Diversity of extracellular vesicles addressed by immunoaffinity beads

Nina Koliha, Yvonne Wiencek, Ute Heider, Andreas Bosio, and Stefan Wild
Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

Introduction

Exosomes are extracellular vesicles (EVs) that are released by a variety of cell types either constitutively or in a stimulation-induced manner. 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 assay consisting of capture and detection antibodies to analyze the composition of exosome surface proteins in a given sample 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) 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 and analyzed by flow cytometry.

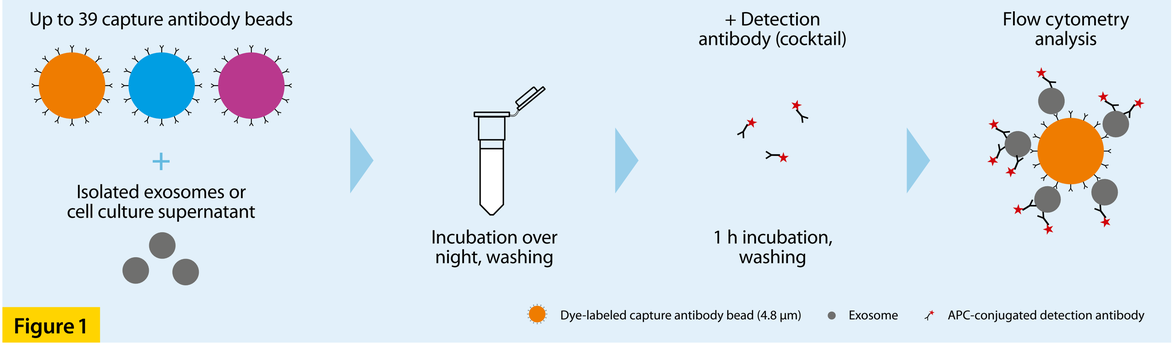

Figure 1

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.

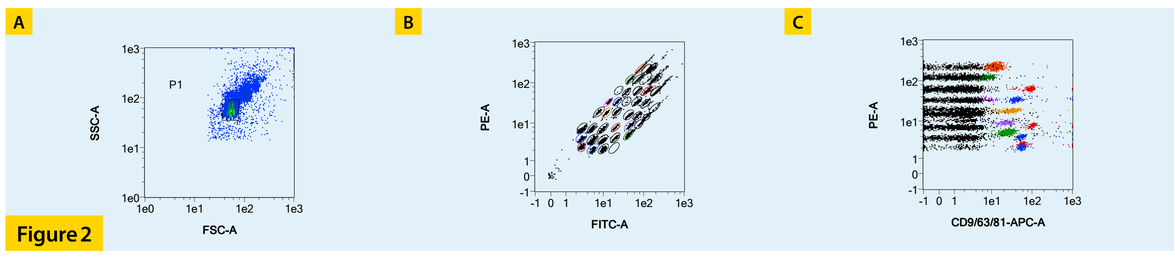

Figure 2

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.

Immunomagnetic separation of EVs

Plasma or isolated EVs from cell culture supernatant were incubated with 50 µL CD9, CD61, CD63, or CD81 MicroBeads (Miltenyi Biotec) for 1 h. Magnetically labeled EVs were applied to a MACS® Column placed in a magnetic field. Labeled EVs were retained in the column, washed, and eluted after removal of the column from the magnetic field. For the multiplex platform analysis, EVs and beads were co-eluted with 100 µL PAP buffer. 100 µL hot (95 °C) 1× SDS Loading Buffer was used to elute EVs for Western Blot analysis.

