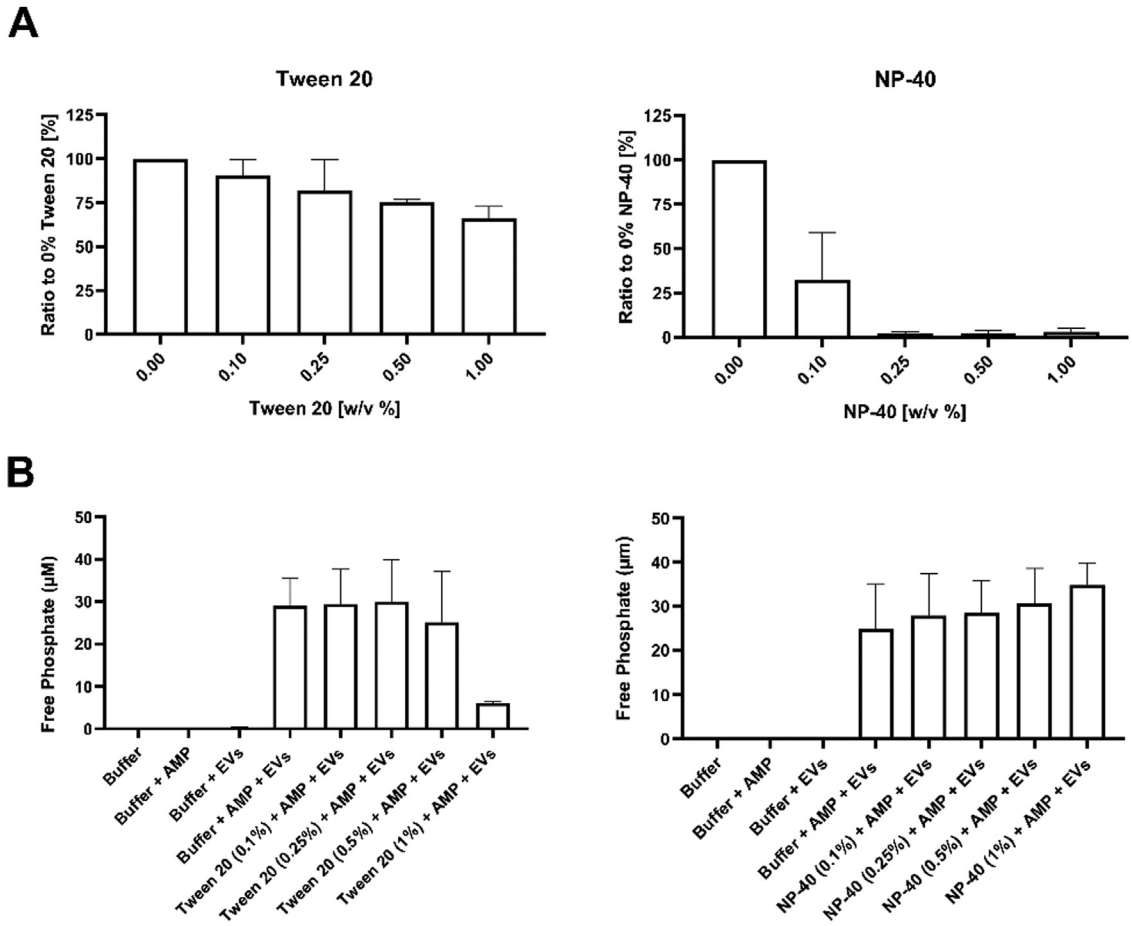

Fig. 5. Ecto-5'-nucleotidase activity in MSC-EV preparations is detergent-resistant. MSC-EV 16.3i was treated with different concentrations of the detergent Tween 20 or NP-40 (0.1%, 0.25%, 0.5% and 1%). (A) Ratio of detectable CD63$^+$ objects in detergent-treated MSC-EV preparation samples compared with that of untreated control samples. (B) Recorded ecto-5'-nucleotidase activity of detergent-treated MSC-EV preparation samples. Data are presented as mean ± standard deviation of duplicates.

their ecto-5'-nucleotidase activity when they are derived from the same donor MSC stocks.

Conclusions

One of the challenges of the next few years is to identify differences among potent and non-potent MSC-EV samples. Thus, even if the authors cannot provide any explanation for the different activities of their different MSC-EV samples, the data underscore the need to set up reliable assays that can monitor the critical therapeutic activity of given MSC-EV products. Although the mdMLR assay may not become qualified as a potency assay as a result of its use of primary human cells, it is definitely a valuable surrogate for a number of animal experiments. In the future, the mdMLR assay may help to identify other assays that will provide comparable functional predictions and that, because of their characteristics, will be more easily qualified as potency assays.

