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- Supplemental Information -

Title: Accumulation of T-cell-suppressive PD-L1^{high} extracellular vesicles is associated with GvHD and might impact GvL efficacy

Material and Methods

EV isolation

For subsequent FACS stainings and ELISAs isolation was performed using the Human Pan Exosome Isolation Kit (Miltenyi Biotec). For cell culture experiments we chose dual-mode chromatography (DMC) to prevent possible impact of residual magnetic beads. DMC combines size-exclusion (SEC), to remove free proteins and smaller particles, and cation exchange (CEX) chromatography, to deplete lipoproteins. This method uses biofluid as a mobile phase and a porous gel filtration polymer as a stationary phase (e.g., Sepharose). We prepared size exclusion columns using 10 ml syringes filled with Sepharose CL-4B (GE Healthcare) sealed with a 11 µm Nylon filter (Merck Millipore). Briefly, 1 ml patient plasma per column was added in drops on top of the column. After washing the columns carefully with PBS and discharging the first 3 ml, 2 ml of EV sample was collected. EVs were further purified using density gradient ultracentrifugation. EVs were diluted in homogenization media and placed on top of a linear Iodixanol gradient (Axis-Shield). Samples were centrifuged at 100,000 × *g*, at 4°C for 16 hours (TH-641 Rotor, Thermo Fisher Scientific). The gradient fractions were collected, and the refraction index was determined (Refractometer Abbemat 200). After diluting fractions in 10 mL PBS, ultracentrifuged (3 hours, 100,000 × *g*, 4°C) EV pellets were resuspended. Concentration and size were determined by a Zeta View PMX110 (Particle Metrix GmbH) device.

Transmission electron microscopy (TEM)

Fixed EV solutions (4% PFA in PBS mixed 1:1 with EV) were placed onto UV-irradiated 300-mesh formvar/carbon-coated grids and allowed to absorb to the formvar for 5 minutes. Grids were incubated 5 minutes in a dry environment, rinsed one time with PBS and placed in 1% glutaraldehyde (in PBS) for 5 minutes. After rinsing in distilled water (7x), grids were stained for contrast using a 2% uranyl oxalate solution (at pH 7 for 5 minutes in dark). Afterwards grids were incubated in drops of methyl cellulose–uranyl acetate for 10 min[8]. Subsequently, grids were removed with stainless steel loops and excess fluid was blotted by

gentle pushing on Whatman filter paper. After air-drying, samples were examined and photographed with a Zeiss EM10 electron microscope (Zeiss) and a Gatan SC1000 Orius™ CCD camera (GATAN) in combination with the DigitalMicrograph™ software 3.1 (GATAN). Images were adjusted for contrast and brightness using Adobe Photoshop CC 2018 (Adobe Systems).

Western Blot

Protein lysates were prepared from EVs using RIPA solution (Sigma-Aldrich) and the Halt™ Protease/Phosphatase Inhibitor Single-Use Cocktail (Thermo Fisher Scientific). BCA protein assay Kit (Thermo Fisher Scientific) was used to confirm the samples protein concentrations. Proteins were separated on a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) system. Subsequently, gels were transferred to semi-dry blotting. The EV marker CD63 was visualized by a double staining procedure using the primary anti-CD63 mouse antibody (Invitrogen) and a horseradish peroxidase-conjugated goat-anti-mouse IgG (cell signaling technology). Bands were detected using chemiluminescence imaging after 1 minute incubation in the dark with an ECL substrate.

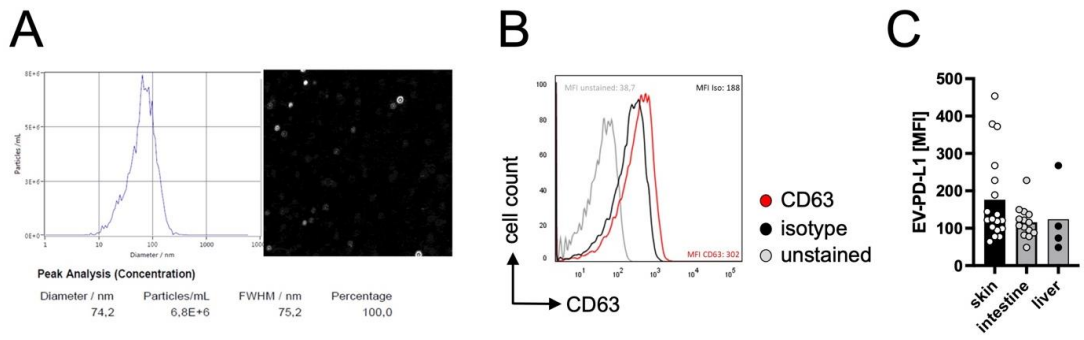
Tables

Supplemental Table 1: FACS antibody list.

