Cell

Supplemental Information

Melanoma Cell-Intrinsic PD-1 Receptor Functions Promote Tumor Growth

Sonja Kleffel, Christian Posch, Steven R. Barthel, Hansgeorg Mueller, Christoph Schlapbach, Emmanuella Guenova, Christopher P. Elco, Nayoung Lee, Vikram R. Juneja, Qian Zhan, Christine G. Lian, Rahel Thomi, Wolfram Hoetzenecker, Antonio Cozzio, Reinhard Dummer, Martin C. Mihm Jr. Keith T. Flaherty, Markus H. Frank, George F. Murphy, Arlene H. Sharpe, Thomas S. Kupper, and Tobias Schatton

SUPPLEMENTAL INFORMATION

Supplemental Experimental Procedures

Melanoma cell lines, culture methods, clinical specimens, and tumor cell isolation. Authenticated human and murine melanoma cell lines were obtained from the American Type Culture Collection, or were provided by Dr. E. Frei (Dana-Farber Cancer Institute, Boston, MA), Dr. M. Hendrix (Children's Memorial Research Center, Chicago, IL), or Dr. U. Schumacher (University Hospital Hamburg-Eppendorf, Hamburg, Germany). Cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin (Gibco), as described (Schatton et al., 2008). Clinical melanoma specimens were obtained from patients and human peripheral blood mononuclear cells (PBMCs) from healthy volunteers in accordance with the Institutional Review Boards of Partners Health Care Research Management, the Dana-Farber Cancer Institute, the University of Zurich, Switzerland, and the University of Bern, Switzerland. Informed consent was obtained from all subjects. Single cell suspensions of clinical melanomas were derived from surgical specimens as described previously (Schatton et al., 2008). PD-1^{+}/PD-1^{-} and ABCB5^{+}/ABCB5^{-} melanoma subpopulations were generated using PD-1 and ABCB5 monoclonal antibody labeling followed by flow cytometric or magnetic bead cell sorting, as described (Schatton et al., 2008; Schatton et al., 2010). Human PBMCs were isolated from whole blood samples by Ficoll-Paque density gradient centrifugation, as described (Schatton et al., 2010).

Human Studies. Stage IV melanoma patients with surgical resection or biopsy of melanoma lesions prior to and after systemic anti-PD1 treatment between February 2013 and May 2015 were included in the study. The data set contained 30 observations on metastatic melanoma patients undergoing anti-PD1 targeted therapy at the Department of Dermatology, University

Hospital Zurich, Switzerland and 4 patients at the Massachusetts General Hospital Cancer Center, Boston, MA, USA. Paraffin-embedded tumor tissue was available from all 34 patients prior to anti-PD1 treatment start. From 11 of the patients, matched tumor tissue from progressive lesions during or immediately after anti-PD1 therapy was also available. Melanoma lesions were morphologically identified by experienced dermatopathologists. Additionally, immunohistochemical melanoma markers (MART-1 and S-100) were used. Studies were conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Boards of the University of Zurich (KEK-ZH-Nr. 2014-0320) and the Dana-Farber/Harvard Cancer Center (IRB# 11-181). All patients agreed to the use of their tumor tissues according to the Biobank project 2014-0320ct (EK No. 647), funded by the University of Zurich Research Priority Program (URPP) in translational cancer biology, or DF/HCC IRB approved protocol 11-181.

RNA extraction, RT-PCR and real-time quantitative PCR. Total RNA was isolated from established native and ABCB5-sorted human and murine melanoma cell lines, human melanoma xenografts, murine melanomas, human PBMCs and murine spleens using the RNeasy Plus Mini Kit (Qiagen). Standard cDNA synthesis reactions were carried out using the Superscript III First-Strand Synthesis System for RT-PCR or SuperScript VILO cDNA Synthesis Kits (Invitrogen) and reverse transcribed products were amplified with the Platinum PCR SuperMix High Fidelity Kit (Invitrogen) using the following gene-specific primer pairs: human *PDCD1* (Gene accession no. NM_005018.2), 5'-ATGCAGATCCCACAGGCGCC-3' (forward) and 5'-TCAGAGGGGCCAAGAGCAGTG-3' (reverse) and murine *Pdcd1* (NM_008798.2), 5'-ATGTGGGTCCGGCAGGTACC-3' (forward) and 5'-TCAAAGAGGCCAAGAACAATGTC-3' (reverse). Human GAPDH (5'-ACCACAGTCCATGCCATCAC-3' (forward) and 5'-

