A multiplex bead platform for protein profiling of exosomes by flow cytometry

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Background

Exosomes or extracellular vesicles (EVs) are loaded with specific sets of proteins, lipids, and nucleic acids. The EV composition depends on the originating cell and different EVs can be distinguished by surface marker profiling. We established a multiplex bead-based assay consisting of capture and detection antibodies to analyze the composition of exosomal surface proteins by flow cytometry.

Methods

Multiplex bead–based assay
Color-coded polystyrene multiplex beads were incubated with isolated EVs in 300 µL PAP (PBS, 0.1% Pluronic® F127, 0.09% azide), cell culture supernatant, or ascites at 4 °C overnight. Beads were washed in PAP and centrifuged at 3,000×g for 5 min. The beads were resuspended in 100 µL PAP and bound EVs were stained with 0.5 µg detection antibody. Stained EVs on the multiplex beads can also be fixed by adding 100 µL Inside Fix (Miltenyi Biotec) for 20 minutes at room temperature.

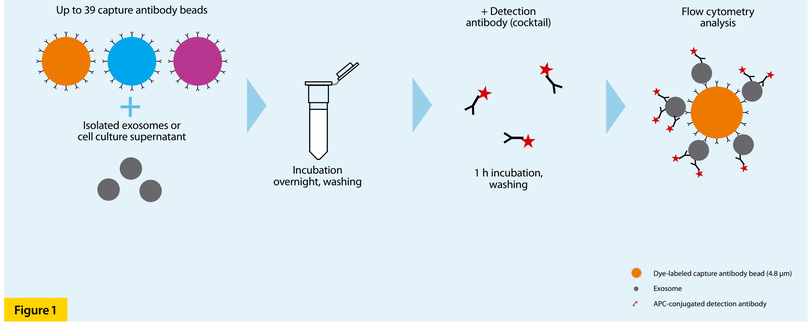

Figure 1

Workflow of the multiplex bead platform: Isolated exosomes were incubated overnight with 39 differently labeled beads each coupled to a different capture antibody. Bound exosomes were detected with APC-conjugated antibodies.

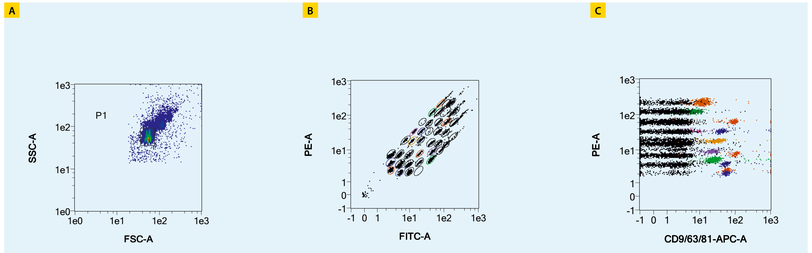

Figure 2

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.

Results

1 Cancer cell EVs from melanoma or colon cancer display different surface markers

EVs isolated from cancer cell lines can be discriminated by differing tumor markers. MSCP-positive EVs were captured from all melanoma samples. In contrast, melanoma EVs hardly showed any CD326 (EpCAM) compared to colon cancer EVs as demonstrated by binding to the respective capture beads (fig. 3).

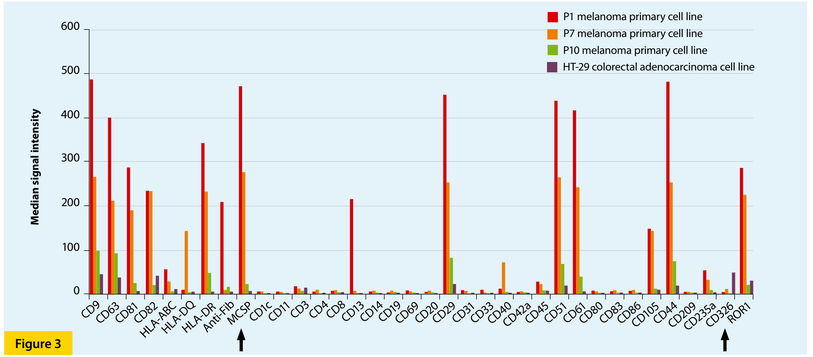

Figure 3

Surface marker profile of EVs isolated from primary melanoma cell cultures or HT-29 colorectal adenocarcinoma cell line. Shown is the APC median signal intensity of different capture antibody bead types after incubation with 4 µg EVs, followed by staining with a cocktail of CD9-APC, CD63-APC, and CD81-APC antibodies.

2 Cancer cell EVs from ovarian cancer display different surface markers

We investigated surface proteins of EVs captured directly from ascites obtained from five ovarian cancer patients. Besides the common exosome markers CD9, CD63, and CD81, we detected markers indicating blood cell–derived vesicles, including CD4 and CD8 for T cells and CD14 for monocytes. Interestingly, samples from different patients showed greatly different levels of key tumor makers such as CD326 (EpCAM), CD133 (PROM1), CD44, and CD49e, which indicate the presence of tumor-derived exosomes in the ascites.

