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# Supplementary Materials for

## Immune evasion mediated by PD-L1 on glioblastoma-derived extracellular vesicles

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**The PDF file includes:**

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- fig. S2. Glioblastoma EVs are bound to the plasma membrane.
- fig. S3. Glioblastoma EVs that contain PD-L1 bind to PD1.
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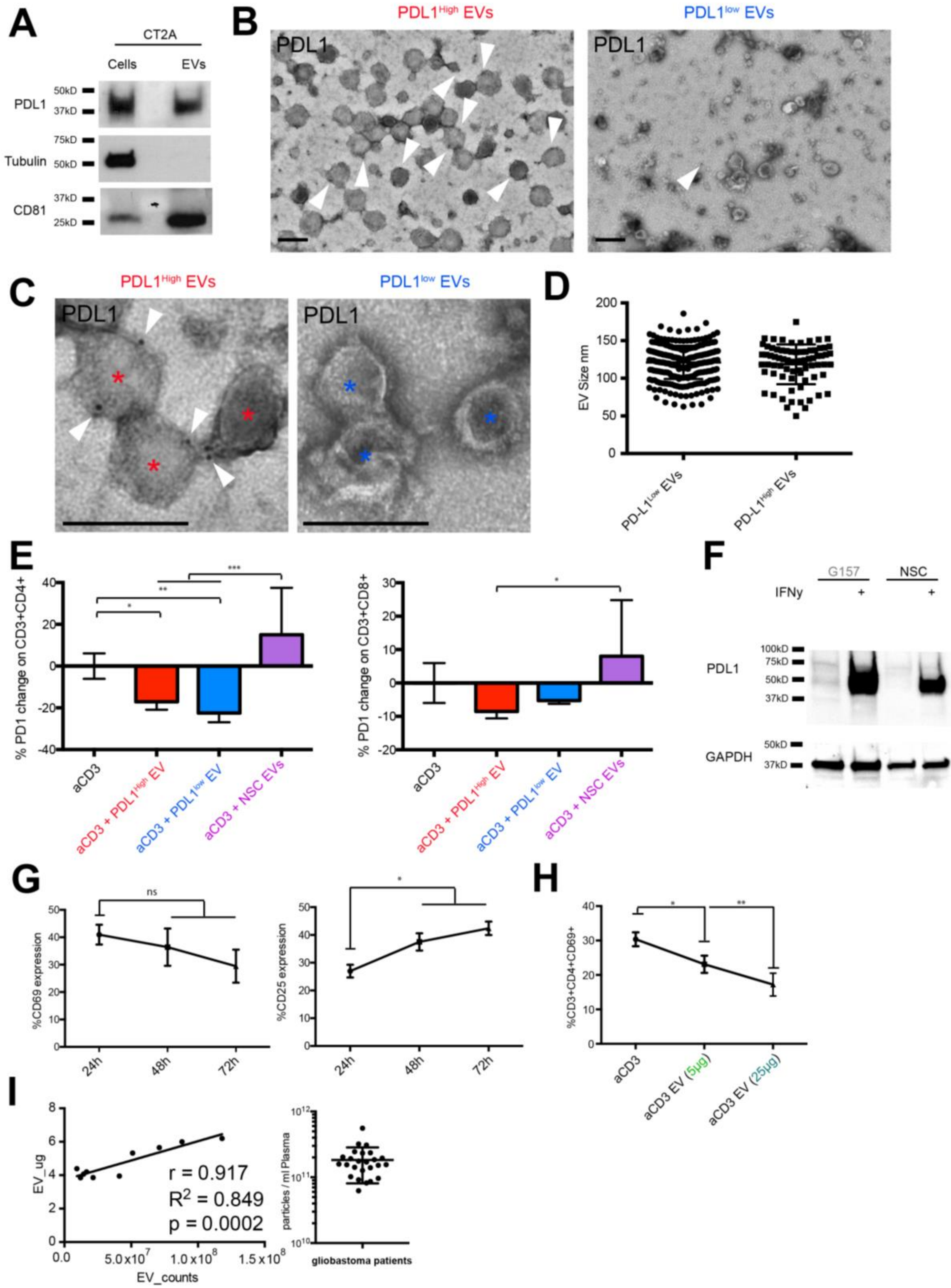
**Other Supplementary Material for this manuscript includes the following:**
(available at advances.sciencemag.org/cgi/content/full/4/3/eaar2766/DC1)

- movie S1 (.mov format). PalmtdT EV bound to CD3$^+$ T cell.



