An Immunogold Single Extracellular Vesicular RNA and Protein (AuSERP) Biochip to Predict Responses to Immunotherapy in Non-Small Cell Lung Cancer Patients

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**SUPPORTING INFORMATION**

**Isolation and activation of T cells**

15 mL of blood was collected in a BD Vacutainer Plastic Blood Collection Tube with K\(_2\)EDTA (#366643, Becton Dickinson, Franklin Lakes, NJ) and lysed with red blood cell lysis buffer (150 mM ammonium chloride, 10 mM sodium bicarbonate, 0.1 mM EDTA, pH = 7.4) for 5 min at room temperature (RT). After centrifugation, a pellet was resuspended in phosphate buffer saline (PBS) and T cells were isolated using an immunomagnetic negative selection kit (EasySep Human CD8+ T Cell Enrichment Kit, #19053, Stemcell Technologies, Vancouver, Canada) according to the manufacturer’s instructions. The cells were subsequently activated with ImmunoCult Human CD3/CD28 T Cell Activator (1:40 dilution, #10971, Stemcell Technologies) and Interleukin 2 (10ng/mL, Human Recombinant IL-2 (CHO-expressed), Stemcell Technologies) in 6 mL of a serum-free and xeno-free medium (ImmunoCult-XF T Cell Expansion Medium, Stemcell Technologies) for 3 days at an initial concentration of 10\(^6\) cells/mL to stimulate PD-1 expression. After incubation, the culture was centrifuged at 2000 × g for 10 min to remove suspended cells and cell debris before EV purification.

**Immunofluorescence staining of cells**

H1568 cells grown on a glass slide in 16-well chambers were first fixed in a 4% formaldehyde solution in PBS for 15 min. Non-specific bindings were blocked with 3% (w/v) bovine serum albumin (BSA) and 0.05% (v/v) Tween 20 in PBS for 1 h at RT. A mouse anti-CD63 monoclonal antibody (MX-49.129.5) – Alexa Fluor 488 conjugate (#sc-5275 AF488, Santa Cruz Biotechnology), a mouse anti-CD9 monoclonal antibody (C-4) – Alexa Fluor 546 conjugate (#sc-13118 AF546, Santa Cruz Biotechnology), and a rabbit anti-PD-L1 monoclonal antibody – Alexa Fluor 647 conjugate (#41726S, Cell Signaling Technology) were diluted in 1% (w/v) BSA and 0.05% (v/v) Tween 20 in PBS, and incubated with the cells for 1 hr at RT. After washing three times with PBS for 5 min each, the glass slide was detached and mounted onto a cover glass using ProLong Gold Antifade Mountant with DAPI. The images were taken with a confocal microscope (Olympus FV3000).
**EV imaging with flow cytometry**

Purified EVs from IFN-γ-stimulated H1568 cells, non-stimulated H1568 cells, MEF, and activated T cells were stained with CD63 (sc-5275 AF488, Santa Cruz Biotechnology) and CD9 (sc-59140, Santa Cruz Biotechnology) antibodies in a 1% (w/v) BSA solution overnight at 4°C. To examine PD-L1 specificity, EVs from IFN-γ-stimulated H1568 cells, non-stimulated H1568 cells, and MEF were stained with a PD-L1 (Rabbit mAb #86744, Cell Signaling Technology) antibody in a 1% (w/v) BSA solution overnight at 4°C. As for PD-1, EVs from activated T cells and MEF were stained with a PD-1 (mAb #53-9969-42, Invitrogen) antibody in a 1% (w/v) BSA solution overnight at 4°C. For mRNA detection, PD-L1 and PD-1 MBs were incubated in 1x tris EDTA for 2 hr at 37°C. PBS was utilized as the negative control. EVs were then imaged using ImageStream® mark II.

