Appendix 1

Cell lines

Carcinoma-derived cell lines MCF7 and MDA-MB-231 (breast cancer), and LNCaP acquired from ATCC (ATCC, Manassas, VA, USA) were maintained in cell culture media as a monolayer at 37 ℃ with 5% CO_2 in humidified air. LNCaP cells were cultured in RPMI 1640 (ThermoFisher Scientific, Waltham, MA, USA), containing 10% foetal bovine serum (FBS) (ThermoFisher Scientific). MDA-MB-231 and MCF-7 cell lines were cultured in DMEM (ThermoFisher Scientific) containing 10% FBS. Cells were harvested at 80% confluency for flow cytometry and immunofluorescent staining. Some MCF7 cells were incubated with 100 ng/mL IFN-γ for 24 hours to induce PD-L1 expression.

Antibody purification, immunomagnetic beads coupling, and recovery assessment

Anti-EpCAM antibody (Ber-EP4, ab7504, Abcam) was purified using the NAb Spin Kits, 0.2 mL (ThermoFisher Scientific) following the manufacturer's instructions. Purified Anti-EpCAM antibody was covalently bound to magnetic beads using a Dynabead Antibody Coupling Kit (ThermoFisher Scientific) following the manufacturer's instructions. Purified antibody was quantified using a NanoDrop One (ThermoFisher Scientific); 3 µg of antibody was used per mg of Dynabeads M-270 Epoxy. Antibody coupling with the Dynabeads was confirmed using flow cytometry. For this, two microlitres of coated beads were added to 500 µL of phosphate-buffered saline (PBS) and incubated for 15 minutes at room temperature with a donkey anti-mouse IgG antibody conjugated to Alexa Fluor 488 (Abcam USA) diluted 1/500. After washing, beads were analysed using a Gallios Flow Cytometer (Beckman Coulter, Brea, CA, USA). Unlabelled EpCAM-coated beads were used as a negative control.

We assessed the performance of the EpCAM-coated magnetic beads and obtained 82% recovery efficiency using LNCaP cell lines (expressed EpCAM and CK), pre-labelled with 1 µL of CellTracker Red (ThermoFisher Scientific) spiked into peripheral blood mononuclear cells (PBMCs) obtained from healthy donors.

Assessment of PD-L1 expression by flow cytometry

PD-L1 expression for each cell line was initially assessed by flow cytometry using a Gallios Flow Cytometer (Beckman Coulter) with the 28.8 PD-L1 antibody clone (Abcam, Cambridge, UK). Ten thousand cells were suspended in 100 µL of stain buffer [1% bovine serum albumin (BSA)/10% normal donkey serum (NDS) in PBS] containing PD-L1 diluted 1/100 for 30 minutes. The cells were then washed once with 0.5% BSA in PBS before resuspending in stain buffer containing a secondary donkey anti-rabbit IgG antibody conjugated to Alexa Fluor® 488 (Abcam) diluted 1/500 for 30 minutes. The cells were once again washed once in 0.5% BSA in PBS before being resuspended in 100 µL of stain buffer and analysed using a Gallios Flow Cytometer (Beckman Coulter). Fluorescence values obtained for each cell line were then compared to form a relative scale which was utilised to identify high, low, and negative PD-L1 expressing cell lines which were subsequently used as controls Figure S1.

MDA-MB-231 cells constitutively expressed high levels of PD-L1, with a 9.2-fold shift in median fluorescence intensity relative to the isotype control. LnCAP cells had no apparent shift in median fluorescence intensity. MCF7 cells incubated with 100 ng/mL of IFN-γ for 24 hours had a 3.7-fold shift in median fluorescence intensity relative to the primary control. Therefore, MDA-MB-231 cell line was selected as the high expression control, IFN-γ induced MCF7 was selected as the low expression control and LnCAP was selected as the negative control.