In the meantime, as exemplified here, appropriate potency testing remains one of the major hurdles in translating MSC-EV products to the clinic.

Funding

This study was supported by the LeitmarktAgentur.NRW and the European Union (European Regional Development Fund 2014-2020, EFRE-0800396), Stem Cell Network North Rhine-Westphalia and the European Union (ERA-NET EuroTransbio 11: EVTrust [031B0332B]).

Author Contributions

Conception and design of the study: FNB, TT and BG. Acquisition of data: FNB, TT, OS, CW, RD, and SS. Analysis and interpretation of data: all authors. Drafting or revising the manuscript: FNB and BG. All authors have approved the final article.

Declaration of Competing Interest

BG is a scientific advisory board member of Innovex Therapeutics SL and Mursla Ltd, a consultant of Fujifilm and a founding director of Exosla Ltd.

Acknowledgments

The authors thank the Westdeutsche Spender Zentrale for providing bone marrow samples of healthy donors. The authors are also grateful to all healthy blood donors whose cells were used in the MLR assay.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2022.09.006.

References

[1] Gimona M, Brizzi MF, Choo ABH, Dominici M, Davidson SM, Grillari J, Hermann DM, Hill AF, de Kleijn D, Lai RC, Lai CP, Lim R, Monguio-Tortajada M, Muraca M, Ochiya T, Ortiz LA, Toh WS, Yi YW, Witwer KW, Giebel B, Lim SK. Critical considerations for the development of potency tests for therapeutic applications of mesenchymal stromal cell-derived small extracellular vesicles. Cytotherapy 2021;23(5):373–80.

[2] Börger V, Bremer M, Ferrer-Tur R, Gockeln L, Stambouli O, Becic A, Giebel B. Mesenchymal Stem/Stromal Cell-Derived Extracellular Vesicles and Their Potential as Novel Immunomodulatory Therapeutic Agents. Int J Mol Sci 2017;18(7). https://doi.org/10.3390/ijms18071450.

[3] Kordelas L, Rebmann V, Ludwig AK, Radtke S, Ruesing J, Doeppner TR, Epple M, Horn PA, Beelen DW, Giebel B. MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease. Leukemia 2014;28(4):970–3.

[4] Van Hoecke L, Van Cauwenberghe C, Börger V, Bruggeman A, Castelein J, Van Imschoot G, Van Wonterghem E, Dittrich R, Claeys W, Xie J, Giebel B, Vandenbroucke RE. Anti-Inflammatory Mesenchymal Stromal Cell-Derived Extracellular Vesicles Improve Pathology in Niemann-Pick Type C Disease. Biomedicines 2021;9(12):1864.

[5] Wang C, Borger V, Sardari M, Murke F, Skuljec J, Pul R, Hagemann N, Dzyubenko E, Dittrich R, Gregorius J, Hasenberg M, Kleinschnitz C, Popa-Wagner A, Doeppner TR, Gunzer M, Giebel B, Hermann DM. Mesenchymal Stromal Cell-Derived Small Extracellular Vesicles Induce Ischemic Neuroprotection by Modulating Leukocytes and Specifically Neutrophils. Stroke 2020;51(6):1825–34.