TCCACCACCCTGTTGCTGTA-3' (reverse)) and murine Gapdh (5'-ACCACAGTCCATGCCATCAC-3' (forward) and 5'-TCCACCACCCTGTTGCTGTA-3' (reverse)) were used as normalizing controls, respectively. Thermocycling was carried out at 94°C for 2 min, followed by 40 cycles at 94°C for 15 s, 60°C for 20 s and 68°C for 1 min. The 867-base *PDCD1* PCR products were sequenced using the amplification primer pairs, respectively. The full coding sequence (CDS) of *PDCD1* (NM_005018.2) expressed by the human A375, C8161, and G3361 melanoma cell lines and of *Pdcd1* (NM_008798.2) expressed by the murine B16-F0 and B16-F10 lines were submitted to the GenBank database under the following accession numbers: KJ865859 (A375), KJ865860 (C8161), KJ865861 (G3361), KJ865857 (B16-F0), and KJ865858 (B16-F10). The primers for detection of human *PDCD1* by real-time quantitative RT-PCR were: 5'-GACAGCGGCACCTACCTCTGTG-3' (forward) and 5'-GACCCAGACTAGCAGCACCAGG-3' (reverse), for human *PDCD1LG1* (*CD274*) detection 5'-TGCCGACTACAAGCGAATTACTG -3' (forward) and 5'-CTGCTTGTCCAGATGACTTCGG-3' (reverse), for human *PDCD1LG2* detection 5'-CTCGTTCCACATACCTCAAGTCC-3' (forward) and 5'-CTGGAACCTTTAGGATGTGAGTG-3' (reverse), for human 18s rRNA detection 5'-GAT GGGCGGCGGAAAATAG-3' (forward) and 5'-GCGTGGATTCTGCATAATGGT-3' (reverse), for murine *Pdcd1* detection 5'-CGGTTTCAAGGCATGGTCATTGG-3' (forward) and 5'-TCAGAGTGTCGTCCTTGCTTCC-3' (reverse), for murine *Pdcd1lg1 (Cd274*) 5'-TGCGGACTACAAGCGAATCACG-3' (forward) and 5'-CTCAGCTTCTGGATAACCCTCG -3' (reverse), for murine *Pdcd1lg2* 5'- CTGGGACTACAAGTACCTGACG -3' (forward) and 5'-CTCTAGCCTGGCAGGTAAGCTG -3' (reverse), and for murine β-actin detection 5'-CATCGTACTCCTGCTTGCTG-3' (forward) and 5'-AGCGCAAGTACTCTGTGTGG-3'

(reverse). Samples were assayed using the Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA) and kinetic PCR was performed on a StepOne Plus Real-Time PCR System (Applied Biosystems). All samples were run in triplicate. The relative amounts of *PDCD1/Pdcd1*, *PDCD1LG1/Pdcd1lg1* and *PDCD1LG2/Pdcd1lg2* transcripts were analyzed using the $2^{(-\Delta\Delta Ct)}$ method as described previously (Schatton et al., 2008).

Antibodies and biologic reagents. The following monoclonal antibodies (mAbs) were used for flow cytometry: PE-conjugated anti-human PD-1 (MIH4, BD Pharmingen), PD-L1 (29E.2A3), PD-L2 (24F.10C12), CD45 (Beckman Coulter), and respective isotype controls (Biolegend), PerCP-eFluor710-conjugated anti-human PD-1 (MIH4) and isotype control (eBioscience), FITC-conjugated anti-human CD31, PE-conjugated anti-mouse PD-1 (29F.1A12), PD-L1 (10F.9G2), PD-L2 (TY25), CD45.1, FITC-conjugated anti-mouse F4/80, PerCP-conjugated anti-mouse PD-1 (29F.1A12), CD11b, APC-conjugated anti-mouse PD-L1 (10F.9G2), CD11c, CD49b (Biolegend) and secondary Ab (eBioscience), and unconjugated anti-ABCB5 (3C2-1D12, Schatton et al., 2008; Schatton et al., 2010) and isotype control (MOPC-31C, BD Pharmingen). The following antibodies were used for Western blotting: Unconjugated mouse anti-human PD-1 (192106, R&D Systems), goat anti-mouse PD-1 (Abcam) and mouse anti-β-actin (BD Biosciences), IRDye 800CW-conjugated goat anti-mouse and goat anti-rabbit, IRDye 680LT-conjugated donkey anti-rabbit and IRDye 680RD-conjugated donkey anti-goat (LI-COR Biosciences), unconjugated rabbit anti-phospho (p)-S6 ribosomal protein (Ser235/236), -S6 ribosomal protein, -p-AKT (Ser473), -AKT (pan), -p-ERK1/2 (Thr202/Tyr204), -ERK, and HRP-conjugated goat anti-rabbit (Cell Signaling Technology). The following antibodies were used for immunohistochemistry staining: unconjugated goat anti-human PD-1 (AF-1086, R&D Systems), rat anti-mouse PD-1 (RMP1-14, Biolegend), rabbit anti p-S6 (Ser235/236, 91B2) (Cell