Assessment of PD-L1 expression on cells captured using anti-EpCAM coated magnetic beads

Peripheral blood mononuclear cells (PBMCs) were isolated from blood by density gradient centrifugation over Ficoll-Paque (GE Healthcare Biosciences, Uppsala, Sweden) and resuspended in 1 mL MACS buffer (0.5% BSA, 2 mM EDTA in PBS, pH 7.2). Healthy control PBMC spiked MCF-7, induced with IFNγ to express PD-L1, was then isolated using 3 µL EpCAM antibody conjugated magnetic beads. Captured cells were quenched for endogenous peroxidase activity with 0.3% H_2O_2 for

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20 minutes before incubated for 1 hour at room temperature with anti-pan cytokeratins, WBC marker, and unconjugated anti-PD-L1 antibody (Table S1) and then placed on a magnetic for 2 minutes. The resulting pellet was washed twice with PBS then incubated in stain buffer containing anti-rabbit horseradish peroxidase (HRP) (1/200, Perkin Elmer) for 30 minutes. Cells were again washed with PBS before incubating in TSA Plus working solution (TSA plus Cy5 kit, Perkin Elmer) for 5 minutes. The cells were once again washed with PBS and then placed on a magnetic. The resulting pellet was mounted with Fluoromount Gold plus DAPI (ThermoFisher Scientific). Tyramide signal amplification (TSA) was introduced to increase the signal for PD-L1 detection. Slides were visualised and scanned using a Nikon Eclipse Ti-E inverted fluorescent microscope. Images were analysed using the NIS-Elements Analysis software, version 5.21. Examples in Figure S2.

Carcinoma immunocytochemistry assay for PD-L1 expression on cytospun cells

MDA-MB-231 (strong PD-L1 expression), IFN-γ induced MCF-7 (weak PD-L1 expression), MCF7 (negative PD-L1 expression) cell line spikes were analyzed for PD-L1 expression. After collection, cell line spikes were immediately fixed in 4% paraformaldehyde (PFA) for 10 minutes. After that, cells were cytospun using Cytospin™ 4 (Thermo Fisher Scientific) onto glass slides at 2,000 rpm for 5 minutes at medium acceleration. Cells were then dried, and slides stored in a desiccator at 4 ℃ or progressed straight to staining. Cells were incubated in blocking buffer (10% NDS/10% Glycine/5% Human FcR block/3% BSA/0.2% TX in PBS) for 15 minutes before incubating in stain buffer (10% NDS/3% BSA/0.2% TX in PBS) containing pan-cytokeratins, WBCs markers (Table S1) for 1 hour. Following this incubation cells were washed in 1% BSA in PBS followed by washes in PBS. They were then incubated with 2 µL/mL of the nuclei staining dye solution, Hoechst 33342 (Thermo Fisher Scientific), for 15 minutes and finally wash with PBS.

A silicon isolator was immediately placed on the glass slide encircling the area where the cells were located. PBS (200 µL) was added to the space containing the cells, and the cells were immediately visualized and scanned using an inverted fluorescent microscope (Eclipse Ti-E, Nikon®, Japan). Images were analysed using the NIS-Elements High Content Analysis software, version 4.2.

After microscopy, the silicon isolator was removed, slides were washed five times for 5 minutes each in PBS before incubation in freshly prepared 1 mg/mL NaBH4 in PBS solution for 180 minutes, with the NaBH4 in PBS solution being replaced with fresh solution after 90 minutes. Slides were then washed five times for 5 minutes each in PBS before incubating in 100 mM tris solution for 1 hour. Slides were once again washed three times for 5 minutes each in PBS before quenching endogenous peroxidase activity with 0.3% H2O2 for 20 minutes. Slides were incubated in blocking buffer for 15 minutes before incubating in stain buffer containing PD-L1 (clone 28.8, Abcam) and Alexa Fluor 647 labelled anti-vimentin for 1 hour.

Slides were then washed three times with 1% BSA in PBS for 5 minutes each and then incubated in stain buffer containing anti-rabbit HRP (1/200, Perkin Elmer) for 30 minutes. Slides were again washed three times with 1% BSA in PBS for 5 minutes each before incubating in TSA Plus working solution (TSA plus Cy5 kit, Perkin Elmer) for 5 minutes. Finally, slides were once again washed three times with 1% BSA in PBS for 5 minutes each, washed once in PBS for 5 minutes, dried and mounted with Fluoromount Gold plus DAPI (ThermoFisher Scientific) for re-imagining.

