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Supporting Information

A Simple, Rapid and Low-cost qPCR Assay for Evaluation the Severity of Exosomal PD-L1-mediated T cell Exhaustion in Blood Samples

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Experimental Procedures

Cell Lines and Culture

A375 cell line (human melanoma) was purchased from Procell Life Science & Technology (Wuhan, China). The cells were cultured in DMEM (Gibco, USA) with 10% exosome-depleted FBS (Gibco, USA) and 100 U/mL penicillin-streptomycin at 37°C with 5% CO_2. For stimulation with IFN-γ, cells Pwere incubated with 100 ng/ml of recombinant human (Peprotech, USA) for 48 h. For stimulation with GW 4869, cells were incubated with 10 μM of GW 4869 (Selleck, USA) for 24 h. The cell supernatants were collected and centrifuged (4000 g, 4°C) for 10 min and stored at −80°C until use. Jurkat cell line (T cell acute lymphoblastic leukemia) was purchased from Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in 1640 medium (Gibco, USA) with 10% fetal bovine serum (Gibco, USA) and 100 U/mL penicillin-streptomycin at 37°C with 5% CO_2.

Purification of exosomes by standard differential ultracentrifugation

For exosome purification from cell culture supernatants, cells were cultured in media supplemented with 10% exosome-depleted FBS. Supernatants were collected from 48 h cell cultures and exosomes were purified by a standard differential centrifugation protocol. Briefly, culture supernatants were centrifuged at 2,000 g for 20 min to remove cell debris and dead cells (Beckman Coulter, Allegra X-15R). Microvesicles were pelleted and removed after centrifugation at 16,500 g for 45 min (Beckman Coulter, J26). The supernatants were then ultra-centifugated at 100,000 g for 2 h at 4 °C (Beckman Coulter, Optima XPN-100). The pelleted exosomes were washed once with filtered PBS and collected by ultracentrifugation at 100,000 g for 2 h. Finally, the exosomes were resuspended in filtered PBS and stored at −80°C until use.

Characterization of exosomes

The purified exosomes were characterized by transmission electron microscopic (TEM) imaging. Briefly, the collected exosomes were dropped on carbon-coated nickel grids, and then stained with 2% uranyl acetate for 1 min at room temperature. After drying in air, samples were observed on a transmission electron microscope (JEOL JEM1230, Japan). The size distribution of purified exosomes was measured by nanoparticle tracking (Particle Metrix, Zeta View, Germany).

Preparation of exosome-conjugated Ni-NTA agarose beads

Ni-NTA agarose beads (Smart-lifesciences, China) with uniform size in the range of 50~150 μm have multiple Ni-NTA groups that can be used to attached His-tagged recommbinant proteins to the bead surface. Firstly, 10 μg recombinant Human PD-1 (Sino Biological, China) and 10 μg recombinant Human CD62L (Cusabio, China) were incubated with 100 μL Ni-NTA agarose beads for 4 h at room temperature, followed by three washing steps in PBS buffer containing 20 mM imidazole. Exosomes (5×10^4 particles per μL) were then incubated with the modified beads by adding 1 mL

filtered PBS for 2 h. Afterwards, the resultant exosome-coated beads were washed twice by centrifugation (150 g, 5 min), and resuspended in 40 μL PBS (0.5% BSA).

Gel electrophoresis analysis of PDLA products

The positive sample (10 μL of exosome-Ni-NTA bead complexes) were incubated with 200 nM of two affinity probes for 40 min in 100 μL binding buffer (PBS with 0.55 mM $MgCl_2$ and 0.5% BSA). After washing twice, the beads were incubated with 200 nM connector and T4 ligase from a T4 DNA Ligation Kit (Accurate Biology, China) in a ligation volume of 20 μL according to the instructions. Then the resultant solution was heated to 90 °C for 10 min and rapidly centrifuged for disaggregation of the affinity probes from targets. Finally, 10 μL of the as-obtained supernatant was analyzed by 4% agarose gel. Controls were treated identically, but with negative samples (exosome-Ni-NTA bead complexes without one aptamer probe or connector groups, exosomes or Ni-NTA beads alone with dual-affinity probes and connector groups).

