Supplementary information

Title: Profound functional suppression of tumor-infiltrating T-cells in ovarian cancer patients can be reversed using PD-1 blocking antibodies or DARPin® proteins

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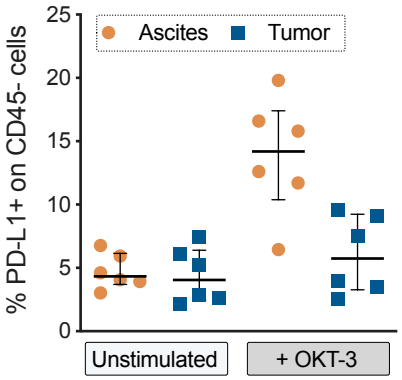
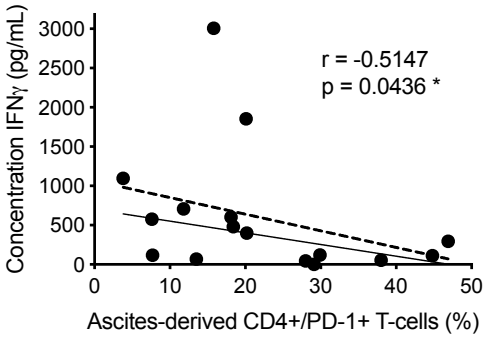
Supplementary Table I. Antibodies used for flow cytometric characterization post-PD-1 blockade.

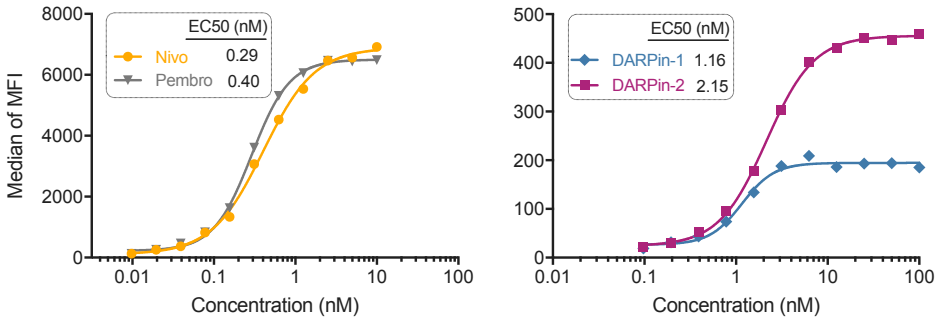
Target	Fluorochrome	Clone
CD326/EpCAM	FITC	EBA-1
CD25	PE	M-A251
CD3	PE-Cy7	SK7
CD274/PD-L1	APC	MIH1
CD8	APC	RPA-T8
CD45	Alexa700	HI30
CD8	APC-Cy7	SK1
CD69	APC-Cy7	FN50
HLA-DR	APC-H7	G46-6
CD279/PD-1	Bv421	MIH4
CD4	V500	RPA-T4
IgG1 isotype ctrl	FITC/APC/Bv421 PE	X40 MOPC-21



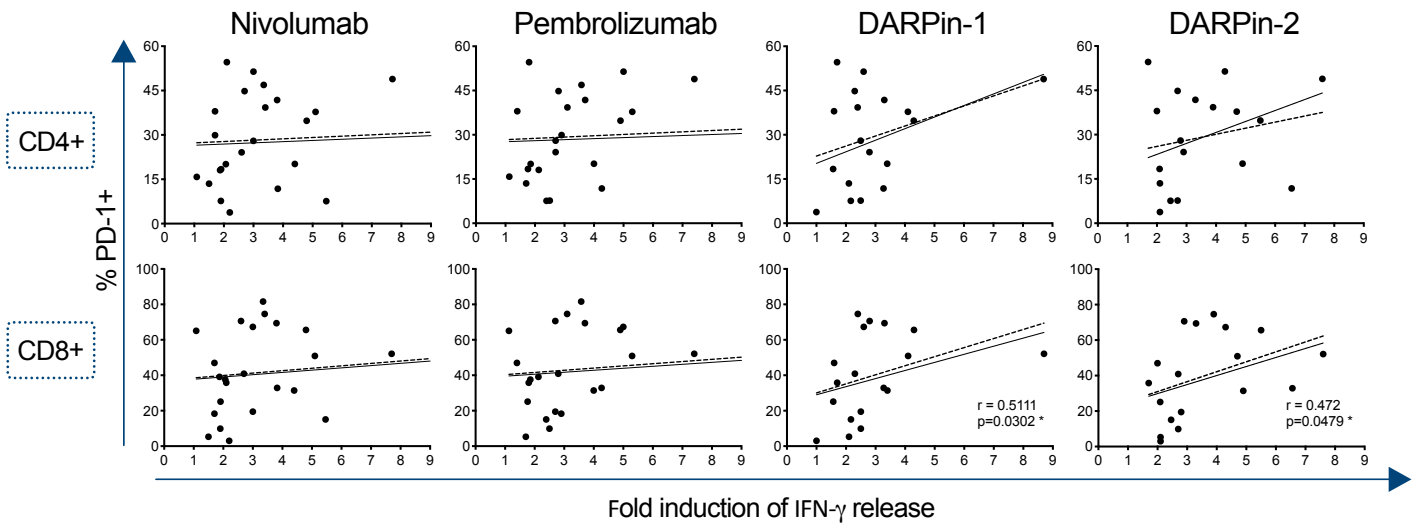
Supplementary Figure 1. PD-L1 expression on CD45- cell fractions from paired ascites and tumor samples (n=6). PD-L1 expression was assessed by flow cytometry on unstimulated and stimulated (addition of OKT-3) cell fractions after 48h incubation.