Signaling Technology), rabbit anti-mouse (Leica), rabbit anti-rat, biotinylated rabbit anti-goat and mouse anti-rabbit, HRP-conjugated goat-anti-rabbit and rabbit anti-rat (Dako). For immunofluorescence staining, the following antibodies were used: unconjugated goat anti-human PD-1 (AF-1086, R&D Systems), mouse anti-human MART-1 (M7196, Dako), mouse anti-human CD45 (HI30, Biolegend), goat anti-mouse PD-1 and rabbit anti-mouse MART-1 (Abcam), biotinylated rabbit anti-goat (Dako), AF594-conjugated goat anti-mouse, Streptavidin, AF488-conjugated goat anti-mouse, donkey anti-rabbit, and donkey anti-goat (Invitrogen). For *in vitro* and *in vivo* PD-1 blocking experiments, the following mAbs were used: anti-human PD-1 (J116) and MOPC-21 isotype control (BioXcell), anti-mouse PD-1 (29F.1A12), anti-mouse PD-L1 (10F.9G2) and isotype controls (Biolegend). Recombinant human and mouse B7-H1 Fc (PD-L1 Ig) and control Ig chimera were purchased from R&D Systems and Bethyl Laboratories, respectively. Anti-asialo GM1 serum (Wako Pure Chemical Industries), rat anti-mouse Ly-6G mAb (RB6-8C5, BioXcell), and clodronate liposomes (Encapsula Nano Sciences) were used for *in vivo* depletion of NK cells, neutrophils and macrophages, respectively. The pharmacologic mTOR pathway inhibitors, rapamycin and PP242, and the PI3K inhibitors, wortmannin and LY294001, were purchased from SelleckChem, and dimethyl sulfoxide (DMSO) from Sigma.

Flow cytometry. The analysis of PD-1, PD-L1, and PD-L2 cell surface expression by established human and murine melanoma cell lines was performed by single-color flow cytometry, PD-1 and ABCB5 co-expression by established melanoma lines, PD-1, CD31 and CD45 surface expression by patient-derived melanoma single cell suspensions, and assessment of NK cell-, macrophage-, and neutrophil depletion efficiency in the circulation of NSG mice was analyzed by multi-color flow cytometry, as described previously (Schatton et al., 2008; Schatton et al., 2010). Fluorescence emission at the Fl1 (FITC), Fl2- (PE), Fl3- (PerCP and

PerCP-eFluor 710), and/or Fl4 (APC) spectra was acquired on a FacsCanto (Becton Dickinson), as described (Schatton et al., 2008) and analyzed using the FlowJo software (Tree Star). Statistical differences were determined using the Student's t-test. A two-sided *P* value of $P<0.05$ was considered significant.