Specificity test of TARACET-PCR to the ligation products

The positive sample (5 μL of the ligation product generated from exosome-Ni-NTA bead complexes with dual-affinity probes and connector) and the negative samples (5 μL of the ligation products generated from: exosome-Ni-NTA bead complexes without one aptamer probe or connector, exosomes alone or Ni-NTA beads alone with dual-affinity probes and connector) and blank control sample were mixed with 1 μL of forward/reverse primers (10 μM) and 4 μL of Taqman probe (1 μM), and then added to the Probe qPCR Mix (Accurate Biology, China) with a final volume of 20 μL. Finally, the Taqman quantitative PCR was processed by thermal cycling: 600 s at 95°C, followed by 45 cycles at 95°C for 5 s and 62°C for 60 s using a LightCycler 96 (Roche, USA).

TARACET-PCR for analysis of exosomal PD-L1 on Jurkat cells

1×10^4 Jurkat cells were co-incubated with supernatant from A375 cells treated by different methods for 2 h, then the cells were harvested by centrifugation and washed twice with ice-cold PBS buffer. The collected cells were incubated with 200 nM of two affinity probes for 40 min in 40 μL binding buffer (PBS with 0.55 mM $MgCl_2$ and 0.5% BSA). Subsequently, the solution was diluted 25 times for ligation. The ligation volume was 20 μL with the T4 ligase and connector in ligation buffer. After ligation, 5 μL of the ligation product was mixed with forward/reverse primers and Taqman probe, and then added to the Probe qPCR Mix with a final volume of 20 μL. Finally, the Taqman quantitative PCR was processed by thermal cycling: 600 s at 95°C, followed by 45 cycles at 95°C for 5 s and 62°C for 60 s using a LightCycler 96.

TARACET-PCR for analysis of exosomal PD-L1 on T cells in a humanized mouse model

All animal experiments were conducted and agreed with the Principles of Laboratory Animal Care (People's Republic of China) and the Guidelines of the

Animal Investigation Committee, Binzhou Medical University, China. Seven-week-old huPBMC-BRGSF mice (BALB/c $Rag2^{-/-}$ $IL2R\gamma^{-/-}$ $Sirpa^{NOD}$ $Flk2^{-/-}$) were purchased from Cyagen Biosciences (China). After adaptation for 7 days, six mice were subcutaneously injected with 5×10^6 A375 cells in the right axillary region to form primary tumors. A group of mice injected with the same volume of PBS served as a negative control. After 14 days, the mice were sacrificed when tumor growth to suitable size (~5×5 mm^2). The peripheral blood of mice were collected in EDTA tubes, and then 200 μL of the collected blood was used for isolation of mice peripheral blood mononuclear cells (PBMCs) by red blood cell lysis (Sigma-Aldrich, Germany). Subsequently, the collected cells were performed for PDLA and TARACET-PCR procedures as the same as the exosome-coated Jurkat cells.

Flow cytometry analysis

For analysis of the expression of CD62L, PD-1 and PD-L1 on Jurkat cells, about 1×10^6 jurkat cells were harvested by centrifugation (1,500 rpm) and washed with PBS twice. Then, the cells were stained in PBS buffer using the following antibodies: anti-human CD62L-FITC, anti-human PD-1-PE, and anti-human PD-L1-APC (BioLegend, USA) according to the instructions. Finally, cells were washed once, resuspended in 500 μL PBS buffer, and analyzed by flow cytometry (BD FACSCanto II, USA).

For analysis of the percentage of T cells and CD8+ T cells in the PBMCs of humanized mice. The PBMCs of humanized mice were isolated by the method as described above. The collected cells were stained in PBS buffer using the following antibodies: anti-human CD3-FITC and anti-human CD8-BV421 (BD Biosciences, USA) according to the instructions. Finally, cells were washed once, resuspended in 500 μL PBS buffer, and analyzed by flow cytometry (BD FACSCanto II, USA).