Results

1 EVs from NK cells and platelets differ in their tetraspanin composition

NK cell– or platelet-derived EVs were incubated with the 39-plex beads and stained with APC-conjugated antibodies detecting the tetraspanins CD9, CD63, or CD81 (Miltenyi Biotec, Germany) or with a cocktail of these antibodies.
For the NK cell–derived EVs, no signals were detected on anti-CD9 beads (fig. 3A). Additionally, no CD9-APC antibody staining was observed on any bead type. We conclude that NK cell–derived EVs carry hardly any CD9. Platelet-derived EVs appeared to carry only low amounts of CD81, i.e., hardly any EVs were detected on anti-CD81 beads or on any other bead type after CD81-APC antibody staining (fig. 3B). Based on this finding we prepared a mixture of NK cell and platelet EVs to mimic two distinct EV populations in a single sample. After staining with a cocktail of CD9-, CD63-, and CD81-APC antibodies, each capture antibody bead type gave a signal indicating that EVs bound to all three bead types. Using a staining cocktail, $CD9^-CD81^+$ EV populations could not be distinguished from $CD9^+CD81^-$ populations (fig. 3C). However, separate stainings with CD9 or CD81 antibody gave very low $CD9^+CD81^+$ double-positive signals demonstrating that CD9 and CD81 do not co-exist on the same EV, i.e., the sample comprises two subpopulations of $CD9^-CD81^+$ (NK cell) EVs and $CD9^+CD81^-$ (platelet) EVs.

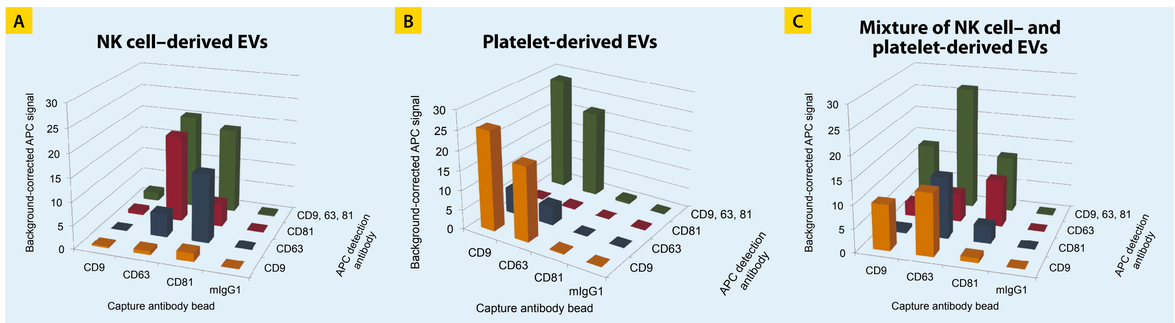

Figure 3

(A–C) Background-corrected APC median signal intensities of anti-CD9-, anti-CD63-, anti-CD81 beads and isotype control beads after incubation with (A) 16 µg NK cell–derived EVs, (B) 16 µg platelet-derived EVs, and (C) a mixture of both NK cell–derived and platelet-derived EVs (8 µg each) followed by staining with CD9-APC, CD63-APC, or CD81-APC antibodies or with a cocktail of these antibodies. mIgG1 indicates isotype control beads.

2 EV subpopulations can be separated using superparamagnetic MicroBeads

We used the same mixture of NK cell and platelet EVs and separated the respective subpopulation using CD9 MicroBeads or CD81 MicroBeads. Western blot analysis of the mixture of NK cell and platelet EVs gave no indication of the two subpopulations as CD29, CD81, and CD9 were detected (fig. 4 middle). However, using CD9 MicroBeads and CD81 MicroBeads, the $CD9^+$ EVs (platelet origin) and $CD81^+$ NK cell EVs could be separated from each other. CD63 MicroBeads were used as control.

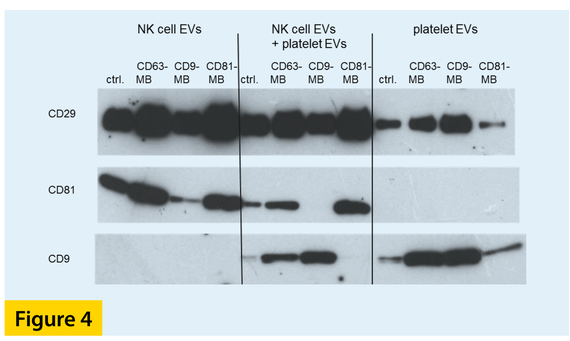

Figure 4

Western blot analysis of NK cell EVs (left) a mixture of NK cell and platelet EVs (middle) and platelet EVs (right). Ctrl.: Loading control of 1.7 µg NK cell EVs (left), a mixture of 0.8 µg NK cell EVs and 1.7 µg platelet EVs (middle), and 3.3 µg platelet EVs (right). CD63-MB, CD9-MB, CD81-MB: EVs isolated with CD63, CD9, or CD81 MicroBeads (25 µg NK cell EVs, a mixture of 12.5 µg NK cell EVs and 25 µg platelet EVs, and 50 µg platelet EVs were used as starting material for magnetic sorting). CD29, CD81, and CD9: detection antibodies.