MCF7 cells demonstrated strong CK/EpCAM staining and no detectable vimentin staining while MDA-MB-231 cells demonstrated weak CK/EpCAM staining and strong vimentin staining. PD-L1 was expressed at low levels in the IFN-γ induced MCF7 cells and strongly expressed in the MDA-MB-231 cells. All MCF7 cells, both IFN-γ induced and not, and all MDA-MB-231 cells were negative for the WBC markers CD16, CD66b, and CD45. This protocol was applied for the analysis of CTCs enriched using the Parsortix system (Figure S3).

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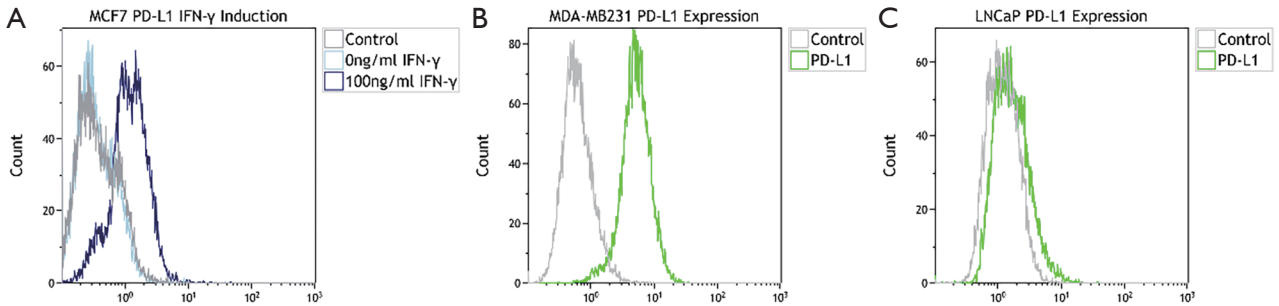

Figure S1 Histogram plots from flow cytometric analysis of PD-L1 expression on MCF7 cells (A), MDA-MB-231 cells (B) and LNCaP cells (C) using the 28.8 antibody diluted 1/100 with AF488 conjugated donkey anti-rabbit secondary antibody diluted 1/500. Cells stained with donkey anti-rabbit secondary antibody, but no primary antibody, were used as controls. MCF7 cells were analysed with and without induction of PD-L1 expression by incubation with IFN-γ for 24 hours.

Table S1 Antibodies used for immunocytochemistry staining

Antibody	Host species	Conjugate	Clone	Antigen location	Supplier (CTLG no.)	Dilution	Use
CD16	Mouse	AF647	3G8	Membrane	BioLegend USA, (302008)	1/50	WBC identification
CD45	Mouse	AF647	HI30	Membrane	BioLegend USA (304018)	1/50	WBC identification
CD66b	Mouse	AF647	G10F5	Membrane	BioLegend USA (305110)	1/100	WBC identification
Cytokeratins	Mouse	FITC	CK3-6H5	Cytoskeleton	Miltenyi Biotech Gladbach, Germany (130-118-964)	1/50	CTC identification
Cytokeratins	Mouse	AF488	C11	Cytoskeleton	Cell Signalling Technology, USA (4523S)	1/100	CTC identification
Cytokeratins	Mouse	AF488	AE1/AE3	Cytoskeleton	ThermoFisher Scientific, USA, (53-9003-80)	1/200	CTC identification
EpCAM	Mouse	PE	VU-1D9	Membrane	ThermoFisher Scientific, USA, (MA1-10197)	1/100	CTC identification
Vimentin	Mouse	AF647	V9	Cytoskeleton	Abcam, USA, (ab195878)	1/1,000	CTC identification
PD-L1	Rabbit	n/a	28.8	Membrane	Abcam, USA, (ab205921)	1/400	PD-L1 expression
HRP	Rabbit	n/a		n/a	Perkin Elmer	1/200	Signal amplification
TSA					TSA plus Cy5 kit, Perkin Elmer	1/50	Signal amplification

AF, Alexa Fluor; WBC, white blood cells; CTC, circulating tumour cell; PE, phycoerythrin; HRP, horseradish peroxidase.