Table S1 Sequences in this work

name	Sequence (5’ → 3’)
PD-L1 aptamer	TACAGGTTCTGGGGGGTGGGTGGGGAACCTGTTTGTGGTCTATGTCGTCGTT CGCTAGTAGTTCCTGGGCTGCAC
CD62L aptamer	TCGAGGCGTAGAATTCCCCCGATGCGCGCTGTTCTTGACTGATTTACGTAGCCAA GGTAACCAGTACAAGGTGCTAAACGTAATGGCTTCGGCTTAC
Connector	AAATACGCCTCGAGTGCAGCCCATTT
Forward Primer	TGTGGTCTATGTCGTCGTTCG
Reverse Primer	CAGTCAAGAACAGCGCGCATC
Taqman probe	FAM-CTGCACTCGAGGCGTAGAATTCCCC-BHQ-1
Standard sequence	TACAGGTTCTGGGGGGTGGGTGGGGAACCTGTTTGTGGTCTATGTCGTCGTTCGCTAG TAGTTCCTGGGCTGCACTCGAGGCGTAGAATTCCCCCGATGCGCGCTGTTCTTGACTG ATTTACGTAGCCAAGGTAACCAGTACAAGGTGCTAAACGTAATGGCTTCGGCTTAC

Table S2 The comparison of this work with currently available methods

number	method	LOD (particles/ μL)	reference
1	nanotetrahedron-assisted electrochemical aptasensor	20.9	*ACS Nano 2017,* 11, 3943
2	quantum dot-based electrochemical detection	100	*Analyst 2017,* 142, 2211
3	electrochemical sandwich immunosensor	200	*Anal. Chem.* 2016, 88, 10466
4	paper-based aptasensor	1.1×10^3	*Biosens. Bioelectron.* 2018, 102, 582
5	dual signal amplification method based on DNA dendrimer self-assembly	1.16×10^3	*Analyst.* 2019, 144, 1995
6	B-Chol anchor assay with enzyme-linked HCR	2.2×10^3	*Anal. Chem.* 2017, 89, 12968
7	copper-mediated signal amplification strategy	4.8×10^4	*Anal Chem.* 2018, 90, 8072
8	dual-aptamer activated proximity-induced qPCR assay	54.2	Our method

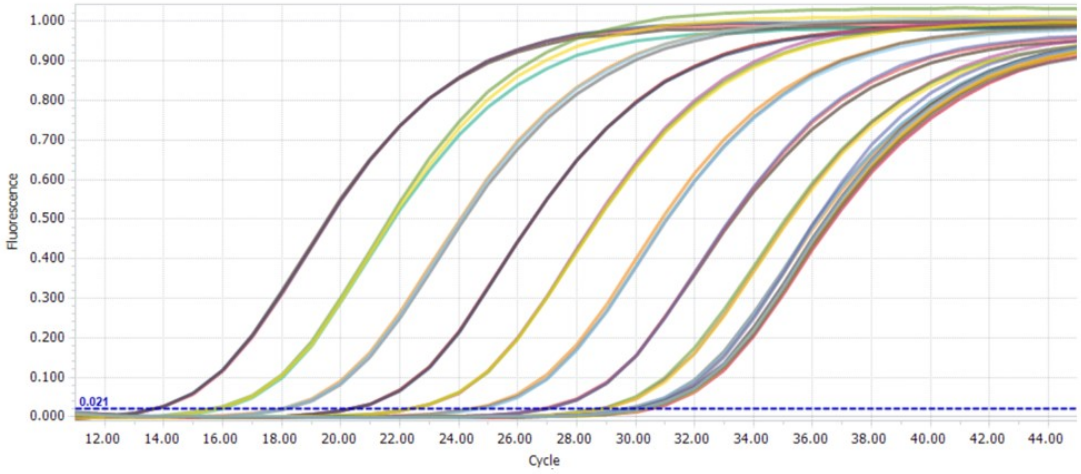

Fig. S1 Raw qPCR traces for validating TaqMan's qPCR specificity and sensitivity by a synthetic standard DNA sequence.