[6] Thery C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, Antoniou A, Arab T, Archer F, Atkin-Smith GK, Ayre DC, Bach JM, Bachurski D, Baharvand H, Balaj L, Baldacchino S, Bauer NN, Baxter AA, Bebawy M, Beckham C, Zavec ABedina, Benmoussa A, Berardi AC, Bergese P, Bielska E, Blenkiron C, Bobis-Wozowicz S, Boilard E, Boireau W, Bongiovanni A, Borras FE, Bosch S, Boulanger CM, Breakefield X, Breglio AM, Brennan MA, Brigstock DR, Brisson A, Broekman ML, Bromberg JF, Bryl-Gorecka P, Buch S, Buck AH, Burger D, Busatto S, Buschmann D, Bussolati B, Buzas EI, Byrd JB, Camussi G, Carter DR, Caruso S, Chamley LW, Chang YT, Chen C, Chen S, Cheng L, Chin AR, Clayton A, Clerici SP, Cocks A, Cocucci E, Coffey RJ, Cordeiro-da-Silva A, Couch Y, Coumans FA, Coyle B, Crescitelli R, Criado MF, D'Souza-Schorey C, Das S, Chaudhuri ADatta, de Candia P, De Santana EF, De Wever O, Portillo HADel, Demaret T, Deville S, Devitt A, Dhondt B, Vizio DDi, Dieterich LC, Dolo V, Rubio APDominguez, Dominici M, Dourado MR, Driedonks TA, Duarte FV, Duncan HM, Eichenberger RM, Ekstrom K, Andaloussi SEl, Elie-Caille C, Erdbrugger U, Falcon-Perez JM, Fatima F, Fish JE, Flores-Bellver M, Forsonits A, Frelet-Barrand A, Fricke F, Fuhrmann G, Gabrielsson S, Gamez-Valero A, Gardiner C, Gartner K, Gaudin R, Gho YS, Giebel B, Gilbert C, Gimona M, Giusti I, Goberdhan DC, Gorgens A, Gorski SM, Greening DW, Gross JC, Gualerzi A, Gupta GN, Gustafson D, Handberg A, Haraszti RA, Harrison P, Hegyesi H, Hendrix A, Hill AF, Hochberg FH, Hoffmann KF, Holder B, Holthofer H, Hosseinkhani B, Hu G, Huang Y, Huber V, Hunt S, Ibrahim AG, Ikezu T, Inal JM, Isin M, Ivanova A, Jackson HK, Jacobsen S, Jay SM, Jayachandran M, Jenster G, Jiang L, Johnson SM, Jones JC, Jong A, Jovanovic-Talisman T, Jung S, Kalluri R, Kano SI, Kaur S, Kawamura Y, Keller ET, Khamari D, Khomyakova E, Khvorova A, Kierulf P, Kim KP, Kislinger T, Klingeborn M, Klinke 2nd DJ, Kornek M, Kosanovic MM, Kovacs AF, Kramer-Albers EM, Krasemann S, Krause M, Kurochkin IV, Kusuma GD, Kuypers S, Laitinen S, Langevin SM, Languino LR, Lannigan J, Lasser C, Laurent LC, Lavieu G, Lazaro-Ibanez E, Lay SLe, Lee MS, Lee YXF, Lemos DS, Lenassi M, Leszczynska A, Li IT, Liao K, Libregts SF, Ligeti E, Lim R, Lim SK, Line A, Linnemannstons K, Llorente A, Lombard CA, Lorenowicz MJ, Lorincz AM, Lotvall J, Lovett J, Lowry MC, Loyer X, Lu Q, Lukomska B, Lunavat TR, Maas SL, Malhi H, Marcilla A, Mariani J, Mariscal J, Martens-Uzunova ES, Martin-Jaular L, Martinez MC, Martins VR, Mathieu M, Mathivanan S, Maugeri M, McGinnis LK, McVey MJ, Meckes Jr. DG, Meehan KL, Mertens I, Minciacchi VR, Moller A, Jorgensen MMoller, Morales-Kastresana A, Morhayim J, Mullier F, Muraca M, Musante L, Mussack V, Muth DC, Myburgh KH, Najrana T, Nawaz M, Nazarenko I, Nejsum P, Neri C, Neri T, Nieuwland R, Nimrichter L, Nolan JP, Nolte-'t Hoen EN, Hooten NNoren, O'Driscoll L, O'Grady T, O'Loghlen A, Ochiya T, Olivier M, Ortiz A, Ortiz LA, Osteikoetxea X, Ostergaard O, Ostrowski M, Park J, Pegtel DM, Peinado H, Perut F, Pfaffl MW, Phinney DG, Pieters BC, Pink RC, Pisetsky DS, von Strandmann EPogge, Polakovicova I, Poon IK, Powell BH, Prada I, Pulliam L, Quesenberry P, Radeghieri A, Raffai RL, Raimondo S, Rak J, Ramirez MI, Raposo G, Rayyan MS, Regev-Rudzki N, Ricklefs FL, Robbins PD, Roberts DD, Rodrigues SC, Rohde E, Rome S, Rouschop KM, Rughetti A, Russell AE, Saa P, Sahoo S, Salas-Huenuleo E, Sanchez C, Saugstad JA, Saul MJ, Schiffelers RM, Schneider R, Schoyen TH, Scott A, Shahaj E, Sharma S, Shatnyeva O, Shekari F, Shelke GV, Shetty AK, Shiba K, Siljander PR, Silva AM, Skowronek A, Snyder 2nd OL, Soares RP, Sodar BW, Soekmadji C, Sotillo J, Stahl PD, Stoorvogel W, Stott SL, Strasser EF, Swift S, Tahara H, Tewari M, Timms K, Tiwari S, Tixeira R, Tkach M, Toh WS, Tomasini R, Torrecilhas AC, Tosar JP, Toxavidis V, Urbanelli L, Vader P, van Balkom BW, van der Grein SG, Van Deun J, van Herwijnen MJ, Van Keuren-Jensen K, van Niel G, van Royen ME, van Wijnen AJ, Vasconcelos MH, Vechetti Jr. IJ, Veit TD, Vella LJ, Velot E, Verweij FJ, Vestad B, Vinas JL, Visnovitz T, Vukman KV, Wahlgren J, Watson DC, Wauben MH, Weaver A, Webber JP, Weber V, Wehman AM, Weiss DJ, Welsh JA, Wendt S, Wheelock AM, Wiener Z, Witte L, Wolfram J, Xagorari A, Xander P, Xu J, Yan X, Yanez-Mo M, Yin H, Yuana Y, Zappulli V, Zarubova J, Zekas V, Zhang JY, Zhao Z, Zheng L, Zheutlin AR, Zickler AM, Zimmermann P, Zivkovic AM, Zocco D, Zuba-Surma EK. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J Extracell Vesicles 2018;7(1):1535750.