**Western blot**

EVs from IFN-γ-stimulated H1568 cells, non-stimulated H1568 cells, MEF, and activated T cells were first isolated and enriched using TFF. All samples were then concentrated via the Total Exosome Isolation Kit (ThermoFisher Scientific) according to the manufacturer's protocol. The total protein extracted from EV and cell lysates was quantified using the Pierce® BCA Protein Assay Kit (ThermoFisher Scientific), where 20 µg of the total protein for each sample was loaded into a sodium dodecyl sulfate-polyacrylamide gel for electrophoresis. The separated proteins were transferred onto 0.2-μm nitrocellulose membranes (Bio-Rad Laboratories), where primary antibodies against CD63 (ab231975, Abcam), CD9 (MA1-19301, ThermoFisher Scientific), PD-1 (86163T, Cell Signaling Technology), and PD-L1 (13684T, Cell Signaling Technology) were utilized. Later, horseradish peroxidase-conjugated goat anti-rabbit (Abcam) or goat anti-mouse IgG (Abcam) was added as secondary antibodies and were subsequently imaged.

**Viral detection**

Moloney murine leukemia viruses (MV-M-V5, ViroFlow Technologies, Ontario, CA) were captured with AuSERP via a V5 capture antibody (ab-9116, Abcam) and detected with a V5 antibody conjugated with FITC (R963-25, Invitrogen) following the same protocol for protein detection as previously described.
**Dynamic light scattering.**