A
CT2A
Cells
EVs
PDL1
Tubulin
CD81
50kD
37kD
75kD
50kD
37kD
25kD
B
PDL1High EVs
PDL1low EVs
PDL1
PDL1
C
PDL1High EVs
PDL1low EVs
PDL1
PDL1
D
EV Size nm
PD-L1Low EVs
PD-L1High EVs
E
% PD1 change on CD3+CD4+
% PD1 change on CD3+CD8+
aCD3
aCD3 + PDL1High EV
aCD3 + PDL1low EV
aCD3 + NSC EVs
F
G157
NSC
IFNy
PDL1
GAPDH
100kD
75kD
50kD
37kD
G
ns
%CD69 expression
%CD25 expression
24h
48h
72h
H
%CD3+CD4+CD69+
aCD3
aCD3 EV (5μg)
aCD3 EV (25μg)
I
EV_ug
EV_counts
r = 0.917
R2 = 0.849
p = 0.0002
particles / ml Plasma
gliobastoma patients

**fig. S1. Glioblastoma EVs contain PD-L1.** (**A**), Murine glioblastoma CT2A cells and EVs carry PD-L1 (**B** & **C**), PD-L1 immunolabeled PD-L1$^{High}$ and PD-L1$^{low}$ glioblastoma EVs are shown, triangle indicates gold-immunolabeled PD-L1, scale bar = 200 nm, different magnifications. (**D**), size distribution between PD-L1$^{High}$ and PD-L1$^{Low}$ vesicles. (**E)**, PD-L1$^{high/low}$ EVs lead to a significant percentage decrease of PD1 in CD3+CD4+ (left panel) and CD3+CD8+ cells (right panel) (n=4, mean ± sd). (**F**). NSCs do not express detectable PD-L1, but it is inducible by IFN-γ (**G**). CD69 expression does not change significantly after 48 and 72 h while CD25 expression increases at 48h. CD69 and CD25 expression were measured by flow cytometry after anti-CD3 treatment (n=2). (**H**), GSC EVs inhibit T-cell activation in a dose-dependent manner as assessed by CD69 levels (n=3, mean ± sd). Datasets include EVs from four different GSCs. (**I**). EVs from plasma of human glioblastoma patients were analyzed for number and protein content (μg). ***left panel,*** Correlation between EV number and protein content (n= 10); ***right panel,*** EV amount per ml plasma of glioblastoma patients (n=24). Analysis was performed by one-way ANOVA, with post-hoc Bonferroni's correction (**** = $p<0.0001$, *** = $p<0.001$, ** = $p<0.01$, * = $p<0.05$).