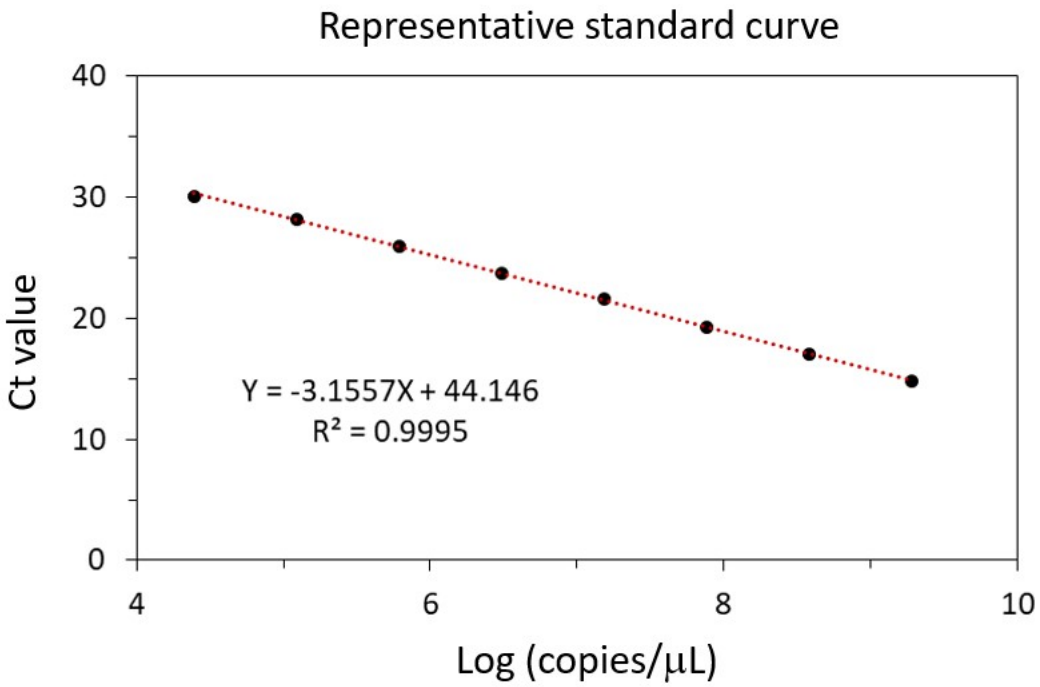

Fig. S2 The linear correlation between the Ct values of qPCR and logarithm of the standard DNA's raw copy numbers.

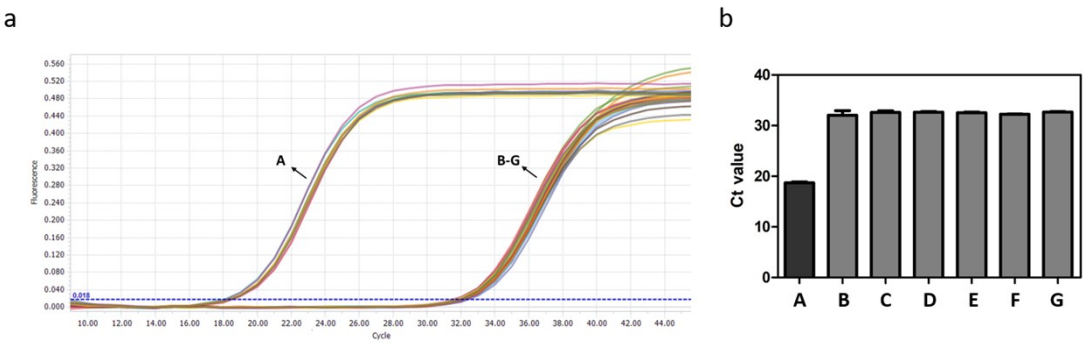

Fig. S3 Specificity test of TARACET-PCR to the ligation products. (a) Raw qPCR traces for testing the specificity of TARACET-PCR to the different ligation products. A. the ligation product generated from exosome-Ni-NTA bead complexes with dual-affinity probes and connector; B. the ligation products generated from exosome-Ni-NTA bead complexes without MJ5C-L; C. the ligation products generated from exosome-Ni-NTA bead complexes without LD201t1-L; D. the ligation products generated from exosome-Ni-NTA bead complexes without connector; E. the ligation products generated from exosomes alone with dual-affinity probes and connector; F. the ligation products generated from Ni-NTA beads alone with dual-affinity probes and connector; G. blank control sample. (b) Statistical results of the qPCR experiments