Size distributions were determined in purified serum diluted 10X in DI water with a Nano Zetasizer Zen3600 dynamic light scattering (Malvern Instruments Ltd., Worcestershire, United Kingdom). Briefly, the samples were illuminated at 637 nm at a 90° angle at 22°C.
**Figure S1.** Characterization of AuSERP coated with different-sized gold nanoparticles (5, 30, and 50 nm). a 3D height atomic force microscopy (AFM) images. b Representative total internal reflection fluorescence (TIRF) microscopic images of CD63 protein expression on the surface of H1568 single extracellular vesicles (EVs) in comparison with blank controls (PBS). c Distributions of fluorescence intensity of CD63 protein signals on single EVs. a.u., arbitrary units.
Figure S2. Morphological differences between single EVs and EV clusters. a EVs derived from U251 cells, a glioblastoma cell line, tend to secrete EV clusters as demonstrated by SEM, which produce large, aggregate fluorescent signals in TIRF images. b EVs derived from H1568 cells, an NSCLC cell line, tend to secrete single EVs as demonstrated by SEM, which produce smaller, localized fluorescent signals in TIRF images.
Figure S3. *In vitro* model and characterization of cellular and single-EV PD-L1 protein. **a** Immunofluorescence staining of PD-L1 protein in H1568 cells with/without interferon-gamma (IFN-γ) stimulation. Cell nuclei and PD-L1 protein were stained blue (by DAPI) and magenta (by anti-PD-L1 antibody), respectively. **b** Original TIRF microscopic images for Fig. 2c. The insets show the PD-L1 protein signals on a single EV. **c** A comparison of two different anti-PD-L1 antibodies provided by Cell Signaling Technology and Abcam to detect PD-L1 proteins on the surface of single EVs derived from IFN-γ-stimulated H1568 cells with AuSERP. The H1568 EVs were spiked in healthy donor EVs at a 1:1 ratio with $10^9$ particles/mL each. The data were expressed as mean ± SD; n = 2. TFI, total fluorescence intensity; a.u., arbitrary units. **d** Representative TIRF microscopic images of PD-L1 protein expression of the H1568 single EVs stained by the two different antibodies. The insets show the PD-L1 protein signals on a single EV.
Figure S4. Size and morphological characterization of EVs produced by H1568 cells. a-b Size distributions of EVs derived from H1568 cells with/without IFN-γ stimulation measured with Tunable Resistive Pulse Sensing (TRPS). c A comparison of their EV concentrations. The data were expressed as mean ± SD; n = 3. d Cryo-TEM images of EVs derived from IFN-γ-stimulated H1568 cells.
**Figure S5.** Large-scale SEM of single CLN-MB and single EV fusion. The arrows indicate fusion events and the numbers correspond to the enlarged panels at the bottom.
Figure S6. *In vitro* model and characterization of single-EV PD-L1 protein and mRNA with AuSERP. a Original TIRF microscopic images for Fig. 3e. The insets show the PD-L1 mRNA signals on a single EV. b Multiplex imaging of PD-L1 protein and mRNA from single EVs derived from IFN-γ-stimulated H1568 cells. PD-L1 protein and mRNA were stained green and red, respectively. The colocalized single-EV PD-L1 protein and mRNA signals appear yellow.
Figure S7. Upper limit and linear range of AuSERP. a Upper limit of single-vesicle detection for AuSERP. EVs spiked into healthy donor EVs derived from IFN-γ-stimulated H1568 cells at a concentration of $10^{11}$ particles/mL, demonstrated the saturation of the AuSERP biochip surface and aggregation of EVs as shown by detecting CD63 (right), which is not clearly represented by the low abundance biomarker, PD-L1 (left). b The average intensities of the histogram for various concentrations of spiked tumor EVs. The data were expressed as mean ± SD; n = 2. a.u., arbitrary units.
Figure S8. *In vitro* model and characterization of single-EV PD-1 protein and mRNA. **a** The size distribution of EVs produced by activated T cells measured with TRPS. **b** Representative TIRF microscopic images and their corresponding histograms of PD-1 protein expression on the surface of single EVs derived from activated T cells in comparison with negative controls. The single-EV PD-1 protein signals were characterized with AuSERP using an anti-PD-1 antibody and the TSA method. T cell-derived EVs were spiked in healthy donor EVs with $5 \times 10^{10}$ particles/mL each. TFI, total fluorescence intensity; a.u., arbitrary units. **c** Quantitative detection of PD-1 protein with AuSERP. T cell-derived EVs were spiked in healthy donor EVs at different concentrations ranging from 0 to $5 \times 10^{10}$ particles/mL, while the healthy donor EV concentration was kept constant at $5 \times 10^{10}$ EVs/mL. The limit of detection (LOD) of AuSERP for PD-1 protein was $\sim 10^6$ spiked T cell EVs. The data were expressed as mean ± SD; n = 3. **d** Representative TIRF microscopic images and their corresponding histograms of PD-1 mRNA in single EVs derived from activated T cells in comparison with negative controls. The single-EV PD-1 mRNA signals were characterized with AuSERP using PD-1 CLN-MBs. T cell-derived EVs were spiked in healthy donor EVs with $5 \times 10^{10}$ particles/mL each.
Figure S9. Specificity of AuSERP probes to PD-L1+/PD-1+ EVs. a Quantitative fluorescence intensities of PD-L1/PD-1 protein and mRNA and CD63 protein expression levels on EVs derived from IFN-γ-stimulated H1568 cells, non-stimulated H1568 cells, activated T cells, and mouse embryonic fibroblasts (MEF, negative control for PD-L1/PD-1) as measured by AuSERP (*p < 0.05, ***p < 0.001, ****p < 0.0001, Tukey’s HSD test with respect to MEF). b Counts of PD-L1+/PD-1+ EVs via protein and mRNA detection and CD63+ EVs via protein detection from EVs derived from IFN-γ-stimulated H1568 cells, non-stimulated H1568 cells, activated T cells, and mouse embryonic fibroblasts (MEF, negative control for PD-L1/PD-1).
activated T cells, and MEF as measured by high-resolution flow cytometry (**p < 0.001, ****p < 0.0001, Tukey’s HSD test with respect to MEF). c Representative scatter plots for PD-L1 mRNA cargo via high-resolution flow cytometry. d Qualitative expression of PD-L1/PD-1/CD63 proteins on EVs derived from IFN-γ-stimulated H1568 cells, non-stimulated H1568 cells, activated T cells, and MEF as measured by western blot. The data were expressed as mean ± SD; n = 3. RFI, relative fluorescence intensity; a.u., arbitrary units.
Figure S10. Expression levels of CD63/CD9 on the model EVs. a Quantitative fluorescence intensities of CD63/CD9 protein and mRNA expression levels on EVs derived from IFN-γ-stimulated H1568 cells, non-stimulated H1568 cells, and activated T cells as measured by AuSERP (*p < 0.05, Tukey’s HSD test). b Counts of CD63+/CD9+ EVs via protein detection from EVs derived from IFN-γ-stimulated H1568 cells, non-stimulated H1568 cells, and activated T cells as measured by high-resolution flow cytometry (*p < 0.05, **p < 0.01, Tukey’s HSD test). The data were expressed as mean ± SD; n = 3. RFI, relative fluorescence intensity; a.u., arbitrary units.
**Figure S11.** Original TIRF microscopic images for Fig. 4c. The insets show the PD-1/PD-L1 protein and mRNA signals on a single EV.
**Figure S12.** Viral detection with AuSERP to prove sEV detection. 