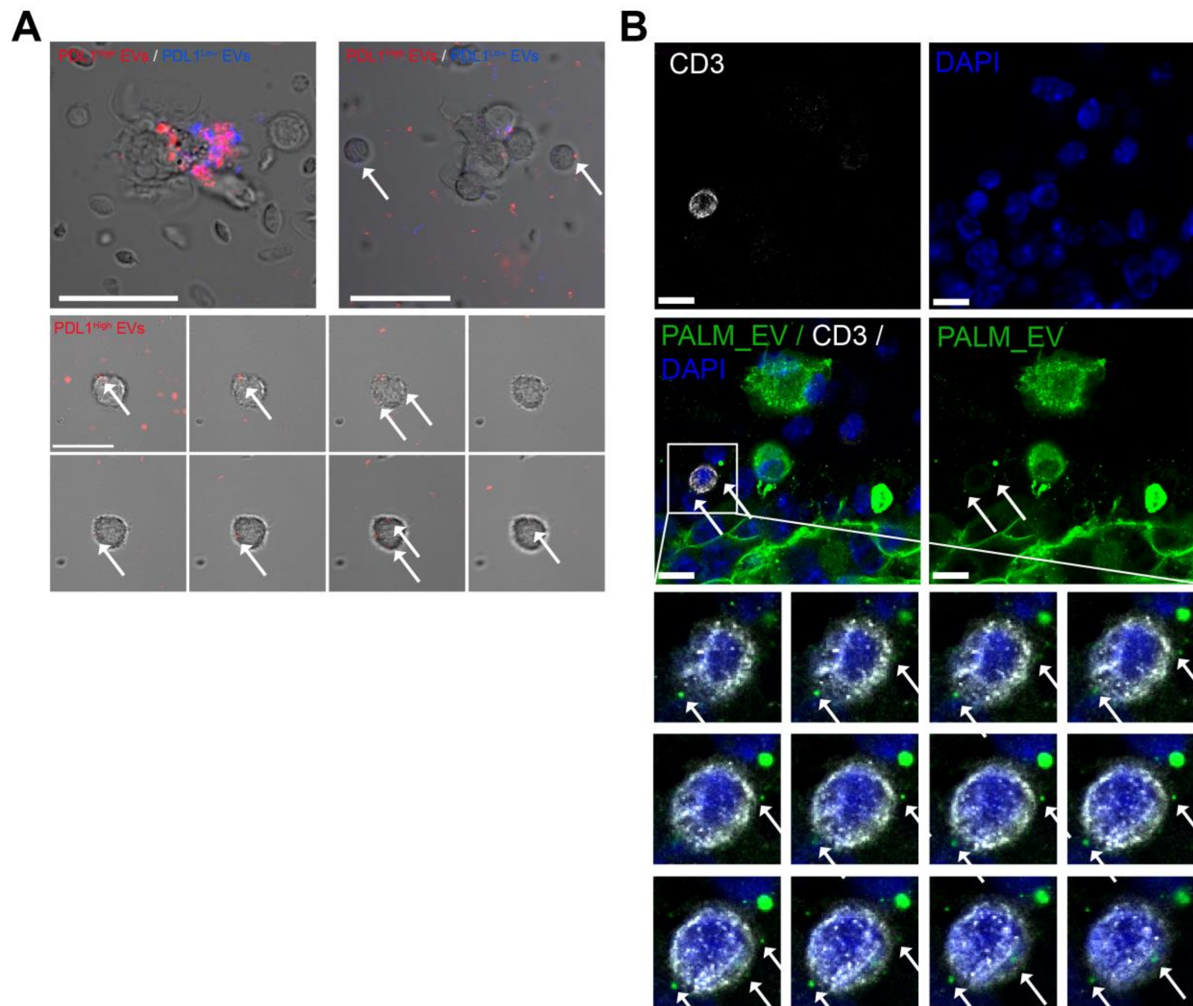



**fig. S2. Glioblastoma EVs are bound to the plasma membrane.** (**A**), GSC EVs can either be internalized or stick to the outer membrane of PBMCs. Confocal microscopy of PD-L1$^{high}$ palmtdT (red) and PD-L1$^{low}$ palm-GFP labeled EVs (blue) is shown. White arrows indicate EVs bound to the outer membrane. Scale bar = 20µm. lower panel: z-stack of CD3+ isolated lymphocytes with palmtdT-PD-L1$^{high}$ EVs. (**B**). Glioblastoma EVs co-localize with CD3+ cells *in vivo*. 100,000 CT2A-PALM-GFP labeled mouse syngeneic glioma cells were injected and established in the brains of C57BL/6 mice. 18 days later, mice were euthanized when symptomatic, their brains were harvested and stained for GFP (to detect CT2A tumor cells and

CT2A EVs) and for CD3 (white) to detect T-cells. Confocal images for PALM-GFP-EVs (green) and CD3+ (white) cells are shown. scale bar = 10µm.

