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A multiplex bead platform for the characterization of cell culture–and plasma-derived exosomes

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Introduction

Exosomes are released by a variety of cell types either constitutively or in a stimulation-induced fashion. Depending on the originating cell, exosomes are loaded with a specific set of proteins, lipids, and nucleic acids. To investigate the origin, composition, and function of exosomes in biological fluids (e. g. plasma), specific markers are needed. We established a multiplex bead-based platform consisting of capture and detection antibodies to analyze the composition of exosome surface proteins in a given sample by flow cytometry.

Methods

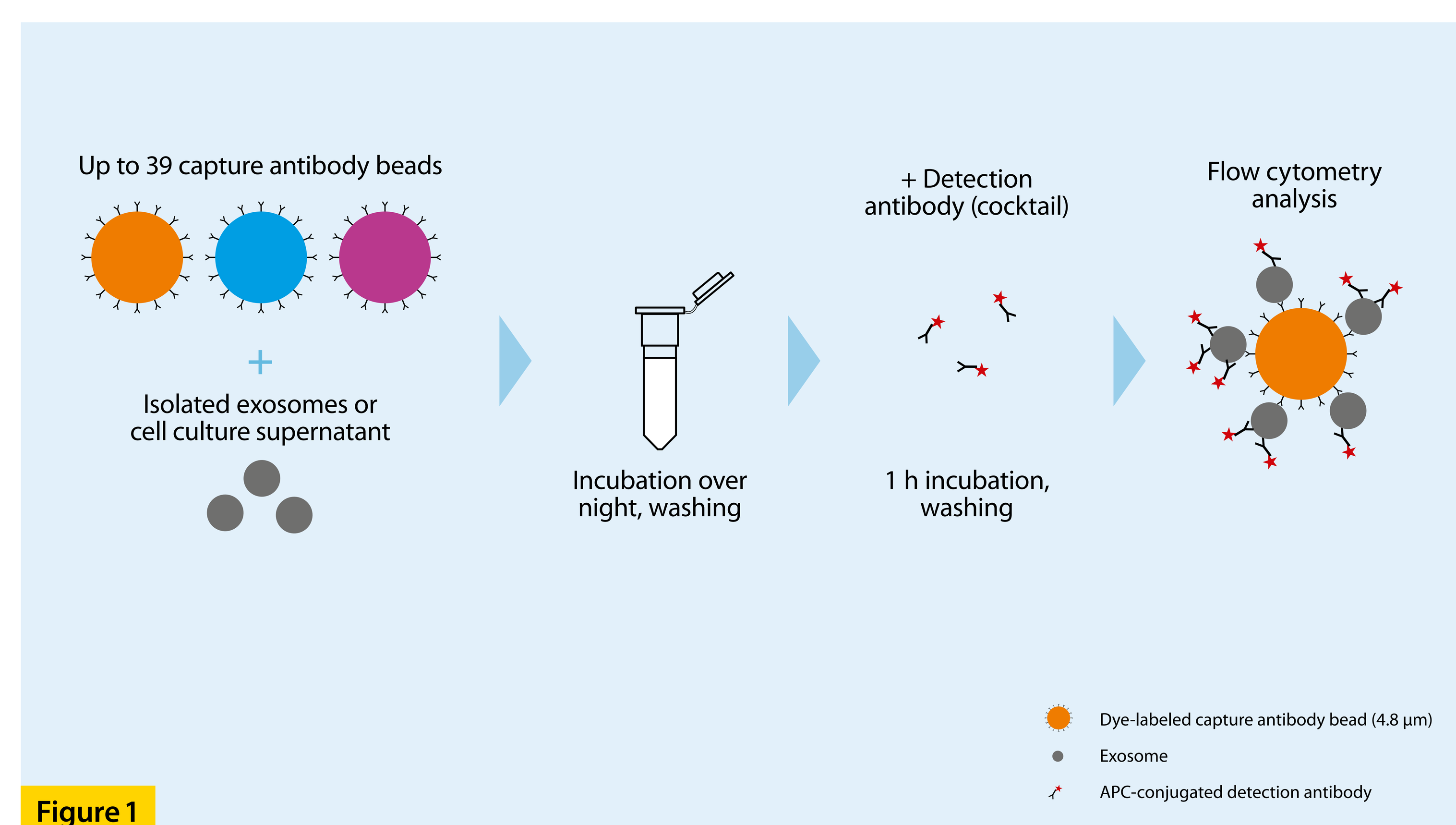

Figure 1: Workflow of the multiplex bead platform. Isolated exosomes or cell culture supernatant were incubated overnight with up to 39 different bead types, each coupled to a different capture antibody. The different bead types were labeled with one of two dyes or different proportions of both dyes, so that all bead types were distinguishable by flow cytometry. Exosomes bound to the beads were detected with CD9-APC, CD63-APC, or CD81-APC antibodies or with a cocktail of these antibodies.

Figure 2

Figure 2: Analysis example showing **(A)** gating according to bead size, **(B)** discrimination of differently labeled bead populations, and **(C)** measurement of signal intensities of the single bead populations.

One possibility of comparing signal intensities from different exosome populations is the normalization of the median signal intensities from the different capture antibody bead populations to the mean signal intensity of the beads coupled to the antibodies detecting exosome markers, i.e., CD9, CD63, and CD81.