**a** Size distribution of sEVs from a purified serum sample. 

**b** Dynamic light scattering of EVs from a purified serum sample. The dotted box indicates the presence of EVs < 50 nm. 

**c** Moloney murine leukemia virus (MLV) was captured and detected with antibodies targeting the V5 epitope. The inset shows a single virus particle. 

**d** The corresponding histogram for the TIRF images.
**Figure S13.** Repeatability of AuSERP across various serum samples for PD-L1/PD-1 protein and mRNA. Each biochip replicate is represented by a different symbol (■, ●, ▲, and ×). The TFI was normalized to the average TFI of the sample to demonstrate the coefficient of variation as the standard deviation.
Figure S14. Single-EV PD-L1 protein and mRNA characterization in subpopulations. a Comparisons of two different capture antibody cocktails (anti-CD63/CD9 and anti-EGFR/EpCAM) on the measurements of PD-L1 protein and mRNA signals in single EVs isolated from the serum of healthy donors, non-responders, and
responders with AuSERP. Each antibody (anti-CD63, anti-CD9, anti-EGFR, and anti-EpCAM) was used at a concentration of 10 µg/mL. For protein characterization, a cohort including healthy donors (n = 5), non-responders (n = 7), and responders (n = 6) was tested. For mRNA characterization, a cohort including healthy donors (n = 7), non-responders (n = 9), and responders (n = 8) was tested. (*p < 0.05, **p < 0.01, Mann-Whitney U test). RFI, relative fluorescence intensity. b Confocal fluorescence microscopic images of CD63/CD9/PD-L1 proteins and PD-L1 mRNA in IFN-γ-stimulated H1568 cells. CD63/CD9/PD-L1 proteins were stained using the corresponding antibodies, while PD-L1 mRNA was visualized using PD-L1 MB via fluorescent in situ hybridization. Cell nuclei were stained blue using DAPI. c TIRF images of PD-L1 and PD-1 protein colocalization on single EVs from NSCLC patient serum.
Figure S15. A comparison of different capture antibodies on the measurement of PD-1 protein and mRNA signals in single EVs with AuSERP. a A comparison of anti-CD63/CD9 and anti-CD3 capture antibodies on the measurement of PD-1 protein and mRNA signals in single EVs isolated from sera of healthy donors, non-responders, and responders. The cocktail of anti-CD63/CD9 antibodies was used at a concentration of 10 µg/mL each, while the anti-CD3 antibody was used at 20 µg/mL. For protein characterization, a cohort including healthy donors (n = 5), non-responders (n = 7), and responders (n = 6) was tested. For mRNA characterization, a cohort including healthy donors (n = 2), non-responders (n = 4), and responders (n = 3) was tested. (*p < 0.05, Mann-Whitney U test). RFI, relative fluorescence intensity. b Quantitative detection of PD-1 protein with different capture antibodies (anti-CD63/CD9 and anti-CD3). T cell-derived EVs were spiked in healthy donor EVs at different concentrations ranging from 0 to 5 × 10^{10} particles/mL, while the healthy donor EV concentration was kept constant at 5 × 10^{10} EVs/mL. TFI, total fluorescence intensity; a.u., arbitrary units. c A comparison of different capture antibodies (anti-CD63/CD9, anti-CD4, anti-CD8, and anti-CD4/CD8) on the measurement of PD-1 protein on the surface of single EVs isolated from the serum of healthy donors (n = 3), non-responders (n = 5), and responders (n = 5). The cocktail of anti-CD63/CD9 and anti-CD4/CD8 antibodies were both used at a concentration of 10 µg/mL for each antibody, while single anti-CD4 and anti-CD8 antibodies were used at 20 µg/mL. (*p < 0.05, Mann-Whitney U test). RFI, relative fluorescence intensity.
Figure S16. Characterization of EVs from the serum of non-responders (n = 27) and responders (n = 27). a Area under the curve (AUC) values of receiver operating characteristic (ROC) curve analyses for the single-EV immunotherapy biomarkers measured with AuSERP. For NSCLC diagnosis, the sample size consisted of patients (n = 54) vs. healthy donors (n = 20). For prediction of NSCLC patient response to anti-PD-1/PD-L1 immunotherapy, the sample size consisted of responders (n = 27) vs. non-responders (n = 27). An ROC curve analysis was performed for a single biomarker and multiple biomarkers with different permutations. b Representative size distributions of the patient serum-derived EVs measured with TRPS. c Box plots of the concentrations of the patient serum-derived EVs measured with TRPS.
Figure S17. AuSERP distinguishes T-cell status via single-EV PD-1 levels. EVs from activated and non-activated T cells were harvested after 3 days and screened with AuSERP for PD-1 protein and mRNA levels. (*p < 0.05, **p < 0.01, Student’s t-test). The data were expressed as mean ± SD; n = 3. RFI, relative fluorescence intensity.
Table S1. Clinical characteristics of stage IV NSCLC patients.