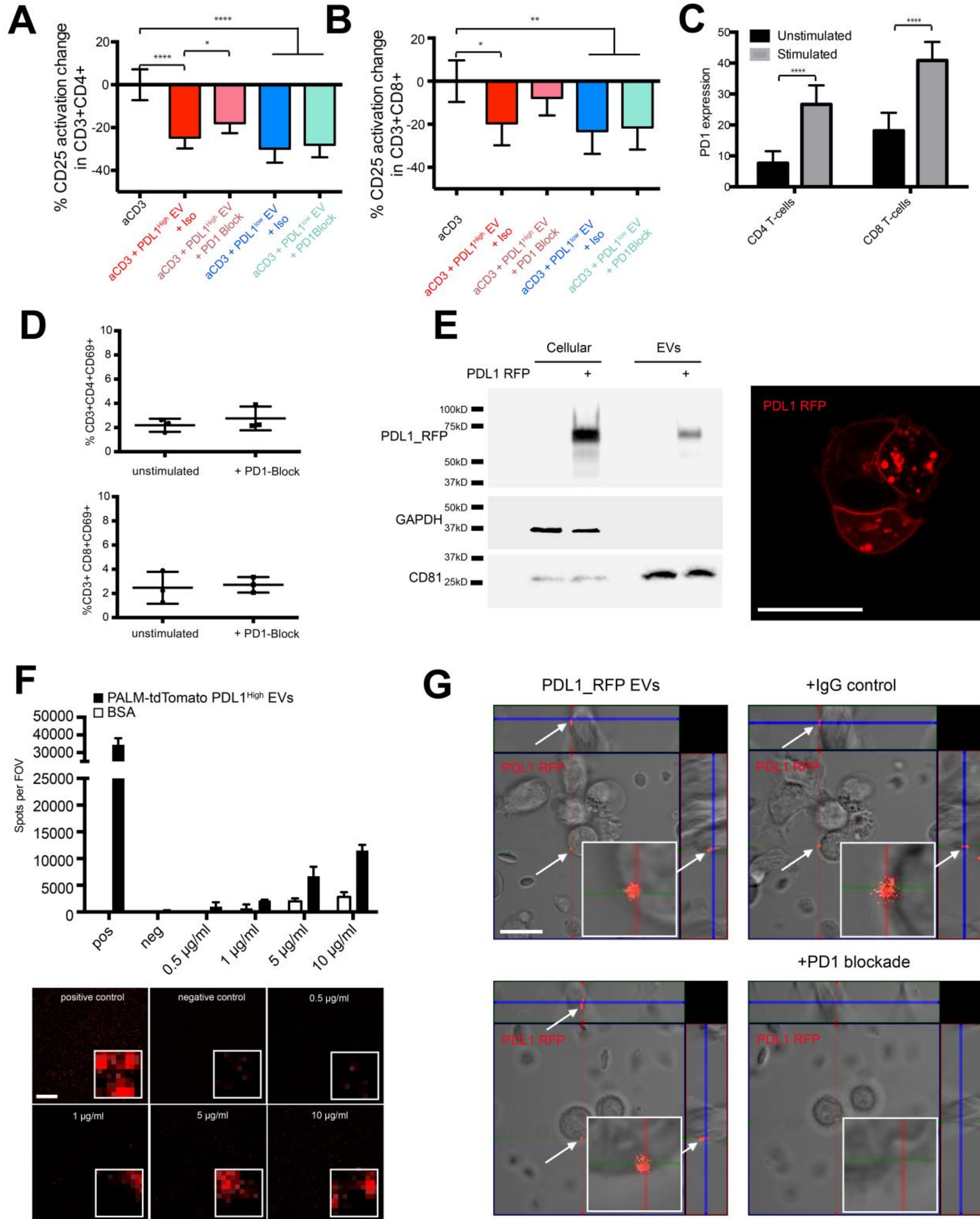

A
% CD25 activation change in CD3+CD4+
aCD3
aCD3 + PDL1High EV + Iso
aCD3 + PDL1High EV + PD1 Block
aCD3 + PDL1low EV + Iso
aCD3 + PDL1low EV + PD1Block
B
% CD25 activation change in CD3+CD8+
C
Unstimulated
Stimulated
PD1 expression
CD4 T-cells
CD8 T-cells
D
% CD3+CD4+CD69+
%CD3+ CD8+CD69+
unstimulated
+ PD1-Block
E
Cellular
EVs
PDL1 RFP
PDL1_RFP
GAPDH
CD81
F
PALM-tdTomato PDL1High EVs
BSA
Spots per FOV
pos
neg
0.5 µg/ml
1 µg/ml
5 µg/ml
10 µg/ml
positive control
negative control
G
PDL1_RFP EVs
+IgG control
+PD1 blockade

**fig. S3. Glioblastoma EVs that contain PD-L1 bind to PD1.** (**A-B**) PD1 blockade prevents the inhibition of PD-L1$^{High}$ GSC EVs on PBMCs. %CD25 expression change for CD4+ (A) and CD8+ (B) T cells. PD1 blocking antibody (10 μg/ml) or Isotype control (10 μg/ml) was added at day 0 (n=7 PBMC donors, mean ± sd). (**C**), PD1 expression increases through TCR activation, PD1 expression measured by flow cytometry is shown (n=6) (**D**), Anti-PD1 blockade does not activate T cells. Anti-PD1 antibody was added to unstimulated PBMCs. CD25 expression was measured in CD3+CD4+ and CD3+CD8+ cells. (**E**). PD-L1$^{high}$ EVs bind to plate-bound purified PD1 in a dose dependent manner. PD-L1 carrying palmtdT-labeled EVs were incubated with plate-bound PD1 or plate-bound BSA as control. Scale bar = 50μm (inserts shown at 500x magnification) (n=4, mean ± sd). (**F**), PD-L1_RFP is found in cells and EVs by immunoblotting and confocal imaging. Scale bar = 10μm. PD-L1 on EVs can bind to the surface of CD3+ sorted T-cells and are displaced by PD1 blockade. Scale bar = 20μm. (**G**), After 30 min incubation at 37°C with either IgG control or PD1-blocking antibody serial images were obtained. Scale bar = 10μm, 500x magnification inserts. High resolution confocal images are shown of PD-L1-RFP carrying EVs (bottom left), Scale bar = 20μm.