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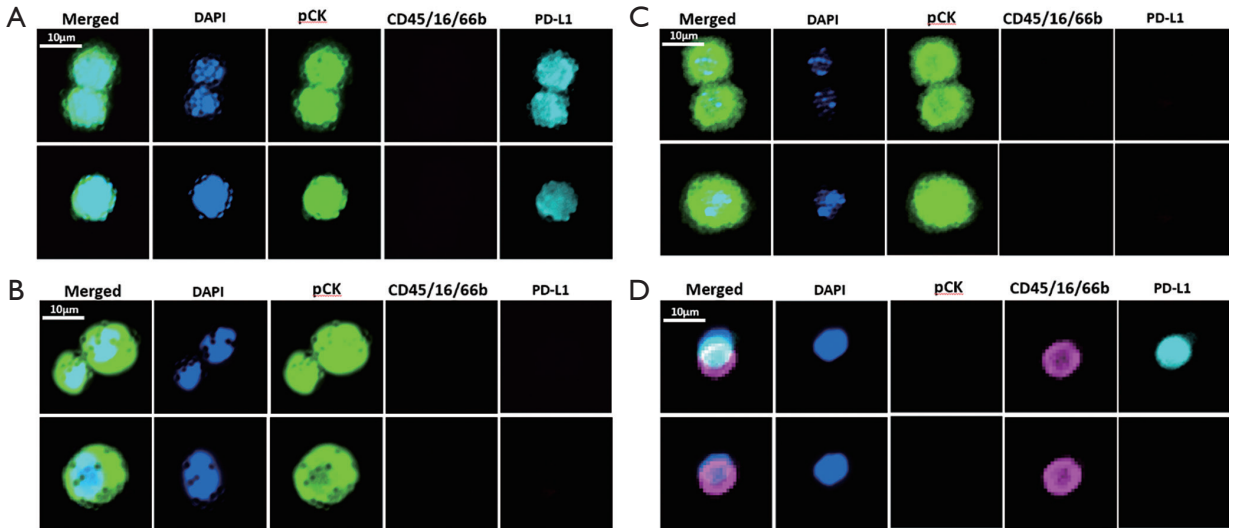

Figure S2 Immunofluorescence staining of beads recovered cells. Representative images of IFN-γ induced MCF7 cells (A), non-induced MCF7 cells (B), LnCaP cells (C) and WBCs (D). Cells were stained with antibodies targeting mixed pan-cytokeratins (pCK, green), AF647 CD45/CD16/CD66b (pink), PD-L1 expression (cyan), DAPI for nuclei staining (blue), Scale bar (top left) represents 10 µm. WBCs, white blood cells.