Originating cell type	Source	Isolation	Purity	Medium	Cultivation time	Exosome isolation
NK cells	Buffy coats	MACSxpress® Technology	95–98%	T cell medium + 5% human AB serum + 500 IU/mL Proleukin + EBV-LCLs	12 days	2,000×g for 30 min, 10,000×g for 45 min, 0.22 µm filtrated supernatant. Exosomes were sedimented at 100,000×g for 2 h, washed with PBS, and then resuspended in PBS. Protein concentration was determined by BCA Assay and BSA as standard.[3]
Platelets	Fresh whole blood	Serial centrifugation	92–99%	Krebs Ringer buffer# + 50 nM calcium ionophore A23187 + 10 mM $CaCl_2$[1]	30 min	
T cells	PBMCs	MACS® MicroBead Technology	96–99%	T cell medium + 5 U/mL IL-2 + 2.5 µg/mL CD28 + CD3 coating[2]	24 h	
Cell lines				RPMI 1640 + 2 mM L-glutamine	72 h	

Table 1: Overview of cell isolation and cultivation for exosome production. # Krebs Ringer buffer (100 mM NaCl, 4 mM KCl, 20 mM $NaHCO_3$, 2 mM Na_2SO_4, 4.7 mM citric acid, 14.2 mM trisodium citrate)

Results

1 Signal intensities are specific and not impaired by the number of beads or the composition of the bead set

In order to verify the specificity of exosome binding to the capture antibody beads, the beads were incubated with NK cell exosomes alone or in combination with soluble mouse IgG1 as isotype control or soluble CD63 antibody to block the binding to the anti-CD63 beads. In contrast to the isotype control, blocking with soluble CD63 antibody specifically inhibited binding of the exosomes to the anti-CD63 beads (fig. 3A), demonstrating that exosome binding to the beads was specific.

Signal intensities of the capture antibody beads were comparable in an 8-plex and 34-plex format (fig. 3B), suggesting that the composition of the bead set or the number of beads used did not affect the signal intensities.