|                        | Non-responder (n= 27) | Responder (n = 27) |
|------------------------|-----------------------|--------------------|
| **Age**                |                       |                    |
| Median (range)         | 63 (47 – 83)          | 65 (45 – 86)       |
| **Gender**             |                       |                    |
| Male                   | 14                    | 18                 |
| Female                 | 13                    | 9                  |
| **Race**               |                       |                    |
| Caucasian              | 23                    | 24                 |
| African American       | 4                     | 3                  |
| **Smoking history**    |                       |                    |
| Current                | 7                     | 6                  |
| Former                 | 19                    | 21                 |
| Never                  | 1                     | 0                  |
| **Performance status** |                       |                    |
| 0                      | 5                     | 6                  |
| 1                      | 18                    | 19                 |
| 2                      | 4                     | 2                  |
| **Histology**          |                       |                    |
| Adenocarcinoma         | 19                    | 18                 |
| Squamous cell          | 7                     | 5                  |
| Adenosquamous          | 0                     | 1                  |
| NOS                    | 1                     | 3                  |
| **PD-L1 IHC**          |                       |                    |
| Positive               | 12                    | 17                 |
| Negative               | 6                     | 4                  |
| Unknown                | 9                     | 6                  |
| **EGFR**               |                       |                    |
| Wild                   | 18                    | 22                 |
| Mutant                 | 5                     | 1                  |
| Unknown                | 4                     | 4                  |
| **Drug**               |                       |                    |
| Nivolumab              | 20                    | 17                 |
| Pembrolizumab          | 7                     | 9                  |
| Atezolizumab           | 0                     | 1                  |
Table S2. Detailed information on the patients enrolled in the study.