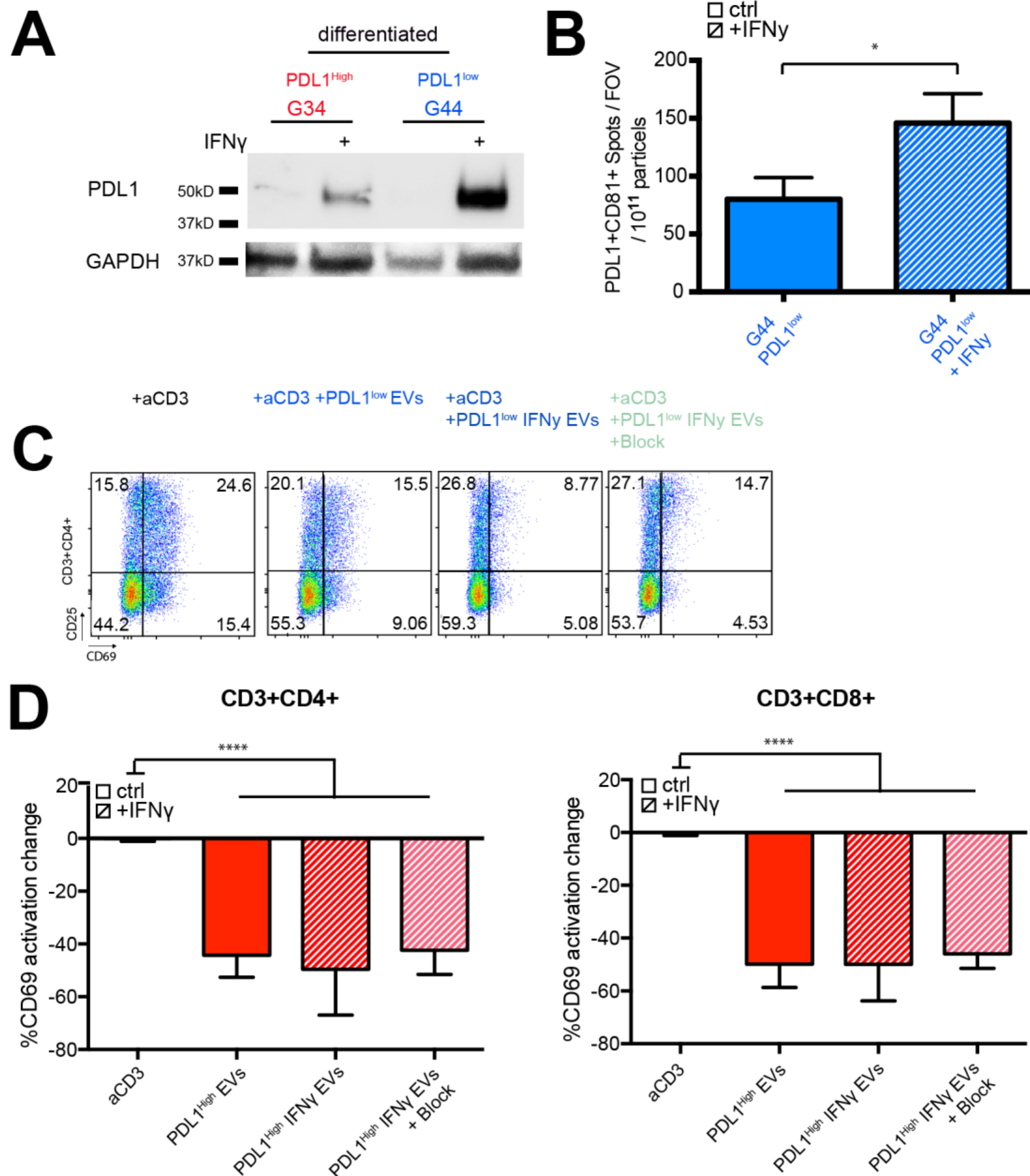



**fig. S4. IFN-γ increases PD-L1–positive EVs in PD-L1$^{low}$ GSCs.** (**A**), Differentiated GSCs upregulate their PD-L1 through IFN-γ (**B**), CD81 positive EVs derived from GSCs have elevated PD-L1 after IFN-γ stimulation as determined by ELISA sandwich fluorescent measurement. (**C**), representative FACS scatter plots are shown for IFN-γ EV treatments and PD1:PD-L1 blockade., (**D**), EVs derived from IFN-γ–treated PD-L1$^{High}$ GSCs did not alter anti-CD3-

stimulated T cell activation and this was not reversed by PD1_PD-L1 blockade. Inhibition potential was measured by the percentage change of CD69+ levels on anti-CD3 stimulated CD3+CD4+ (C) or CD3+CD8+ (D) cells, isolated from 5 human volunteers, mean ± sd). Analysis was performed by one-way ANOVA, with post-hoc Bonferroni's correction (**** = p<0.0001, *** = p<0.001, ** = p<0.01, * = p<0.05). Student's t-test was used to compare 2 groups (* = p < 0,05).

