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ISOLATION AND CHARACTERIZATION OF EXTRACELLULAR VESICLES DERIVED FROM MESENCHIMAL STEM CELLS FOR CLINICAL APPLICATIONS

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INTRODUCTION

- Mesenchymal Stem Cells (MSCs) exert their therapeutic action in a paracrine way by secretion of soluble factors and extracellular vesicles (EVs).
- EVs are a heterogeneous group of cell-derived membrane-surrounded structures comprising different subpopulations that differ in size, composition and biogenic mechanisms: mainly exosomes (30-100 nm) and microvesicles (100-1000 nm).
- In laboratory practice, ultracentrifugation is considered the 'gold standard' method for EV isolation, based on their differential sedimentation rates.
- Non-conventional flow cytometry (FCM) is a promising technique for characterization of EVs, since it adopts technical expedients to detect events falling in areas close to its sensitivity limits (particle diameters < 1µm).

OBJECTIVES

- Culture conditions for MSC expansion and EV collection, both intended for clinical applications, require animal-free culture media because of several safety concerns. Thus, human platelet derivatives are proposed as alternative culture supplement [1].
- Due to overlapping size ranges and the lack of specific markers, heterogeneity of EVs are hardly to be resolved. Given increasing interest in discriminating different-sized EVs, the appropriate setup of non-conventional FCM, together with the use of a fluorescent beads of varying diameters, is critical for correct EV analysis.

MATERIALS AND METHODS

- Primary Bone Marrow-derived MSCs were isolated and expanded in culture medium supplemented with platelet derivatives until passage P2;
- At 60-70% of confluence, MSCs were extensively washed with PBS and incubate with serum-free medium for 24 hours;
- MSC-conditioned medium was harvested to perform EV-isolation by ultracentrifugation protocol as described in figure 1.

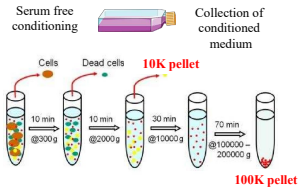

Fig. 1. Illustration of the experimental steps for isolating 2 different subsets (10K and 100K) of extracellular vesicles.

- 10K and 100K pellets were resuspended in EV-suspension buffer (PBS/EDTA) and labelled with CFSE dye and fluorochrome-conjugated antibodies anti-human: CD63, CD81, CD9;
- Sample acquisition was performed with BD FACSAria II using conventional and non conventional approach as described in [2,3] .

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EXPERIMENTAL SET UP

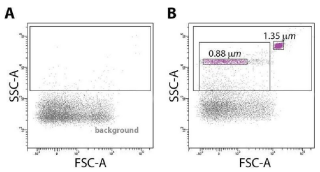

Fig. 2. Conventional FCM strategy adopted to characterize EVs [2]: (A) dot plot of filtered PBS; (B) dot plot of dimensional beads.

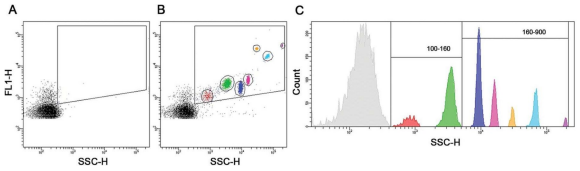

Fig. 3 Non-conventional FCM strategy adopted to characterize Evs [3]: (A) dot plot of filtered PBS/EDTA; (B) dot plot of fluorescent dimensional beads (red: 100 nm, green: 160 nm, blue: 200 nm, pink: 240 nm, light orange: 300 nm, cyan: 500 nm, and purple: 900 nm). (C) Visualization as SSC-H histogram peaks of the various dimensions of beads (gray: background).

System specification:

- Forward Scatter (FSC-Area) vs Side Scatter (SSC-Area) bi-dimensional logarithmic plot;
- 0.88- and 1.35-µm diameter beads.
- Fluorescence (FL1-Height) vs Side Scatter (SSC-Height) bidimensional logarithmic plot;
- Mixture of fluorescent beads of varying diameters.