| ID   | Response   | PD-L1 IHC | Age | Gender | Race            | Smoking history | Performance status | Treatment       |
|------|------------|-----------|-----|--------|-----------------|----------------|-------------------|-----------------|
| N001 | Non-responder | Negative | 70  | Male   | Caucasian       | Former         | 1                 | Nivolumab       |
| N002 | Non-responder | Negative | 77  | Female | Caucasian       | Current        | 1                 | Nivolumab       |
| N003 | Non-responder | Negative | 62  | Male   | Caucasian       | Former         | 0                 | Nivolumab       |
| N004 | Non-responder | Negative | 51  | Male   | African American| Former        | 0                 | Pembrolizumab   |
| N005 | Non-responder | Negative | 61  | Male   | Caucasian       | Former         | 0                 | Nivolumab       |
| N006 | Non-responder | Negative | 47  | Female | Caucasian       | Current        | 1                 | Nivolumab       |
| N007 | Non-responder | Positive  | 71  | Female | Caucasian       | Former         | 1                 | Nivolumab       |
| N008 | Non-responder | Positive  | 69  | Female | Caucasian       | Former         | 1                 | Nivolumab       |
| N009 | Non-responder | Positive  | 64  | Male   | Caucasian       | Former         | 1                 | Nivolumab       |
| N010 | Non-responder | Positive  | 83  | Male   | Caucasian       | Former         | 0                 | Nivolumab       |
| N011 | Non-responder | Positive  | 68  | Female | Caucasian       | Former         | 1                 | Nivolumab       |
| N012 | Non-responder | Positive  | 82  | Female | Caucasian       | Former         | 1                 | Pembrolizumab   |
| N013 | Non-responder | Positive  | 56  | Male   | Caucasian       | Former         | 1                 | Pembrolizumab   |
| N014 | Non-responder | Positive  | 63  | Male   | Caucasian       | Former         | 1                 | Pembrolizumab   |
| N015 | Non-responder | Positive  | 56  | Female | African American| Current       | 2                 | Pembrolizumab   |
| N016 | Non-responder | Positive  | 58  | Male   | African American| Current       | 1                 | Pembrolizumab   |
| N017 | Non-responder | Positive  | 66  | Female | Caucasian       | Never          | 1                 | Pembrolizumab   |
| N018 | Non-responder | Positive  | 63  | Male   | Caucasian       | Former         | 2                 | Nivolumab       |
| N019 | Non-responder | Unknown   | 65  | Female | Caucasian       | Former         | 1                 | Nivolumab       |
| N020 | Non-responder | Unknown   | 51  | Male   | Caucasian       | Former         | 2                 | Nivolumab       |
| N021 | Non-responder | Unknown   | 70  | Female | Caucasian       | Former         | 0                 | Nivolumab       |
| N022 | Non-responder | Unknown   | 58  | Female | Caucasian       | Current       | 1                 | Nivolumab       |
| ID   | Status   | Response | Age | Sex       | Race             | Treatment | Duration | Drug   |
|------|----------|----------|-----|-----------|------------------|-----------|----------|--------|
| N023 | Non-responder | unknown  | 54  | Female    | Caucasian        | Current   | 2        | Nivolumab |
| N024 | Non-responder | Unknown  | 50  | Female    | Caucasian        | Former    | 1        | Nivolumab |
| N025 | Non-responder | Unknown  | 57  | Male      | African American | Former    | 1        | Nivolumab |
| N026 | Non-responder | Unknown  | 74  | Male      | Caucasian        | Former    | 1        | Nivolumab |
| N027 | Non-responder | Unknown  | 69  | Male      | Caucasian        | Current   | 1        | Nivolumab |
| R028 | Responder | Negative | 70  | Male      | Caucasian        | Former    | 1        | Nivolumab |
| R029 | Responder | Negative | 65  | Male      | Caucasian        | Former    | 0        | Nivolumab |
| R030 | Responder | Negative | 83  | Male      | Caucasian        | Former    | 1        | Pembrolizumab |
| R031 | Responder | Negative | 63  | Female    | Caucasian        | Current   | 1        | Nivolumab |
| R032 | Responder | Positive | 76  | Female    | African American | Former    | 1        | Nivolumab |
| R033 | Responder | Positive | 45  | Female    | African American | Current   | 1        | Nivolumab |
| R034 | Responder | Positive | 69  | Male      | Caucasian        | Former    | 1        | Nivolumab |
| R035 | Responder | Positive | 51  | Male      | Caucasian        | Current   | 1        | Atezolizumab |
| R036 | Responder | Positive | 62  | Male      | Caucasian        | Former    | 1        | Nivolumab |
| R037 | Responder | Positive | 86  | Male      | Caucasian        | Former    | 1        | Nivolumab |
| R038 | Responder | Positive | 63  | Female    | Caucasian        | Former    | 1        | Nivolumab |
| R039 | Responder | Positive | 57  | Female    | Caucasian        | Former    | 1        | Nivolumab |
| R040 | Responder | Positive | 75  | Male      | African American | Former    | 2        | Pembrolizumab |
| R041 | Responder | Positive | 55  | Male      | Caucasian        | Former    | 0        | Pembrolizumab |
| R042 | Responder | Positive | 59  | Male      | Caucasian        | Former    | 0        | Pembrolizumab |
| R043 | Responder | Positive | 62  | Male      | Caucasian        | Former    | 0        | Pembrolizumab |
| R044 | Responder | Positive | 65  | Female    | Caucasian        | Former    | 1        | Pembrolizumab |
| R045 | Responder | Positive | 72  | Male      | Caucasian        | Former    | 1        | Pembrolizumab |
| R046 | Responder | Positive | 71  | Male      | Caucasian        | Former    | 1        | Pembrolizumab |
| R047 | Responder | Positive | 67  | Male      | Caucasian        | Current   | 1        | Nivolumab |
| ID   | Status  | Age | Gender | Ethnicity | Stage | Treatment |
|------|---------|-----|--------|-----------|-------|-----------|
| R048 | Responder | 66  | Female | Caucasian | Former | Pembrolizumab |
| R049 | Responder | 65  | Female | Caucasian | Current | Nivolumab |
| R050 | Responder | 60  | Female | Caucasian | Former | Nivolumab |
| R051 | Responder | 73  | Male   | Caucasian | Former | Nivolumab |
| R052 | Responder | 51  | Male   | Caucasian | Current | Nivolumab |
| R053 | Responder | 58  | Male   | Caucasian | Former | Nivolumab |
| R054 | Responder | 65  | Male   | Caucasian | Former | Nivolumab |
Table S3. Average values for the performances of ELISA and AuSERP at detecting EV PD-L1 protein. NA, not applicable; ND, not detected.

