Supplementary material

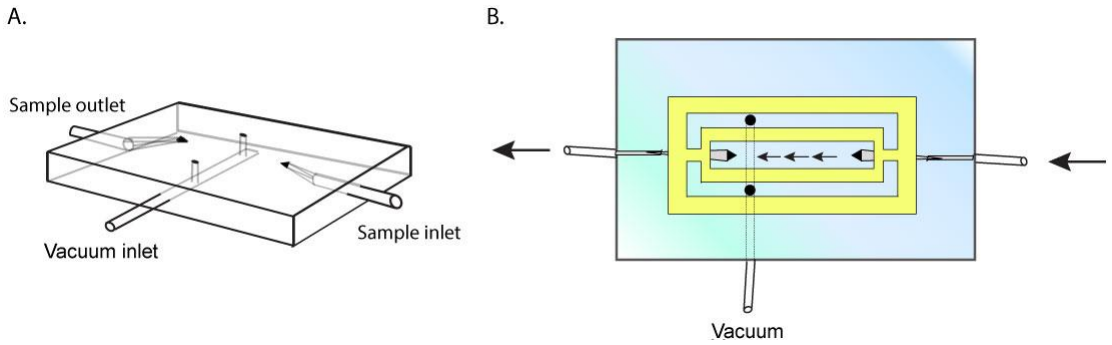
Supplementary Methods

Chemical conjugation of nanobodies with EVs

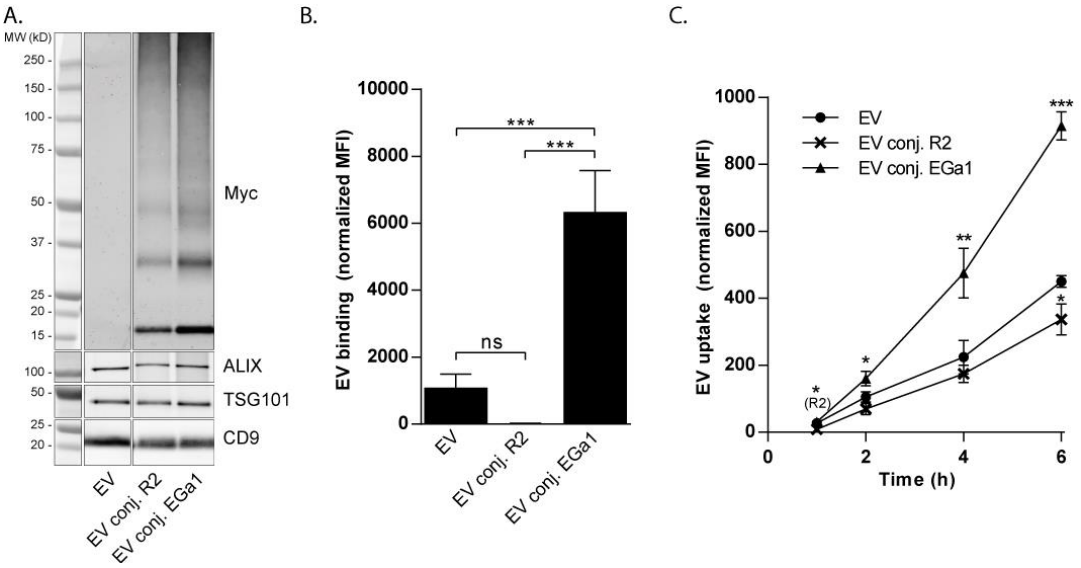
EGa1 and R2 Myc-tagged nanobodies were expressed in BL21 Star (DE3)pLysS chemically competent E. Coli (Invitrogen) and purified as described elsewhere (14). Protected sulfhydryl groups were introduced in nanobodies by reaction with SATA as previously described (14, 32). EVs were isolated from Neuro2A cells using a differential centrifugation protocol. Cells were seeded in medium depleted from EVs by overnight centrifugation at 100 000 x g, and allowed to secrete EVs for 72 hours. Conditioned medium was centrifuged at 300 x g and 2000 x g for 10 min at 4°C to remove cells and debris, respectively, and filtered through 0.22 Steritop 0.22 µm filters (Merck Millipore) to remove large vesicles and debris. EVs were pelleted at 100 000 x g for 70 min at 4°C, resuspended in PBS, and pelleted again at 100 000 x g for 70 min. EVs were resuspended in 100-200 µL PBS, and EV aggregates were removed by centrifugation at 1000 x g for 10 min. For cell association studies, EVs were labeled with 0.125 µg/µL Calcein AM (Molecular Probes) in 0.5% DMSO for 1.5 hours at 37°C, or with 3.75 µM PKH26 (Sigma-Aldrich) in 50% Diluent C for 5 min at room temperature. Immediately after labeling, EVs were purified from unbound label by Sepharose CL-4B size exclusion chromatography and concentrated on 100 kD Vivaspin ultrafiltration tubes. EV protein concentrations were quantified by MicroBCA Protein Assay, and EVs were decorated with nanobodies using sulfo-EMCS (Thermo Fisher Scientific) as a chemical linker. This linker contains an NHS-ester and a maleimide reactive group, allowing it to react with accessible primary amines in EV proteins and with the sulfhydryl groups on the nanobodies. For reaction of the linker with EVs, sulfo-EMCS was dissolved in PBS, immediately mixed with EVs at a final linker concentration of 0.5 mg/mL and incubated for 30 min at room temperature. Simultaneously, SATA-modified nanobodies were deacetylated with deacetylation buffer (50 mM hydroxylamine, 2.5 mM EDTA, 50 mM HEPES, pH 7) for 60 min at room temperature. Nanobodies and EVs were purified from unconjugated sulfo-EMCS