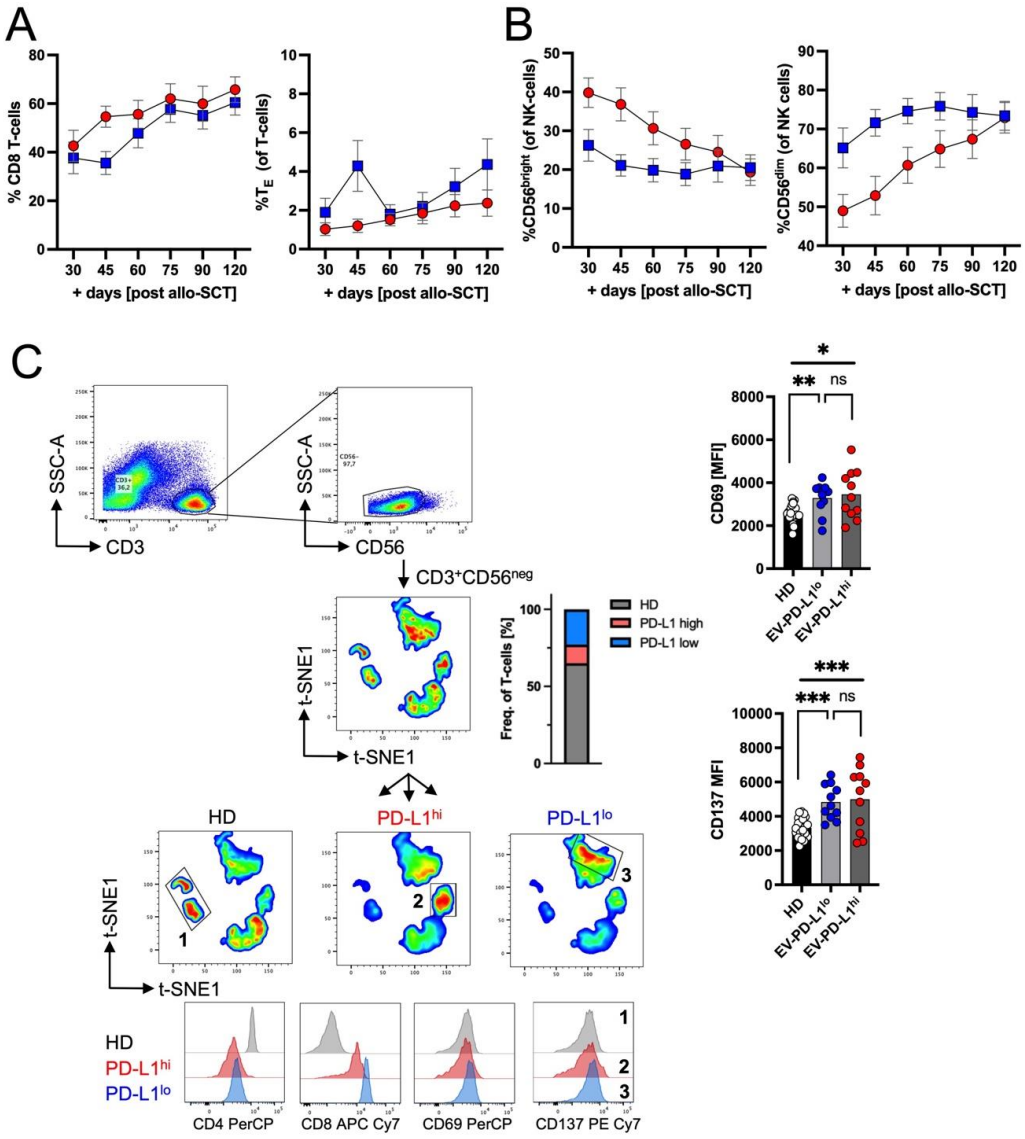
Antibody	Clone	Manufacturer
CD3	UCHT1	Biolegend
CD3	HIT3a	Biolegend
CD3	OKT3	Biolegend
CD3	SK7	Biolegend
CD4	OKT4	Biolegend
CD8	HIT8a	Biolegend
CD8	BW135/80	Biolegend
CD16	3G8	Biolegend
CD25	BC96	Biolegend
CD28	CD28.2	Biolegend
CD45RO	UCHL1	Biolegend
CD56	HCD56	Biolegend