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 if 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.

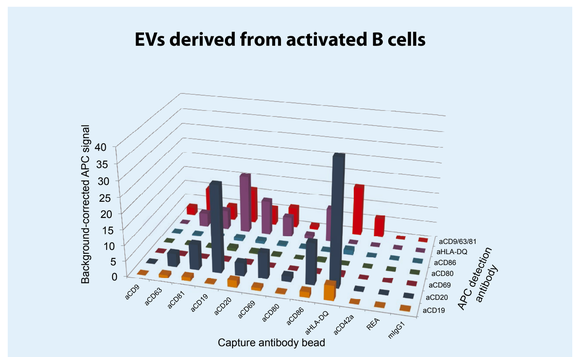

Figure 5

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 Depletion of platelet EVs using superparamagnetic MicroBeads

To purify the B cell EV sample, we depleted platelet EVs using CD61 MicroBeads. Subsequently, the isolated EV fractions were analyzed on the multiplex platform in comparison to the original B cell EV sample (fig. 6). In the flow-through fraction depleted of $CD61^+$ EVs, the signals for each of the three investigated platelet markers (CD41b, CD42a, and CD62P) were drastically reduced. In contrast, the platelet EVs were detected in the eluate of the column-based sorting and were found to be CD9-positive. This supports the hypothesis that B cell EVs are CD9-negative and that the signal on anti-CD9 beads obtained with the original B cell preparation were indeed due to contaminating platelet EVs.

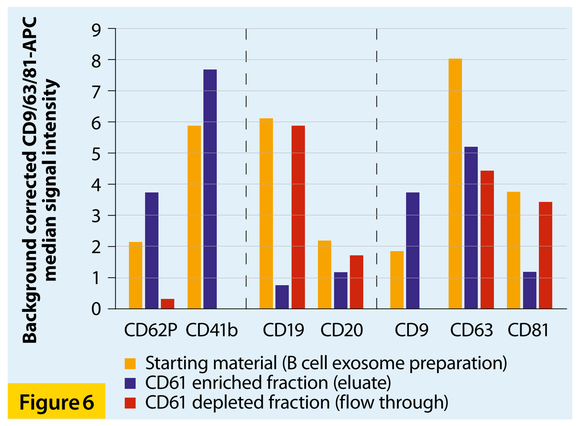

Figure 6

Surface marker profile of EVs from activated B cells before and after depletion of platelet EVs using CD61 MicroBeads. The initial EV sample from activated B cells as well as the EV eluate and flow-through from the $CD61^+$ EV depletion were incubated with 39 capture antibody bead types. Data indicate the corrected APC median signal intensities after staining with a cocktail of CD9-, CD63-, and CD81-APC antibodies. mIgG1 indicates isotype control beads.

5 Analysis of EVs isolated from plasma using superparamagnetic MicroBeads

EVs from 2 mL of plasma were isolated using CD9, CD63, or CD81 MicroBeads and analyzed by the multiplex bead assay (fig. 7). In comparison to EVs isolated by ultracentrifugation, the magnetically isolated EVs showed brighter signals in most cases (amounts used were adjusted to a plasma volume of 2 mL). We conclude that the immunomagnetic isolation of EVs from plasma is efficient even from plasma samples where available volumes might be small.