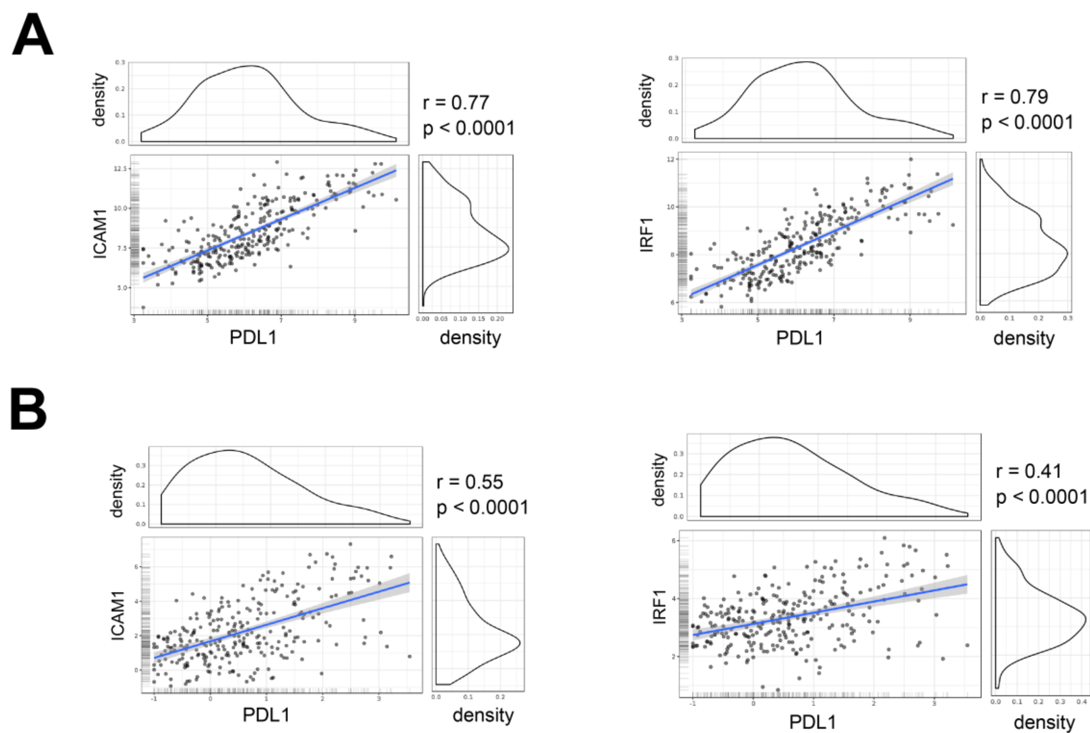
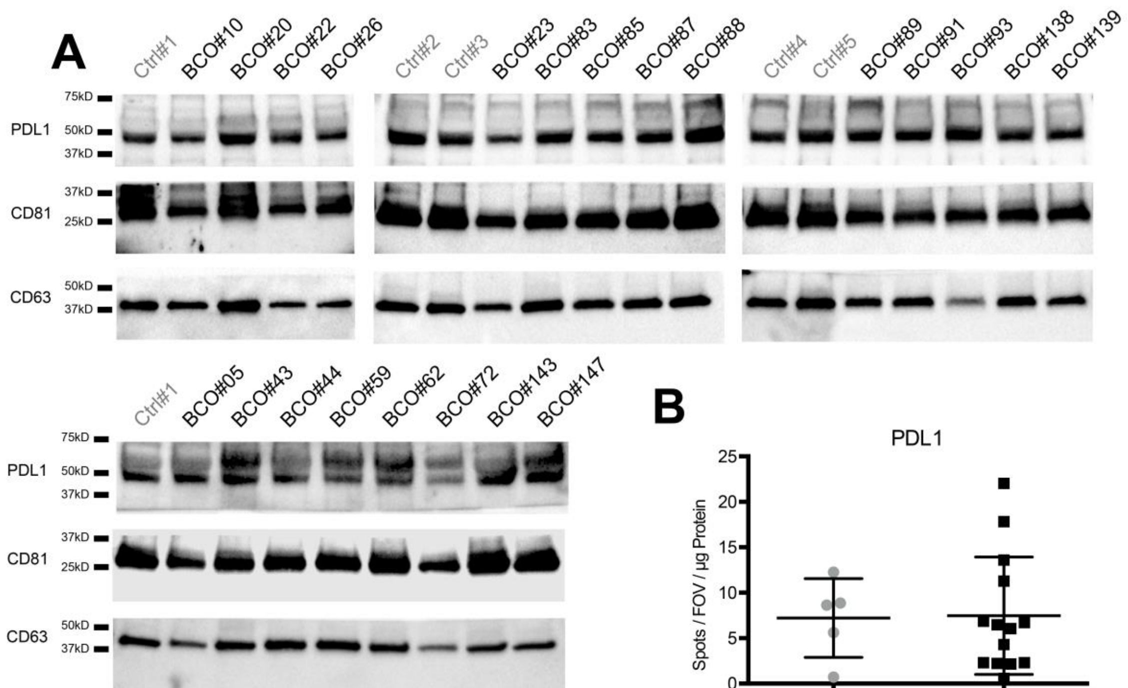
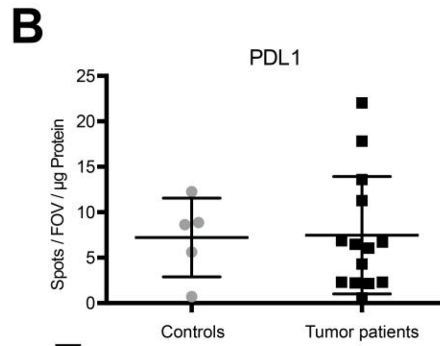