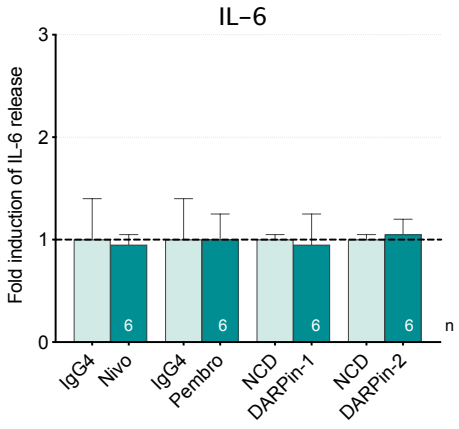
Supplementary Figure 2. Correlation between PD-1 expression and response to α-CD3 stimulation. Correlation between PD-1-expressing CD4+ T-cells in ascites (at time of isolation from patient) and the normalized absolute concentration of IFN-γ (per 500 000 T-cells) after stimulation with α-CD3 for 48h. P- and r- values were obtained by Spearman correlation and visualized using non-linear robust regression (solid line) or least squares regression (dashed line). Significance levels were set to $p<0.05$ (*).



Supplementary Figure 3. Assessment of PD-1 binding using Mirrorball laser scanning imaging cytometry. A titration of PD-1-targeting reagents with a PD-1-expressing cell line (transfected HEK293) were used to assess PD-1 binding measured by median of mean fluorescence intensities (MFI). Half effective concentrations (EC50 values) are presented for each PD-1 binding reagent; nivolumab (nivo) in circles, pembrolizumab (pembro) in reversed triangles, DARPin-1 in diamonds and DARPin-2 in squares.



Supplementary Figure 4. Associations between PD-1 expression and release of IFN-γ after stimulation with α-CD3 and PD-1-blocking reagents. Correlation analysis for PD-1 expression in CD4+ (upper panel) or CD8+ (lower panel) T-cells isolated from ascites and tumor (from fresh characterization after isolation) and the fold induction of IFN-γ after stimulation with α-CD3 and the four different PD-1 targeting reagents. P- and r- values were obtained by Spearman correlation and dashed lines were plotted using non-linear robust regression (solid line) or least squares regression (dashed line). Significance levels were set to $p<0.05$ (*).



Supplementary Figure 5. Fold induction of IL-6 from T-cells isolated from ascites (n=2) or tumor tissue (n=4) in presence of 100 nM of α-PD-1-directed reagents using multiplex assay. Results were generated using 6-plex assay. Fold induction was calculated based on release when adding only α-CD3 with no presence of α-PD-1 reagent or control (represented by dashed line at 1). Four additional ascites samples were initially analyzed but reached the upper detection limit in all conditions (including α-CD3 alone) and were excluded. The number of samples presented is indicated in each bar with presented number of plotted samples presented for each bar (n). Paired Wilcoxon test was used for comparing the response by anti-PD-1 reagent with its corresponding control; IgG4 for mAbs nivolumab (nivo) and pembrolizumab (pembro) or negative control DARPin (NCD) for DARPins. Median values and interquartile ranges are plotted. Significance levels were set to $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).