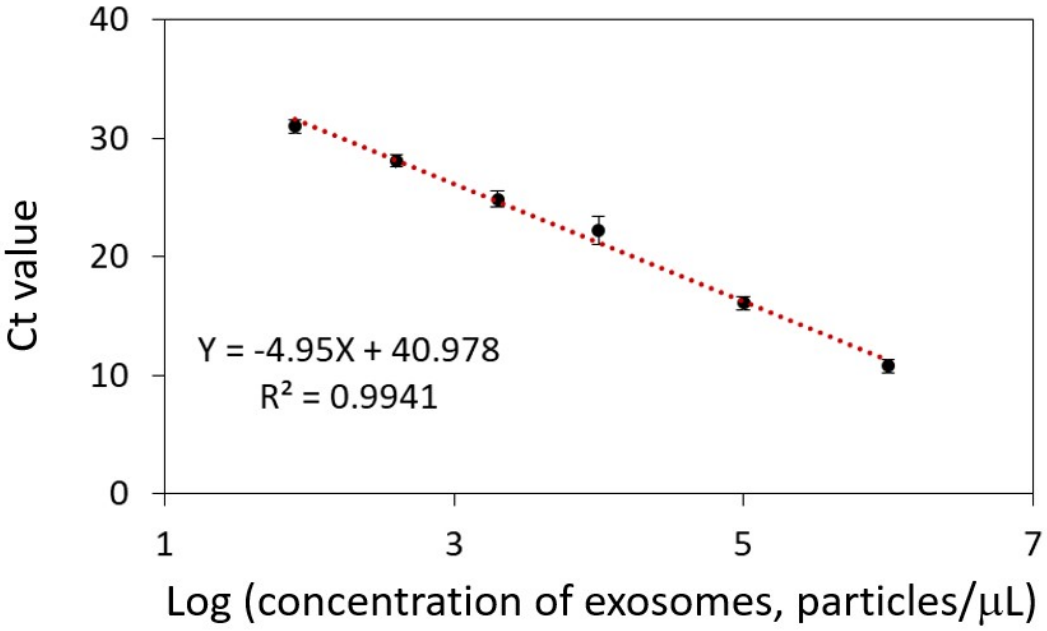

Fig. S4 The linear correlation between the Ct values of qPCR and logarithm of the exosome's concentration.

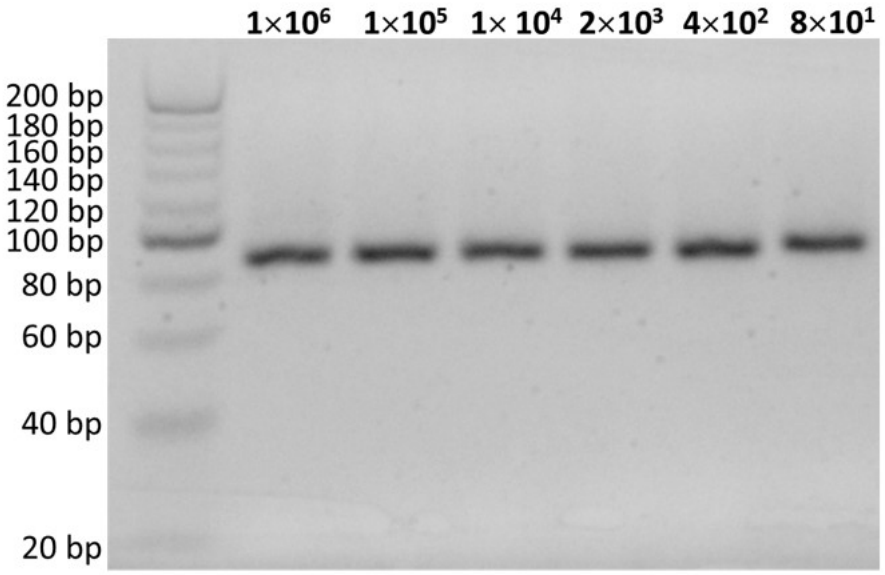

Fig. S5 Gel electrophoresis analysis of qPCR products of Figure 2.