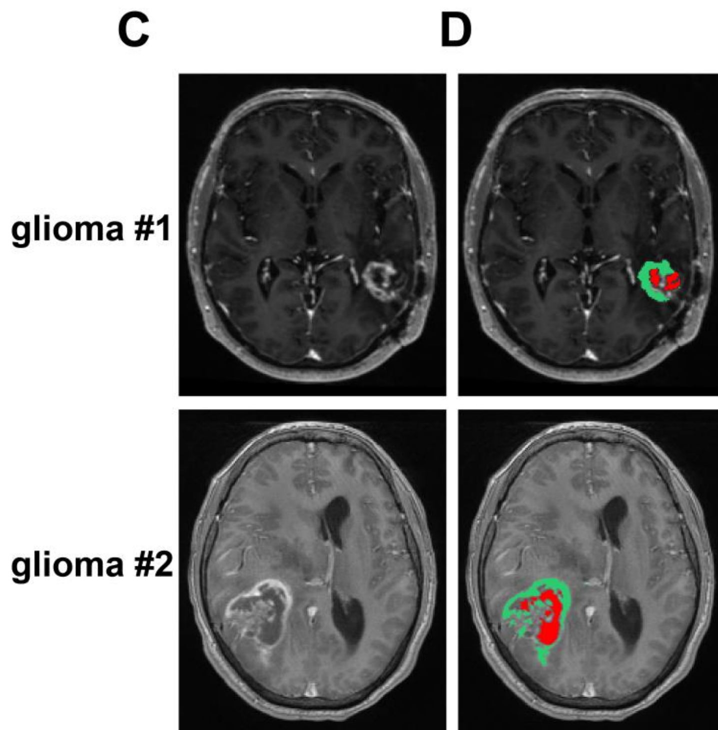
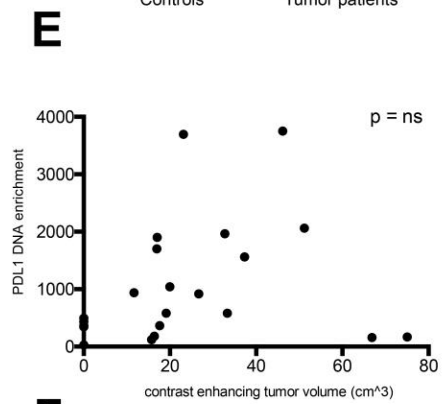