Western blot analysis. Cell lysates were prepared as described (Posch et al., 2013). Protein concentrations were determined using the BCA protein assay kit (Pierce) according to the manufacturer's protocol. Equal amounts of total protein were resolved by SDS/PAGE and transferred to PVDF membranes, as described (Posch et al., 2013). For detection of human and murine PD-1, membranes were blocked in Odyssey Blocking Buffer (LI-COR Biosciences) overnight, incubated with mouse anti-human PD-1 (2µg/ml), goat anti-mouse PD-1 (1.5µg/ml), or mouse anti-β-actin Ab (1:10,000 dilution) in Odyssey Blocking Buffer/0.1% (v/v) Tween-20 for 1 hr at room temperature. Subsequently, blots were washed in tris-buffered saline (TBS)/0.1% (v/v) Tween-20, stained with IRDye-conjugated secondary Abs (1:10,000 dilution, respectively) in Odyssey Blocking Buffer/0.1% (v/v) Tween-20 for 45 min at room temperature, washed in TBS/0.1% (v/v) Tween-20, and then scanned on an Odyssey CLx imaging system (LI-COR Biosciences). Expression levels of phosphorylated vs. total ERK1/2, AKT and S6 ribosomal protein were determined, as described (Posch et al., 2013), in PD-1 variant or wildtype murine B16, human C8161 and G3361 melanoma cell lines cultured under serum-starved conditions (0.1% (v/v) FBS for 12h) in the presence or absence of anti-PD-1 mAb or isotype control mAb (50µg/ml, respectively) following subsequent incubation with or without recombinant PD-L1 Ig or control Ig (5µg/ml, 0% (v/v) FBS for 15 min, respectively) or following incubation in the presence or absence of rapamycin (100nM), pp242 (50-100nM), wortmannin (50-100nM), LY294001 (500nM), or vehicle control (DMSO) for 30 minutes in 0%

(v/v) FBS, as described (Posch et al., 2013), and subsequent addition of recombinant PD-L1 Ig or control Ig, as above. Subsequently, blots were probed overnight at 4 °C with antibody raised against the protein of interest, incubated with HRP- or IRDye-conjugated secondary Ab for 1h, and developed using enhanced chemoluminescence (Pierce) or analyzed using an Odyssey CLx imaging system, as above.

Immunohistochemistry and immunofluorescence staining. Immunohistochemical analysis of p-S6 expression in clinical tumor biopsies obtained from melanoma patients was done as described previously (Schatton et al., 2008; Schatton et al., 2010). Briefly, 3-5 μm thick tumor biopsy sections were deparaffinized in xylene and subsequently rehydrated with 100%, 95%, and 75% ethanol, and deionized H_2O. Sections were then placed in target retrieval solution (Dako) and boiled in a Pascal pressure chamber (Dako) at 125 °C for 30 seconds, 90 °C for 10 seconds, and then cooled down to room temperature. Subsequently, sections were stained with a 1:200 dilution of rabbit anti-p-S6 antibody for 1 hour at room temperature, following incubation with a 1:100 dilution of biotin-conjugated mouse anti-rabbit IgG for 30 min at room temperature and subsequent incubation with streptavidin-alkaline phosphatase (Roche) for 30 minutes at room temperature. p-S6 immunoreactivity was detected using the FAST Red Chromogen System (Biolegend), per the manufacturer's instructions. Nuclear counterstaining (blue) was performed with Meyer's haemalum. p-S6 immunoreactivity by melanoma cells was graded by three independent investigators blinded to the study outcome on a scale of 0-4 (0: no p-S6 expression by melanoma cells; 1: p-S6 expression in 1-25%; 2: 26-50%; 3: 51-75%; 4: >75% of melanoma cells). For each slide, at least two areas with the highest numbers of S-100$^+$ and MART-1$^+$ melanoma cells, as determined in serial sections, were selected for analysis. Immunohistochemical analysis of PD-1 expression by murine B16 melanomas and human A375,

C8161, and G3361 melanoma xenografts was performed as described (Schatton et al., 2008; Schatton et al., 2010), in formalin-fixed, paraffin-embedded tumors harvested 3 weeks (murine B16 melanomas) or 4-5 weeks (human melanoma xenografts) post tumor cell inoculation. Binding of *in vivo* administered rat anti-mouse PD-1 or mouse anti-human PD-1 mAb to tumor target tissue was visualized by secondary anti-rat or anti-mouse IgG staining of experimental tumors harvested 3 hours post intraperitoneal mAb injection, as described (Schatton et al., 2008). For quantification of *in vivo* tumor-bound antibody, representative images (n=5-10) of B16-F10 melanoma grafts (n=2-3) from wildtype C57BL6, PD-1(-/-) KO C57BL6, and NSG mice were analyzed by FIJI/Image J (NIH). Positive staining with DAB was determined as a fraction of total image area. Relative staining per cell was approximated by dividing the DAB-positive fraction by total nuclei counted as a function of image area to correct for any variability in image composition. MART-1/PD-1 and CD45/PD-1 immunofluorescence (IF) double labeling of clinical melanoma specimens and MART/PD-1 IF double labeling of murine B16 melanoma grafts was carried out as described previously (Schatton et al., 2008; Schatton et al., 2010). Sections were analyzed with a Nikon microscope (DXM 1200F). Images were captured using the NIS-Elements software (BR 2.30, Nikon).