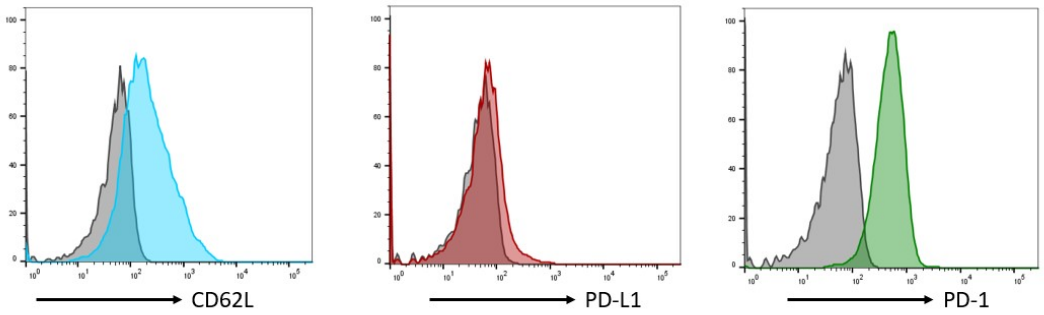

Fig. S6 Flow cytometry analysis of the expression of (Left) CD62L, (Middle) PD-L1 and (Right) PD-1 on Jurkat cells.

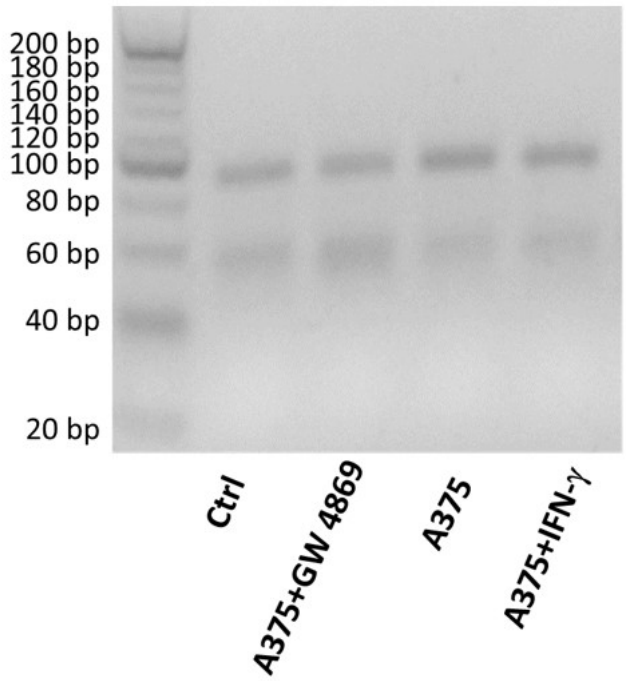

Fig. S7 Gel electrophoresis analysis of qPCR products of Figure 3.

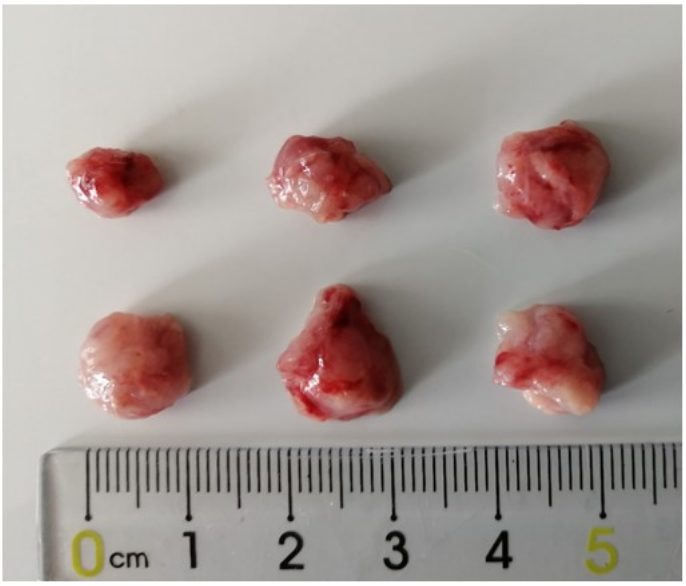

Fig. S8 Image of the tumors after 5×10^6 A375 cells inoculated in huPBMC-BRGSF mice for 14 days.