[7] Madel RJ, Börger V, Dittrich R, Bremer M, Tertel T, Ngo Thi Phuong N, Babo HA, Kordelas L, Buer J, Horn PA, Westendorf AM, Brandau S, Kirschning CJ, Giebel B. Independent human mesenchymal stromal cell-derived extracellular vesicle preparations differentially affect symptoms in an advanced murine Graft-versus-Host-Disease model. bioRxiv 2020. https://doi.org/10.1101/2020.12.21.423658.

[8] Galipeau J. The mesenchymal stromal cells dilemma–does a negative phase III trial of random donor mesenchymal stromal cells in steroid-resistant graft-versus-host disease represent a death knell or a bump in the road? Cytotherapy 2013;15(1):2–8.

[9] Kebriaei P, Hayes J, Daly A, Uberti J, Marks DI, Soiffer R, Waller EK, Burke E, Skerrett D, Shpall E, Martin PJ. A Phase 3 Randomized Study of Remestemcel-L versus Placebo Added to Second-Line Therapy in Patients with Steroid-Refractory Acute Graft-versus-Host Disease. Biol Blood Marrow Transplant 2020;26(5):835–44.

[10] Kurtzberg J, Prockop S, Chaudhury S, Horn B, Nemecek E, Prasad V, Satwani P, Teira P, Hayes J, Burke E, Group MSBS. Study 275: Updated Expanded Access