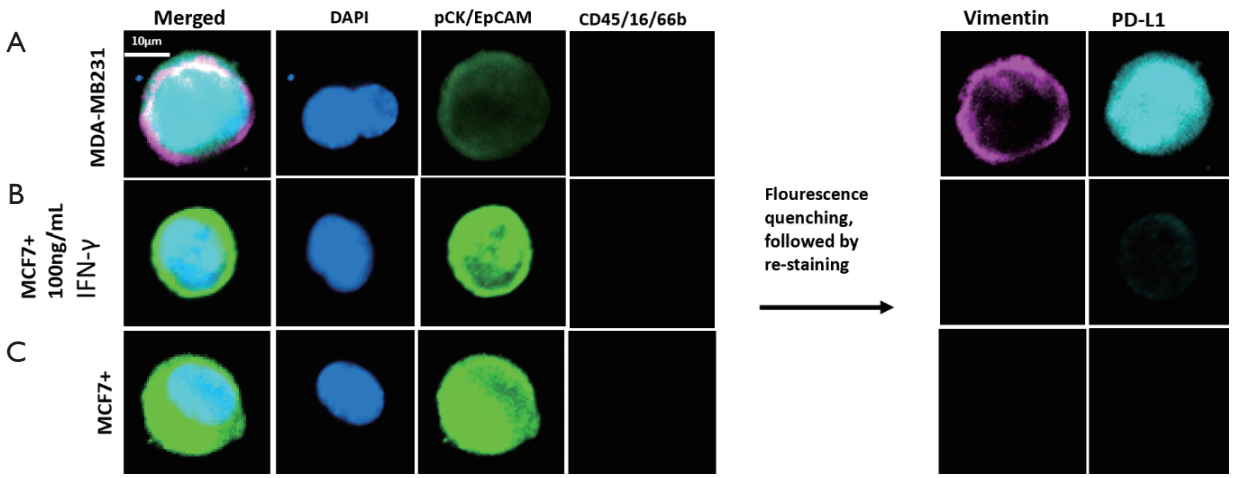

Figure S3 PD-L1 staining controls. Representative images depicting MDA-MB-231 cells (A), IFN-γ induced MCF7 cells (B) and MCF7 cells (C) immune staining with the final carcinoma panel. Cells were stained with FITC/AF488 mixed pan-cytokeratins and EpCAM (green), AF647 CD45, CD16 and CD66b (red), AF647 vimentin (purple) and Cy3 TSA PD-L1 (cyan). Scale bar (top left) represents 10 µm.

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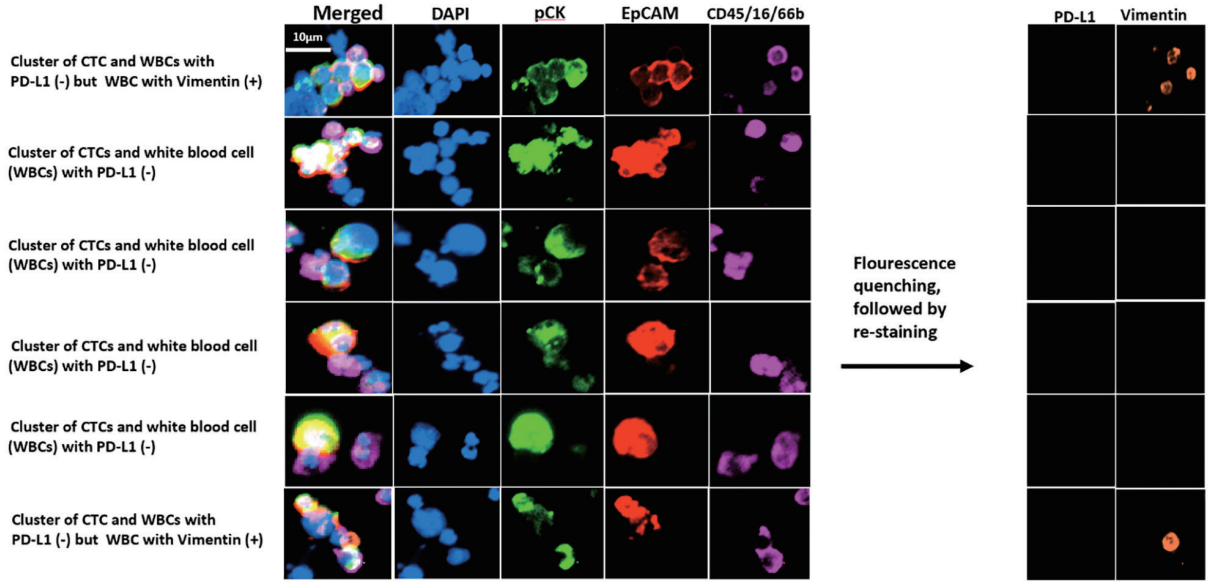

Figure S4 Representative images showing clustered CTC-WBC cells enriched with Parsortix system from SCLC patient blood samples. Cells were stained with FITC/AF488 mixed pan-cytokeratins (green), PE-EpCAM (red), CD45/16/66b AF647 (pink) to identify classical SCLC CTCs, followed by fluorescence quenching and re-immunostained for PD-L1 expression (cyan) and vimentin (orange). Clusters of CTCs and leukocyte, Scale bar (top left) represents 10 μm. CTC, circulating tumour cell; WBCs, white blood cells; SCLC, small-cell lung cancer.