RESULTS AND DISCUSSION

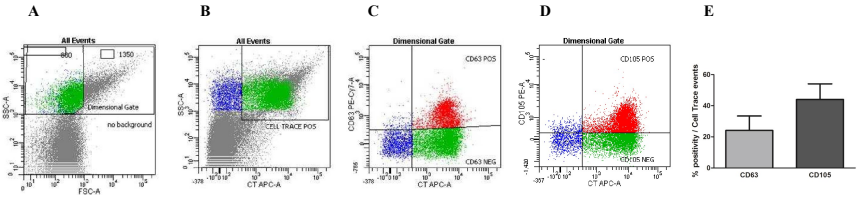

Fig 5. Conventional FCM strategy adopted to characterize EVs (only 100K pellet): dimensional gate setting with beads (A); EVs stained by the membrane-permeant dye Cell Trace (B), the vesicular marker CD63 (C) and the MSC marker CD105 (D); histogram showing the percentage of Cell Trace positive EVs coexpressing CD63 or CD105 (E).

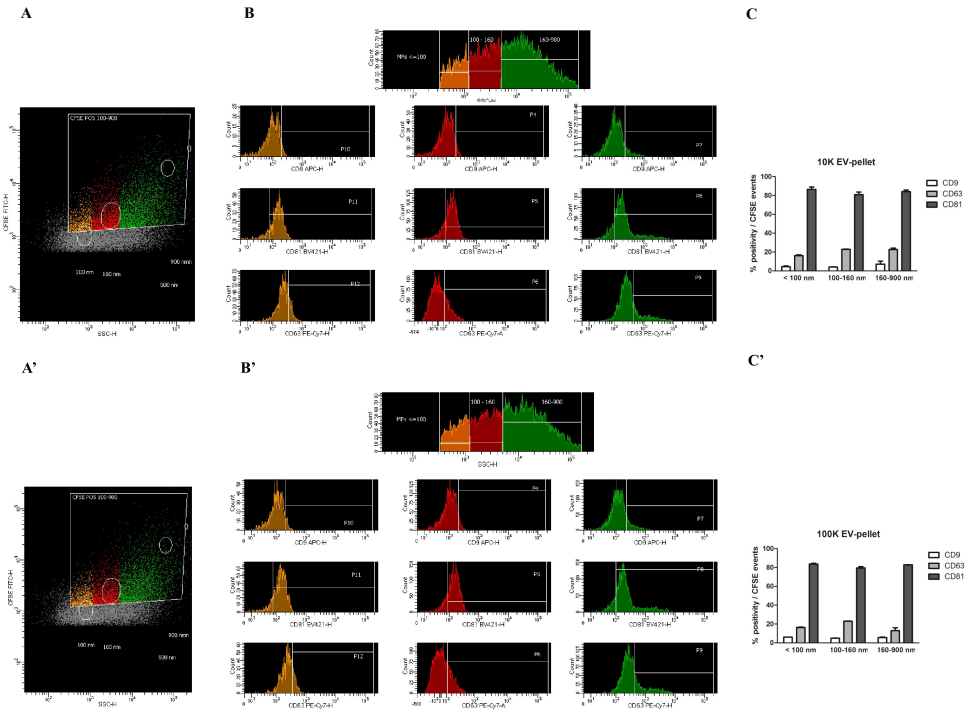

Fig 6. Non-conventional FCM strategy adopted to characterize EVs (upper panel 10K, below panel 100K pellets): dimensional gates setting with fluorescent beads (A, A'); EV-subsets stained by the fluorescent dye CFSE and the vesicular markers CD9, CD63, CD81(B,B'); histogram showing the percentage of CFSE positive EVs coexpressing CD9, CD63 and CD81 (C,C').

DISCUSSION

- Last generation FCM could become a methodology of choice for EV-characterization, based on crucial advantages: relatively low cost, multiparameter phenotyping, and good statistical robustness of the data due to a large number of events being analyzed at the same time;
- Non-conventional FCM method offer a much better resolution than the conventional one and allows to discriminate among several particle subsets during sample acquisition;
- Due to the overlapping size profiles of exosomes and microvesicles, the separation of these two vesicle classes by differential centrifugation is incomplete, and the pellets contain an heterogeneous population of MSC-EVs.

ACKNOWLEDGEMENT

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