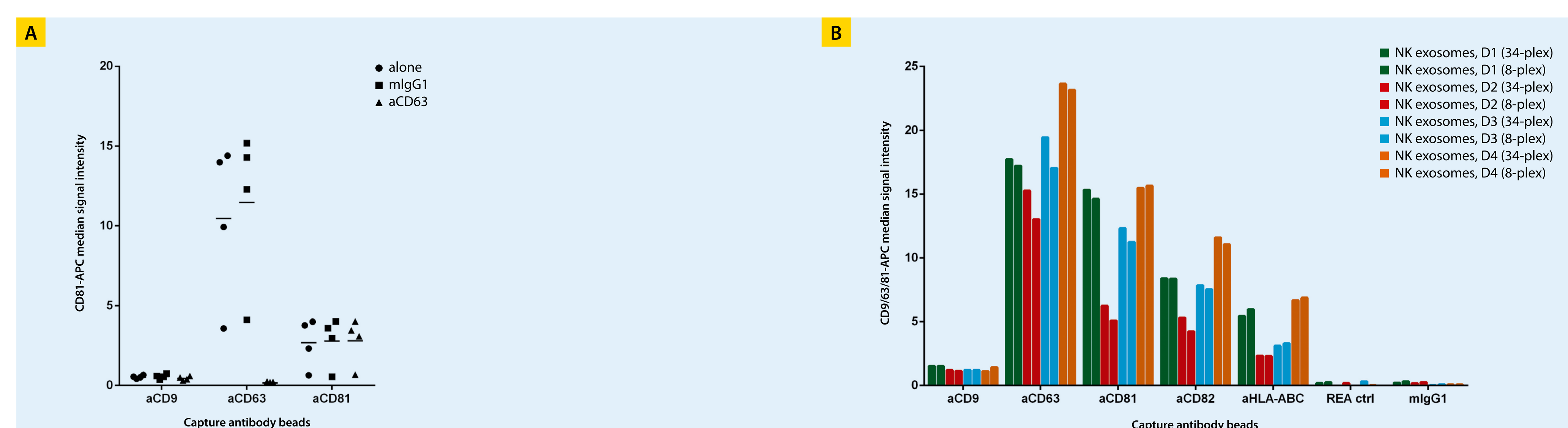

Figure 3: (A) Median signal intensities after overnight incubation of capture antibody beads with 8 µg of NK cell exosomes alone (four donors) or in combination with soluble mouse IgG1 isotype control or soluble CD63 antibody, followed by staining with a CD81-APC antibody for 1 h. **(B)** Median signal intensities after overnight incubation of 8 µg of NK cell exosomes (from four donors, D1–D4) with eight (8-plex) or 34 (34-plex) different capture antibody beads and staining with a cocktail of CD9-APC, CD63-APC and CD81-APC antibodies for 1 h. REA ctrl and mIgG1 indicate isotype control beads, i.e., beads linked to an antibody, which does not bind to exosomes.

2 Exosomes from primary cells carry cell-specific surface proteins and differ in common exosome markers

Surface proteins of exosomes from cultured primary cells were investigated using the multiplex bead platform. Well-established cell markers were detected on the respective secreted exosomes, e.g., CD2, CD3, CD4, and CD8 on T cell exosomes, CD56 on NK cell exosomes, and CD61 and CD42a on platelet exosomes. Exosome surface protein profiles can be used to draw conclusions on their origin or to investigate differences between exosomes and the membrane composition of the originating cells. The common exosome markers CD9, CD63, and CD81 were not equally distributed on the investigated populations. CD9 was underrepresented on NK cell exosomes as well as CD81 on platelet exosomes. In contrast, T cell exosomes seemed to carry comparable amounts of CD9, CD63, and CD81 on their surface (fig. 4).