| Number of spiked tumor EVs | TFI of single EVs (a.u.) | PD-L1 concentration (pg/mL) |
|----------------------------|--------------------------|-----------------------------|
| 2.00E+10                   | Single-EV NA             | 13.79                       |
| 1.00E+10                   | Single-EV NA             | 6.90                        |
| 4.00E+09                   | Single-EV NA             | 2.11                        |
| 1.00E+09                   | 240000000                | 1.33                        |
| 2.00E+08                   | 217000000                | 0.31                        |
| 4.00E+07                   | 129000000                | ND                          |
| 8.00E+06                   | 67100000                 | ND                          |
| 1.60E+06                   | 28500000                 | ND                          |
| 3.20E+05                   | 8070000                  | ND                          |
Table S4. Average values for the performances of qRT-PCR and AuSERP at detecting EV PD-L1 mRNA. NA, not applicable; ND, not detected.

| Number of spiked tumor EVs | TFI of single EVs (a.u.) | Cycle Threshold (Ct) |
|----------------------------|--------------------------|---------------------|
| 4.00E+10                   | Single-EV NA             | 28.09               |
| 5.00E+09                   | Single-EV NA             | 37.26               |
| 1.00E+09                   | 14054377                 | 39.13               |
| 2.00E+08                   | 8508105                  | ND                  |
| 4.00E+07                   | 3406813                  | ND                  |
| 8.00E+06                   | 1145519                  | ND                  |
| 1.60E+06                   | 663258                   | ND                  |
| 3.20E+05                   | 415275                   | ND                  |
| 6.40E+04                   | 359476                   | ND                  |
Table S5. Antibodies used for single-EV capture and detection.

| Antibody        | Catalog number/ Brand name | Supplier                  |
|-----------------|-----------------------------|---------------------------|
| **Capture**     |                             |                           |
| CD63            | MAB5048                     | R&D Systems               |
| CD9             | MAB1880                     | R&D Systems               |
| EGFR (Cetuximab)| Erbitux®                    | ImClone LLC               |
| EpCAM           | AF960                       | R&D Systems               |
| CD3             | MAB100100                   | R&D Systems               |
| Biotinylated CD4| 344610                      | BioLegend                 |
| Biotinylated CD8| 344720                      | BioLegend                 |
| **Detection**   |                             |                           |
| CD63 - Alexa Fluor® 488 | sc-5275 AF488             | Santa Cruz Biotechnology  |
| PD-L1           | 86744S                      | Cell Signaling Technology |
| PD-L1           | ab205921                    | Abcam                     |
| PD-1            | 86163S                      | Cell Signaling Technology |