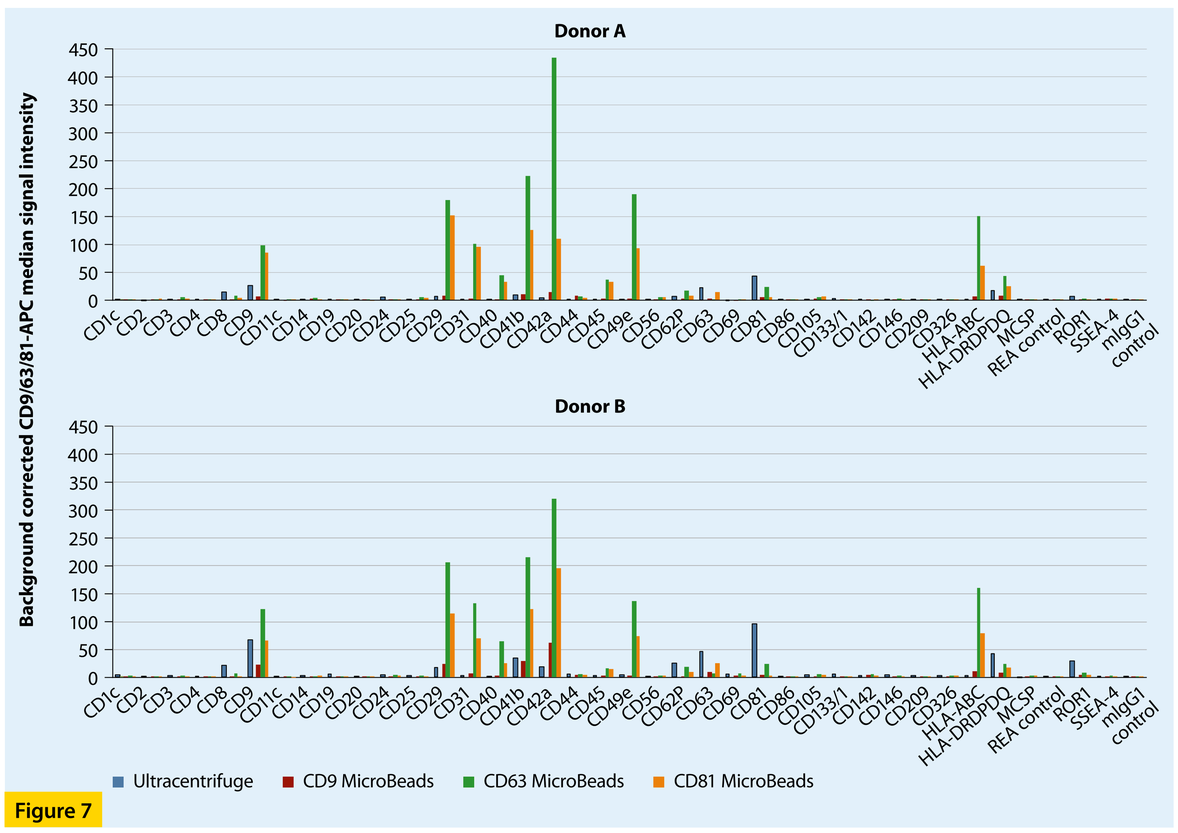

Figure 7

Surface marker profile of EVs isolated from plasma of donor A (top) or B (bottom) by ultracentrifugation or immunomagnetic isolation using CD9, CD63, or CD81 MicroBeads. Data indicate APC median signal intensities of isolated EVs incubated with the 39 capture antibody bead types and stained with a cocktail of CD9-, CD63-, and CD81-APC antibodies. REA and mIgG1 indicate isotype control beads.

Conclusion and outlook

- The multiplex bead platform allows the specific detection of exosome surface proteins and the determination of their relative abundance on exosomes from different sources.
- The common exosome markers were not equally distributed in all exosome populations: NK cell exosomes carried less CD9, while CD81 was underrepresented on platelet exosomes.
- Immunoaffinity-based isolation allows physical separation of EV subpopulations from a sample for further analysis.
- Superparamagnetic MicroBeads can be used for direct isolation of exosomes from plasma.

The relative abundance of epitopes on the surface of exosomes varies depending on the originating cell type and its status. Comprehensive analysis of exosome surface protein compositions will allow the classification of exosome populations according to their origin, for example. Isolation of specific exosome populations with immunoaffinity beads might give further insight into different EV functions.

References
1. Théry, C. *et al.* (2006) Curr. Protoc. Cell Biol. 3.22.1–3.22.29.
2. Koliha, N. *et al.* (2016) J. Extracell. Vesicles 5: 29975. doi: 10.3402/jev.v5.29975.