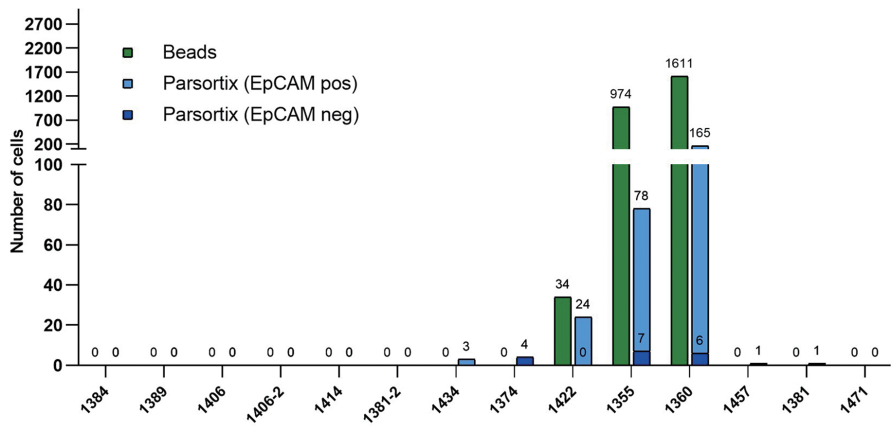

Figure S5 Distribution of EpCAM-positive and negative CTCs enriched by Parsortix. The number of CTCs isolated in matching samples using anti-EpCAM antibody-coated beads were indicated for comparison. CTCs, circulating tumour cells.

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Table S2 Association of CTCs thresholds with clinical characteristics (n=21)

Variables	2-CTCs threshold		50-CTCs threshold	
	<2 CTCs (n=9)	≥2 CTCs (n=12)	<50 CTCs (n=15)	≥50 CTCs (n=6)
Age group (years)				
<67	5 (55.6)	4 (33.3)	6 (40.0)	3 (50.0)
≥67	4 (44.4)	5 (66.7)	9 (60.0)	3 (50.0)
P value	0.284		0.523	
Gender				
Female	6 (66.7)	6 (50.0)	6 (40.0)	3 (50.0)
Male	3 (33.3)	6 (50.0)	9 (60.0)	3 (50.0)
P value	0.377		0.523	
Disease Stage				
Limited	2 (22.2)	0 (0.0)	2 (13.3)	0 (0.0)
Extensive	7 (77.8)	12 (100.0)	13 (86.7)	6 (100.0)
P value	0.171		0.500	
Performance status (ECOG)				
0	3 (33.3)	6 (50.0)	7 (46.7)	2 (33.3)
1	3 (33.3)	5 (41.7)	5 (33.3)	3 (50.0)
≥2	3 (33.3)	1 (8.3)	3 (20.0)	4 (16.7)
P value	0.347		0.773	
Number of metastasis				
1	3 (33.3)	2 (16.7)	4 (26.7)	1 (16.7)
≥2	6 (66.7)	10 (83.3)	11 (73.3)	5 (83.3)
P value	0.353		0.550	
Type of treatment				
Chemotherapy	2 (22.2)	5 (41.7)	4 (26.7)	3 (50.0)
Chemotherapy + ICI	6 (66.7)	7 (58.3)	10 (66.7)	3 (50.0)
Radiation	1 (11.1)	0 (0.0)	1 (6.7)	0 (0.0)
P value	0.373		0.524	

CTCs, circulating tumour cells; ECOG, Eastern Cooperative Oncology Group; ICI, immune checkpoint inhibitor.

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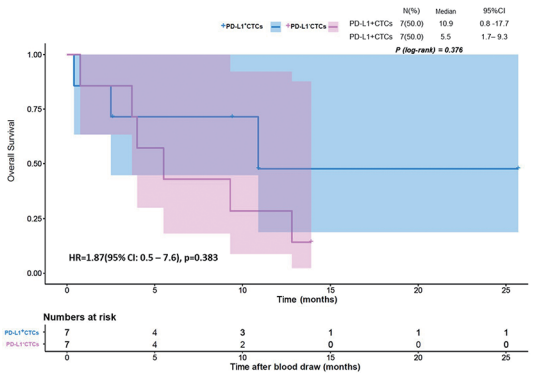

Figure S6 Kaplan-Meier OS curves based on PD-L1 expression on CTCs among SCLC patients. CTCs, circulating tumour cells; CI, confidence interval; HR, hazard ratio; OS, overall survival; SCLC, small-cell lung cancer.

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