and deacetylation buffer using 0.5 mL Zeba Spin Desalting columns with a 7 kD MWCO (Thermo Fisher Scientific) according to the manufacturer's instructions. EVs and nanobodies were eluted in PBS, mixed in a nanobody:EV ratio of 1:75 (nmol:µg), and allowed to react for 30 min at room temperature. EVs were purified from unconjugated nanobodies by size exclusion chromatography and concentrated on 100 kD Vivaspin ultrafiltration tubes. The presence of nanobodies on the EVs was confirmed by Western Blot analysis using mouse anti-Myc antibodies (1:4000, clone 9E10, Sigma-Aldrich).

Supplementary Figures



Supplementary Figure 1: Schematic presentation of perfusion chamber used in perfusion experiments. A. Perspex frame containing in- and outlets for samples (e.g. medium with EVs), and an inlet for a vacuum pump. B. Top view of perfusion chamber during perfusion experiments. A silicon sheet (yellow) with a thickness of 0.125 mm is mounted on the perspex frame, creating a flow channel of 2 x 30 mm (W x L) between sample in- and outlets. A glass coverslip with a near-confluent monolayer of cells is placed on top of the sheet and sealed by applying a vacuum over the vacuum inlet. The entire perfusion chamber is mounted on a fluorescence microscope for imaging and the sample outlet is connected to a syringe pump to generate flow in the direction of the arrows.



Supplementary Figure 2: Chemical conjugation of nanobodies to EVs strongly increases EV binding and uptake by A431 cells. R2 or EGa1 nanobodies were non-specifically conjugated to surface proteins of EVs using a chemical linker (EV conj. R2 and EV conj. EGa1, respectively) to generate EVs with a high nanobody surface density. A. Western blot analysis of nanobody density and EV markers in nanobody-conjugated EVs. Nanobodies (17 kD) contain a Myc tag and were visualized by anti-Myc antibodies. B. Binding of nanobody-conjugated calcein AM-labeled EVs to A431 cells for 1 hour at 4°C, as determined by flow cytometry. C. Uptake of PKH26-labeled nanobody-conjugated EVs by A431 cells for 1-6 hours at 37°C, as determined by flow cytometry. Data are displayed as mean ± SD and are representative of at least two replicate experiments. ns = not significant, * indicates $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with EV control (unless indicated otherwise) as determined by one-way ANOVA with Tukey post-hoc test.