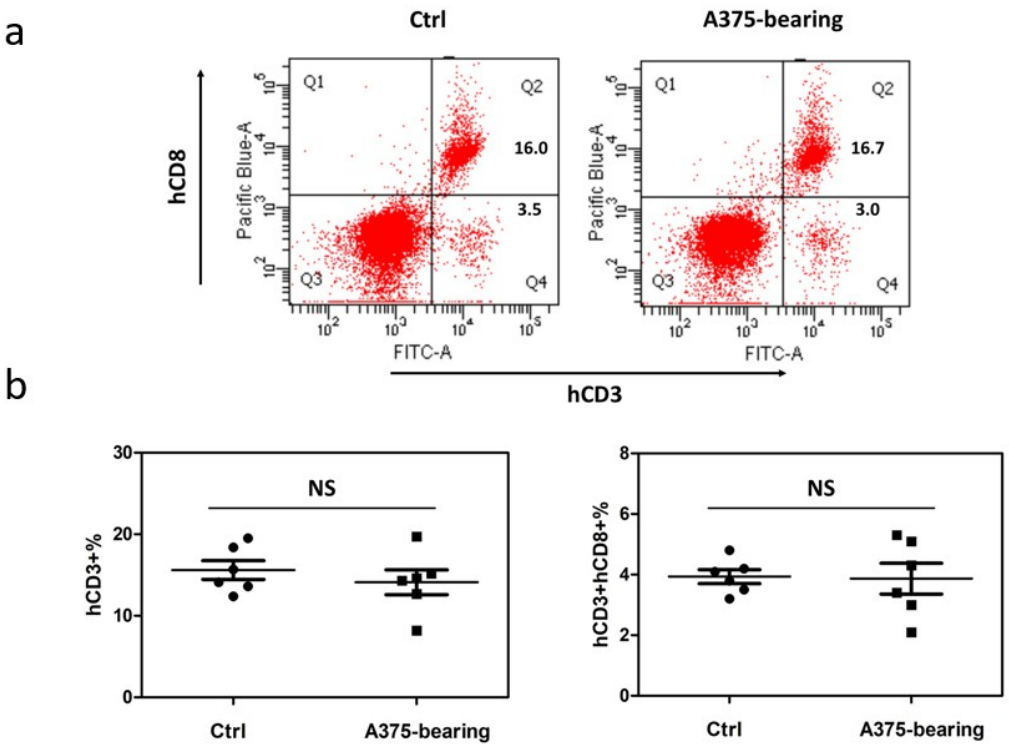

Fig. S9 Flow cytometry analysis of the percentage of the total human T cells (hCD3+) and human CD 8 T cells (hCD3+huCD8+) in the PBMC of Ctrl and A375-bearing huPBMC-BRGSF mice. (a) Raw representative flow cytometry data indicating total human T cells and human CD 8 T cells distribution; (b) Statistical results of flow cytometry experiments.

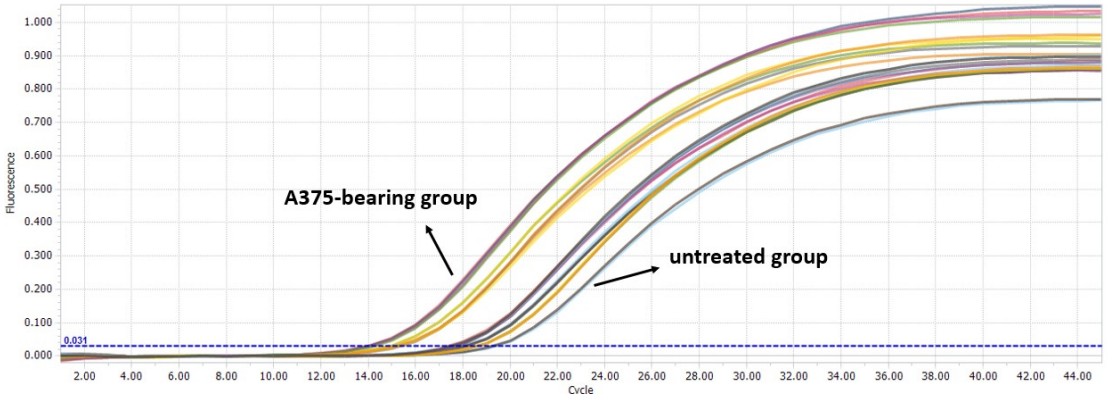

Fig. S10 Raw qPCR traces (TARACET-PCR) for distinction of the PD-L1 levels on tumor-derived exosomes bonded to T cells from tumor-bearing mice and healthy mice.