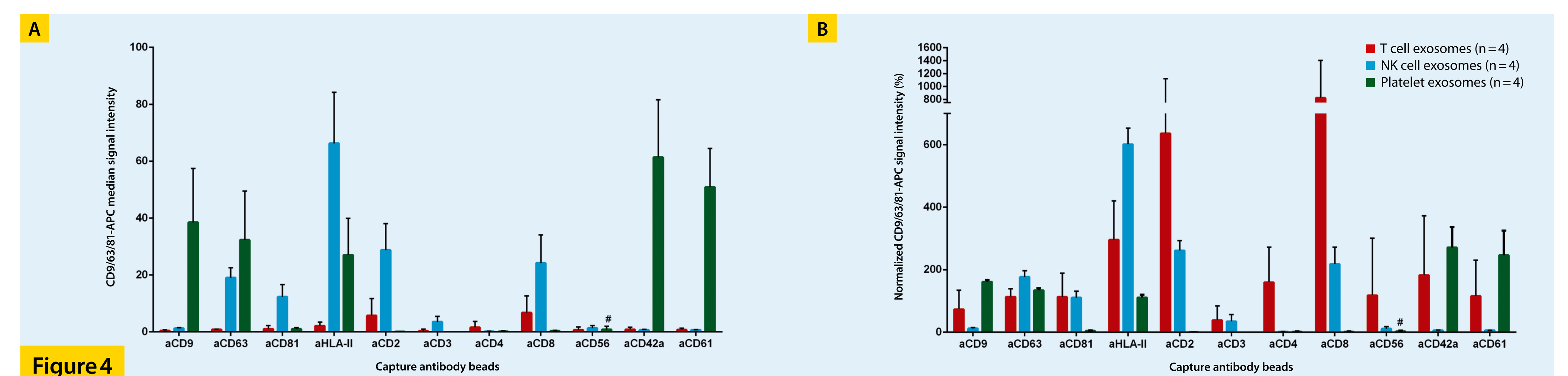

Figure 4: (A) Background-corrected raw data and **(B)** normalized signal intensities of different capture antibody beads after overnight incubation with 8 µg NK cell exosomes, 32 µg platelet exosomes, or 2.5 µg T cell exosomes and staining with a cocktail of CD9-APC, CD63-APC, and CD81-APC antibodies for 1 h. # One outlier was excluded from the analysis.

3 Plasma exosomes have mainly HLA class II and CD61 on their surface

Plasma exosomes from four different donors showed comparable signal intensities for the analyzed epitopes after normalization to the mean signal intensities of anti-CD9, anti-CD63, and anti-CD81 beads. The highest signal intensities were detected on anti-HLA class II beads and anti-CD61 beads (fig. 5). This suggests that HLA class II–expressing cells, such as antigen-presenting cells, and platelets were the main sources of exosomes in peripheral blood.

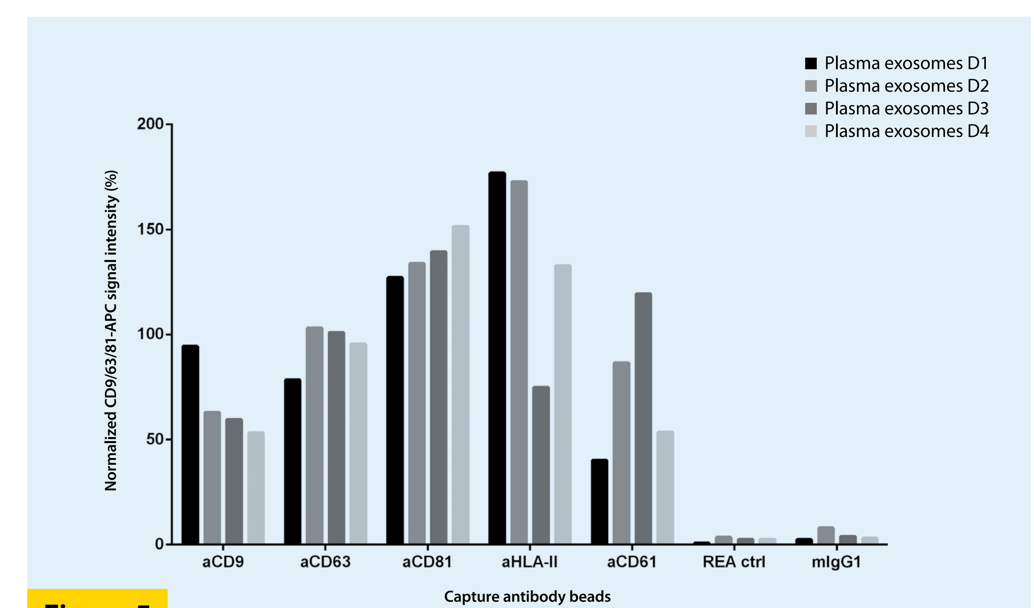

Figure 5: Normalized signal intensities of different capture antibody beads after overnight incubation with 64 µg of plasma exosomes from four donors (D1–D4) and staining with a cocktail of CD9-APC, CD63-APC, and CD81-APC antibodies for 1 h.