CD63	H5C6	Biolegend
CD69	FN50	Biolegend
CD127	A019D5	Biolegend
CD137	4B4-1	Biolegend
CD197 (CCR7)	G043H7	Biolegend
CD274 (PD-L1)	29E.2A3	Biolegend
Ki67	20Raj1	Bioscience
HK2	polyclonal	Abcam
CPT1a	8F6AE9	Abcam
IFNγ	4S.B3	Biolegend
IL-2	MQ1-17H12	Biolegend

Figures

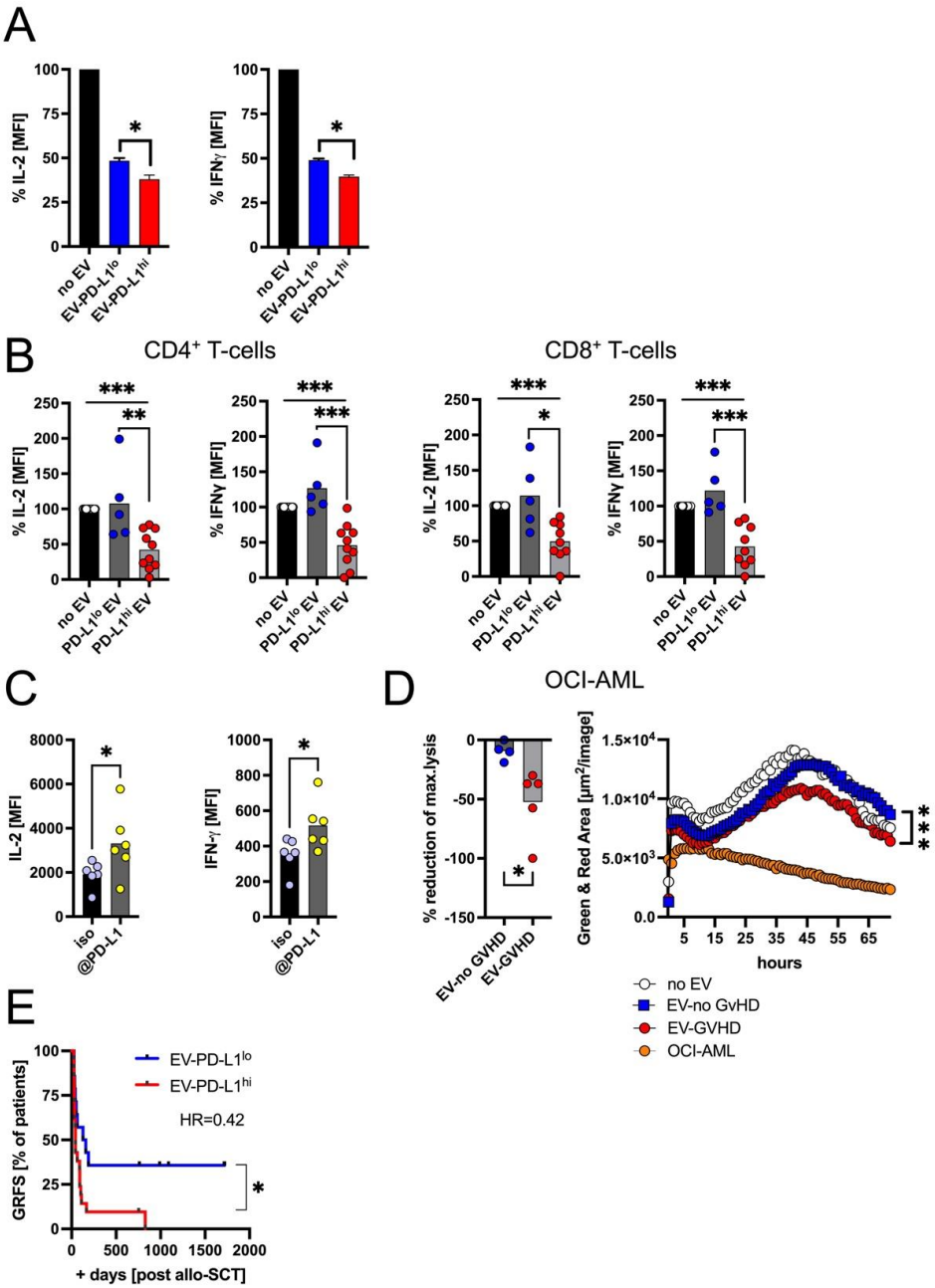


Supplemental Figure 1: (A) Characterization (i.e., size and shape) of allo-HSCT patient-derived EVs by Nanoparticle tracking analysis (Zetaview). (B) FACS based detection of CD63 on latex-bead coupled allo-HSCT EVs. (C) Comparison of EV-PD-L1 levels from GvHD patients with liver- (n=4), skin- (n=19), and intestinal- (n=16) GvHD as assessed by FACS and based on the MFI.



Supplemental Figure 2: (A-B) Time kinetics of $CD8^+$- and T_E-T cells as well as $CD56^{dim}$- and $CD56^{bright}$-NK-cells in EV-PD-$L1^{hi/lo}$ groups are shown for the time points day +30, +45, +75, +90, and +120 after allo-HSCT. Patient categorization is based on EV-PD-L1 MFI at day +45 (hi=>median, n=13 and lo=<median, n=12). (C) t-SNE analysis based on CD3, CD4, CD8, CD69, and CD137 was performed on concatenated PMBCs with subsequent gating on $CD3^+CD56^-$ T-cells as shown in the upper panel. The events were then split into three density plots in line with their matching group: healthy donors (HD, black, n = 22), EV-PD-$L1^{hi}$ (red, n=11) or EV-PD-$L1^{lo}$ (blue, n =11) and plotted according to the calculated variables t-SNE 1 and t-SNE 2 (middle panel). Proportions of T-cells from the HD, PD-$L1^{hi}$, and PD-$L1^{lo}$

groups are shown as stacked bar chart (middle panel, right). Histograms of CD4, CD8, CD69, and CD137 from representative selected areas of each group (gate 1, 2, and 3) are reported (bottom). Abbreviations: 'n' indicates the sample number; bars represent the standard error of the mean; P value: *P < 0.05; **P < 0.01; ***P < 0.001.