**fig. S5. PD-L1 expression correlates with IFN-γ response genes.** (**A**, **B**), PD-L1 expression correlates with IFN-γ response factor (IRF1), human leukocyte antigen A and intercellular adhesion molecule 1 (ICAM1) levels, ((A) Bao RNA-seq dataset; (B) IVY Gap RNA-seq dataset) RNAseq dataset were obtained from http://gliovis.bioinfo.cnio.es


A
Ctrl#1
BCO#10
BCO#20
BCO#22
BCO#26
Ctrl#2
Ctrl#3
BCO#23
BCO#83
BCO#85
BCO#87
BCO#88
Ctrl#4
Ctrl#5
BCO#89
BCO#91
BCO#93
BCO#138
BCO#139
PDL1
CD81
CD63
75kD
50kD
37kD
25kD
BCO#05
BCO#43
BCO#44
BCO#59
BCO#62
BCO#72
BCO#143
BCO#147


B
PDL1
Spots / FOV / µg Protein
Controls
Tumor patients


C
D
glioma #1
glioma #2


E
p = ns
PDL1 DNA enrichment
contrast enhancing tumor volume (cm^3)

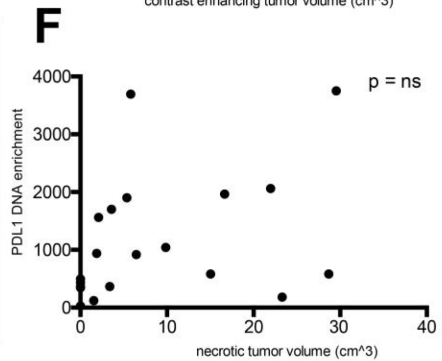

F
p = ns
PDL1 DNA enrichment
necrotic tumor volume (cm^3)

**fig. S6. EV PD-L1 is found in serum from both glioblastoma patients and healthy controls.** (**A**), Western blots show that healthy controls and glioblastoma patients carry PD-L1 positive EVs in circulation (controls = 5, glioma patients = 21). (**B**), Measurements of ELISA-type sandwich analysis show that PD-L1 is not enriched in glioblastoma patients. (**C**), Representative MRI data of glioblastoma patients are shown. (**D**), Measurements of contrast enhancing tumor volumes are labeled in green, and necrotic regions labeling in red. (**E**), PD-L1 DNA enrichment correlates with contrast enhancing tumor volume only for tumors below 60 $cm^3$. When the two tumors larger than 60 $cm^3$ were included in the full cohort, the correlation was lost (p = ns). (**F**), necrotic volumes of glioma tumors did not significantly correlate with PD-L1 DNA enrichment. (p = ns)

**table S1. EV number and their localization: Fluorescently labeled EVs were added to lymphocytes (5 μg/ml) for 3 hours.** High resolution confocal microscopy was used to visualize EVs either being internalized or co-localized with the plasma membrane. Four randomly selected pictures (pic) were analyzed for the amount of EVs.

| icture ID | Internalized | Co-localized with membrane | Total cell |
|---|---|---|---|
| **pic 1** | 3 | 1 | **3** |
| **pic 2** | 13 | 4 | **6** |
| **pic 3** | 6 | 6 | **5** |
| **pic 4** | 8 | 5 | **3** |

**table S2. Short tandem repeat profile of cell lines used in the study.**

| | G34 | G35 | G44 | G157 |
|---|---|---|---|---|
| TH01 | 6 | 9 | 6 | 6 |
| | 8 | 9 | 8 | 7 |
| D5S818 | 12 | 12 | 12 | 11 |
| | 13 | 13 | 13 | 13 |
| D13S317 | 12 | 12 | 12 | 10 |
| | 13 | 12 | 13 | 13 |
| D7S820 | 10 | 10 | 10 | 12 |
| | 12 | 10 | 12 | 12 |
| D16S539 | 8 | 9 | 8 | 9 |
| | 14 | 9 | 14 | 11 |
| CSF1PO | 11 | 12 | 11 | 10 |
| | 11 | 12 | 12 | 12 |
| AMEL | X | X | X | X |
| | Y | X | Y | X |
| vWA | 16 | 15 | 16 | 14 |
| | 18 | 15 | 18 | 19 |
| TPOX | 8 | 9 | 8 | 9 |
| | 11 | 9 | 11 | 11 |

**table S3. Primers used in the study.**

| Primer sequence for qPCR | Gene name |
|---|---|
| AAGACCCAGACATCAAGGCG | IL10 For |
| AGGCATTCTTCACCTGCTCC | IL10 Rev |
| GGGAAGCTTATGACGCCTGT | IDO For |
| CTGGCTTGCAGGAATCAGGA | IDO Rev |
| | |
| **Primer sequence for droplet PCR** | **Gene name** |
| GAGTGGTAAGACCACCACC | PD-L1 For |
| GGTTTTCCTCAGGATCTAATC | PD-L1 Rev |

**movie S1. PalmtdT EV bound to CD3⁺ T cell.** PBMCs were sorted for CD3+ cells and incubated for 2h with PalmtdT-EVs. Frames were taken every 2 seconds over a time period of 1 minute at 63x magnification.