4 Exosomes from different cancer cell lines reflect markers of the originating tumor

Two cancer-related antigens were tested for their presence on cancer exosomes. The melanoma-associated chondroitin sulfate proteoglycan antigen (MCSP) is expressed on the majority (>90%) of human melanoma tissues and melanoma cell lines, but not on carcinoma cells[4–6]. In contrast, the epithelial cell adhesion molecule (EpCAM) is broadly expressed on the basolateral surface of carcinomas, but not on melanoma[7]. Consequently, MCSP could be detected on exosomes derived from three primary melanoma cell lines, but hardly on exosomes from the colon carcinoma cell line HT-29 (fig. 6). Likewise, EpCAM was detected on the colon carcinoma exosomes, but barely on the three melanoma exosome preparations (fig. 6).

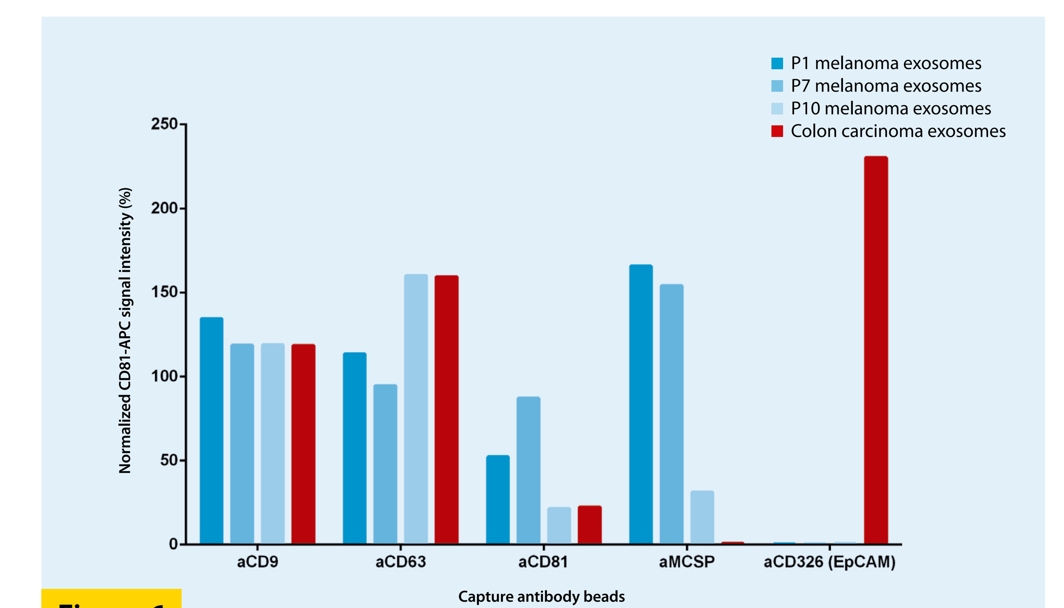

Figure 6: Normalized signal intensities of different capture antibody beads after incubation with 4 µg of exosomes from melanoma or colon carcinoma cell lines and staining with a CD81-APC antibody.

Conclusion & outlook

- The multiplex bead platform allows the specific detection of exosome surface proteins and the assessment of their relative abundance on exosomes from different sources.
- The composition of the multiplex bead set did not affect signal intensities.
- The common exosome markers were not equally distributed in all exosome populations: NK cell exosomes had less CD9, while CD81 was underrepresented on platelet exosomes.
- Well-established cell markers were detectable on the secreted exosomes, i.e., CD3 on T cell exosomes, CD56 on NK cell exosomes, and CD61 on platelet exosomes.
- The presence of HLA class II and CD61 on plasma exosomes suggests that HLA class II–expressing cells, such as antigen-presenting cells, and platelets are major sources of exosomes in blood.
- Tumor-associated markers were specifically detected on tumor cell line–derived exosomes, namely MCSP on melanoma exosomes and EpCAM on colon carcinoma exosomes.

The relative abundance of epitopes on exosome surfaces varies depending on the originating cell type and its status. A comprehensive analysis of exosome surface protein composition will enable the classification of exosome populations, e.g., according to their origin, and might give insight into their different functions. In our ongoing research we investigate the impact of cell stimulation and cancer status on the exosome protein profile.

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