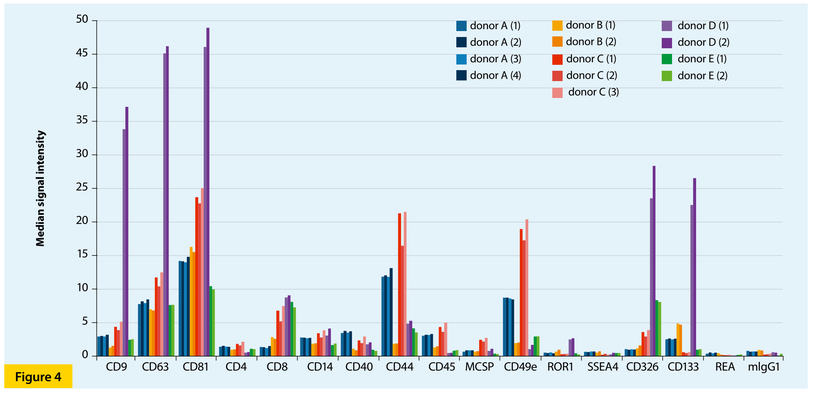

Figure 4

Surface marker profile of EVs captured from ascites obtained from ovarian cancer patients. APC median signal intensity of selected capture antibody bead types after incubation with ascites, followed by staining with a cocktail of CD9-APC, CD63-APC, and CD81-APC antibodies. REA and mIgG1 indicate isotype control beads.

3 Analysis of surface markers on B cell EVs suggests presence of EV subpopulations

EVs from activated B cells were incubated with the 39-plex beads and stained with a cocktail of CD9, CD63, and CD81-APC antibodies or with selected single antibodies (fig. 5). With regard to B cell–specific markers, anti-CD19 beads showed stronger signals than anti-CD20 beads after staining with the antibody cocktail (APC median signal 11.3 and 5.4, respectively). We therefore conclude that less EVs were positive for CD20 than for CD19. Conversely, on all bead types CD20-APC signals were stronger than CD19-APC signals, suggesting that the amounts of CD20 per EV were higher compared to CD19. We propose a subpopulation of B cell EVs carrying high levels of CD20.
The anti-CD42a beads and anti-CD9 beads exhibited signals exclusively after staining with the CD9/63/81-APC antibody cocktail. The signals on the anti-CD42a beads indicated the presence of platelets during cell culture. Therefore, we performed cell stainings after B cell isolation and indeed detected 90.5% CD42a-positive events when no gates and triggers were set (data not shown), confirming the presence of platelets within the isolated B cell fraction. We conclude that the signal on the anti-CD9 beads was most likely due to platelet EVs and that B cell EVs most likely are CD9-negative.
For the analysis of *in vitro* activated B cell exosomes, we used specific staining antibodies to investigate potential exosome subpopulations. The signals for the activation markers CD80 and CD86 demonstrate that the activation status of the cells is reflected by the secreted vesicles.

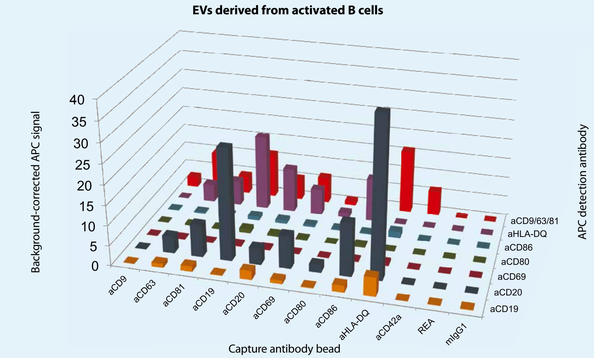

Figure 5

Matrix profile of B cell EVs. Background-corrected APC median signal intensity of different capture antibody bead types after incubation with 32 µg EVs from activated B cells, followed by staining with CD19-APC, CD20-APC, CD69-APC, CD80-APC, CD86-APC, Anti-HLA-DQ-APC, or a cocktail of CD9-APC, CD63-APC, and CD81-APC antibodies. REA and mIgG1 indicate isotype control beads.

4 EVs bound to multiplex beads via different surface markers can be fixed

To enable the analysis of EVs from potentially infectious material, we established a protocol to fix the stained vesicles bound to multiplex beads for later flow cytometric analysis. Fixed and unfixed samples gave comparable results (fig. 6).

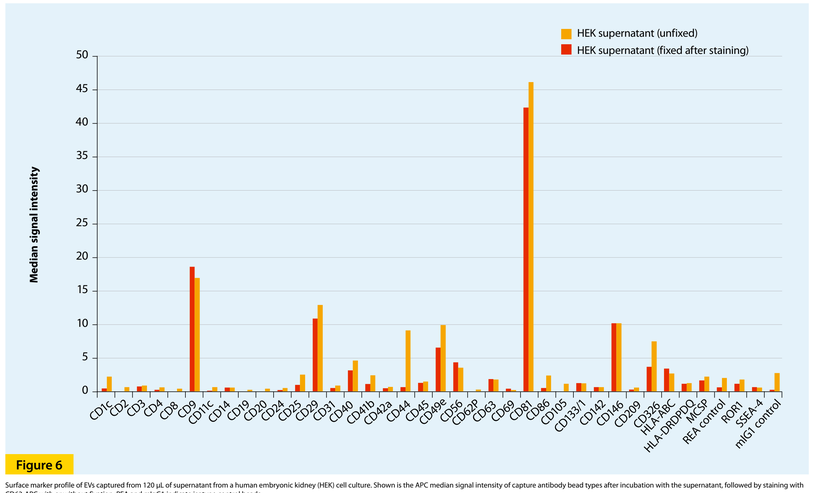

Figure 6

Surface marker profile of EVs captured from 120 µL of supernatant from a human embryonic kidney (HEK) cell culture. Shown is the APC median signal intensity of capture antibody bead types after incubation with the supernatant, followed by staining with CD63-APC with or without fixation. REA and mIgG1 indicate isotype control beads.

Conclusion

- We developed a multiplex bead platform to simplify the analysis of the surface protein composition of extracellular vesicles.
- Vesicles can be captured directly from body fluids like ascites.
- EVs, which are bound to specific beads and stained using common exosome markers, can be detected using standard flow cytometry. Fixation is possible.
- Additional staining antibodies can reveal information on potential exosome subpopulations.