Program for Remestemcel-L in Steroid-Refractory Acute Graft-versus-Host Disease in Children. Biol Blood Marrow Transplant 2020;26(5):855–64.
[11] Kurtzberg J, Abdel-Azim H, Carpenter P, Chaudhury S, Horn B, Mahadeo K, Nemecek E, Neudorf S, Prasad V, Prockop S, Quigg T, Satwani P, Cheng A, Burke E, Hayes J, Skerrett D, Group M-GS. A Phase 3, Single-Arm, Prospective Study of Remestemcel-L, Ex Vivo Culture-Expanded Adult Human Mesenchymal Stromal Cells for the Treatment of Pediatric Patients Who Failed to Respond to Steroid Treatment for Acute Graft-versus-Host Disease. Biol Blood Marrow Transplant 2020;26(5):845–54.
[12] Zhang S, Chuah SJ, Lai RC, Hui JHP, Lim SK, Toh WS. MSC exosomes mediate cartilage repair by enhancing proliferation, attenuating apoptosis and modulating immune reactivity. Biomaterials 2018;156:16–27.
[13] Lai RC, Yeo RW, Tan KH, Lim SK. Mesenchymal stem cell exosome ameliorates reperfusion injury through proteomic complementation. Regen Med 2013;8(2):197–209.
[14] Zhang B, Lai RC, Sim WK, Choo ABH, Lane EB, Lim SK. Topical Application of Mesenchymal Stem Cell Exosomes Alleviates the Imiquimod Induced Psoriasis-Like Inflammation. Int J Mol Sci 2021;22(2). https://doi.org/10.3390/ijms22020720.
[15] Witwer KW, Van Balkom BWM, Bruno S, Choo A, Dominici M, Gimona M, Hill AF, De Kleijn D, Koh M, Lai RC, Mitsialis SA, Ortiz LA, Rohde E, Asada T, Toh WS, Weiss DJ, Zheng L, Giebel B, Lim SK. Defining mesenchymal stromal cell (MSC)-derived small extracellular vesicles for therapeutic applications. J Extracell Vesicles 2019;8(1):1609206.
[16] Dominici M, Blanc KLe, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8(4):315–7.
[17] Eltzschig HK, Sitkovsky MV, Robson SC. Purinergic signaling during inflammation. N Engl J Med 2012;367(24):2322–33.
[18] Idzko M, Ferrari D, Eltzschig HK. Nucleotide signalling during inflammation. Nature 2014;509(7500):310–7.
[19] Tsukamoto H, Chernogorova P, Ayata K, Gerlach UV, Rughani A, Ritchey JW, Ganesan J, Follo M, Zeiser R, Thompson LF, Idzko M. Deficiency of CD73/ecto-5′-nucleotidase in mice enhances acute graft-versus-host disease. Blood 2012;119(19):4554–64.
[20] Radtke S, Gorgens A, Liu B, Horn PA, Giebel B. Human mesenchymal and murine stromal cells support human lympho-myeloid progenitor expansion but not maintenance of multipotent haematopoietic stem and progenitor cells. Cell Cycle 2016;15(4):540–5.
[21] Ludwig AK, De Miroschedji K, Doeppner TR, Borger V, Ruesing J, Rebmann V, Durst S, Jansen S, Bremer M, Behrmann E, Singer BB, Jastrow H, Kuhlmann JD, El Magraoui F, Meyer HE, Hermann DM, Opalka B, Raunser S, Epple M, Horn PA, Giebel B. Precipitation with polyethylene glycol followed by washing and pelleting by ultracentrifugation enriches extracellular vesicles from tissue culture supernatants in small and large scales. J Extracell Vesicles 2018;7(1):1528109.
[22] Borger V, Staubach S, Dittrich R, Stambouli O, Giebel B. Scaled Isolation of Mesenchymal Stem/Stromal Cell-Derived Extracellular Vesicles. Curr Protoc Stem Cell Biol 2020;55(1):e128.
[23] Tertel T, Gorgens A, Giebel B. Analysis of individual extracellular vesicles by imaging flow cytometry. Methods Enzymol 2020;645:55–78.
[24] Tertel T, Bremer M, Maire C, Lamszus K, Peine S, Jawad R, Andaloussi SEL, Giebel B, Ricklefs FL, Gorgens A. High-Resolution Imaging Flow Cytometry Reveals Impact of Incubation Temperature on Labeling of Extracellular Vesicles with Antibodies. Cytometry A 2020;97(6):602–9.
[25] Gorgens A, Bremer M, Ferrer-Tur R, Murke F, Tertel T, Horn PA, Thalmann S, Welsh JA, Probst C, Guerin C, Boulanger CM, Jones JC, Hanenberg H, Erdbrugger U, Lannigan J, Ricklefs FL, El-Andaloussi S, Giebel B. Optimisation of imaging flow cytometry for the analysis of single extracellular vesicles by using fluorescence-tagged vesicles as biological reference material. J Extracell Vesicles 2019;8(1):1587567.
[26] Wiklander OPB, Bostancioglu RB, Welsh JA, Zickler AM, Murke F, Corso G, Felldin U, Hagey DW, Evertsson B, Liang XM, Gustafsson MO, Mohammad DK, Wiek C, Hanenberg H, Bremer M, Gupta D, Bjornstedt M, Giebel B, Nordin JZ, Jones JC, El Andaloussi S, Gorgens A. Systematic Methodological Evaluation of a Multiplex Bead-Based Flow Cytometry Assay for Detection of Extracellular Vesicle Surface Signatures. Front Immunol 2018;9:1326.
[27] Beavis PA, El Stagg J, Darcy PK, Smyth MJ. CD73: A potent suppressor of antitumor immune responses. Trends Immunol 2012;33:231–7. https://doi.org/10.1016/j.it.2012.02.009.
[28] Regateiro FS, El Cobbold SP, Waldmann H. CD73 and adenosine generation in the creation of regulatory microenvironments. Clin Exp Immunol 2013;171:1–7. https://doi.org/10.1111/j.1365-2249.2012.04623.x.
[29] Zimmermann H. Extracellular metabolism of ATP and other nucleotides. Naunyn Schmiedebergs Arch Pharmacol 2000;362:299–309. https://doi.org/10.1007/s002100000309.
[30] Kerkela E. Adenosinergic Immunosuppression by Human Mesenchymal Stromal Cells Requires Co-Operation with T cells. Stem Cells 2016;34:781–90. https://doi.org/10.1002/stem.2280.