Supplemental Figure 3: (A) Jurkat T-cells were co-incubated with PD-L1^{hi} (n=3) - or PD-L1^{lo} (n=4) EVs from allo-HSCT patients and stimulated with anti-CD3, and -CD28 activation beads for 48 hours. IL-2 and IFN-γ production was measured by FACS based on the MFI.

Activated Jurkat T-cells without EV treatment were set as 100%. (B) T-cells were co-incubated with PD-L1^{hi} (n=9) - or PD-L1^{lo} (n=5) EVs from allo-HSCT patients and stimulated with anti-CD3, and -CD28 activation beads for 48 hours. IL-2 and IFN-γ production was measured by FACS based on the MFI. Activated T-cells without EV treatment were set as 100%. (C) Activated T-cells were treated with allo-HSCT patient-derived EVs (n=6) in presence of anti-CD274/PD-L1 blocking antibodies (10 µg, eBioscience, USA) or the according IgG isotype control (R&D Systems, Germany). IL-2 and IFN-γ production was measured by FACS based on the MFI. (D) T-cells were stimulated for 48 hours, treated with/without EVs from patients with (n=5)/without (n=4) GvHD, and co-cultured with calcein-labeled OCI-AML AML cells. Lysis of OCI-AML was calculated based on calcein release and conditions without EVs represented the baseline (left panel). Targeting of OCI-AML AML cells by EV-pretreated CD3^{+} T-cells was also assessed by real-time imaging using an IncuCyte® Zoom device. T-cells were treated with/without EVs from patients with (n=5)/without (n=5) GvHD, and co-cultured with PKH-labeled OCI-AML AML cells (green) in an effector to target ratio of 2:1 in presence of activating anti-CD3 and -CD28 antibodies. Dead cells are identified as cytotox (red). Total green & red area represents total OCI-AML cell killing over time (right panel). (E) Kaplan-Meier analysis of the of GVHD-free/relapse-free survival (GRFS) of allo-HSCT patients according to the EV-PD-L1 level. Categorization into PD-L1^{hi} patients (n=16, >median) and into PD-L1^{lo} patients (n=16, <median) is based on PD-L1 MFI at day 45. Abbreviations: ‘n’ indicates the sample number; bars represent the standard error of the mean; P value: *P < 0.05; **P < 0.01; ***P < 0.001.