Generation of stable PD-1 and PD-L1 knockdown or PD-1-overexpressing melanoma cell line variants. Short hairpin RNAs (shRNAs) against human PD-1 (*PDCD1*) (NM_005018.2, RNAi Screening Facility, Broad Institute, Boston, MA), murine PD-1 (*Pdcd1*) (NM_008798, Mission shRNA, Sigma), murine PD-L1 (*Cd274*, also known as *Pdcd1lg1*) (NM_021893, Mission shRNA, Sigma) or scrambled shRNA-control (Addgene) were packaged into lentiviral particles by HEK293 EBNA packaging cells co-transfected with the viral packaging plasmids pN8e-GagPolΔ8.1 and pN8e-VSV-G, and viral supernatants were harvested 48-72 hours after

transfection. The target 21mers were (5'-3'): GCCTAGAGAAGTTTCAGGGAA (shRNA-1) and CATTGTCTTTCCTAGCGGAAT (shRNA-2) for human *PDCD1*, GACATGAGGATGGACATTGTT (shRNA-1) and GCTCGTGGTAACAGAGAGAAT (shRNA-2) murine *Pdcd1*, and GCGTTGAAGATACAAGCTCAA for murine *Pdcd1lg1* knockdown. Human *PDCD1*- or murine *Pdcd1* expression vectors were generated by amplification of the full *PDCD1/Pdcd1* CDS (TrueClone, Origene) with the following primer sets (5'-3') recognizing human *PDCD1* or murine *Pdcd1*, respectively: CGACAGATCTGCCACCATGCAGATCCCACAGGCGCC (forward) and TCCGAAGCTTTCAGAGGGGCCAAGAGCAGTG (reverse); and CGACAGATCTGCCACCATGTGGGTCCGGCAGGTACC (forward) and TCCGAAGCTTTCAAAGAGGCCAAGAACAATGTC (reverse). Full-length human *PDCD1* or murine *Pdcd1* were digested and ligated into the retroviral plasmid, pLNCX2 (Clontech), using the Rapid DNA Ligation kit (Roche) according to the manufacturer's protocol. Sequences were validated using the human *PDCD1* or murine *Pdcd1* cloning primers described above. Empty vectors were used as controls. HEK293 EBNA cells co-transfected with pN8e-GagPolΔS and pN8e-VSV-G were utilized to package vectors into retroviral particles. Viral supernatants were harvested as above. Human A375, C8161, G3361, or murine B16-F0 or B16-F10 melanoma cells were infected with filtered lentiviral and/or retroviral supernatant and selected in either 0.75-2μg/ml puromycin (Puromycin Dihydrochloride, Life Technologies) and/or 300-500μg/ml neomycin (G418 sulfate, Life Technologies). The stably transduced melanoma cells were further flow-sorted for > 95% purity of human *PDCD1*-, murine *Pdcd1*- or *Pdcd1lg1* knockdown, human *PDCD1*- or murine *Pdcd1*-overexpressing populations and subsequently utilized in functional experiments. Human *PDCD1*- or murine *Pdcd1* knockdown or overexpression were

confirmed by quantitative RT-PCR and flow cytometry prior to all *in vivo* tumorigenicity studies described below. We also co-transduced *Pdcd1*-overexpressing vs. vector control B16-F10 melanoma cells, generated as above, with *Pdcd1lg1*- or scrambled shRNA-control lentiviral particles, as described above.

Site-directed PD-1 mutagenesis and enforced expression in melanoma cells. To abrogate PD-1 signaling into the melanoma cell, tyrosine residues within the cytoplasmic PD-1 signaling motifs 'immunoreceptor tyrosine-based inhibitory motif' (ITIM) and/or 'immunoreceptor tyrosine-based switch motif' (ITSM) were mutated to phenylalanine using the GENEART Site-Directed Mutagenesis System (Invitrogen) according to the manufacturer's protocol. The following mutagenic primers were used to mutate the human PD-1 (*PDCD1*) ITIM site (Y223F): 5'-GCCACTGGAAATCCAGCTCCCCAAAGTCCACAGAGAACAC-3' (forward) 5'-TCTGTGGACTTTGGGGAGCTGGATTTCCAGTGGCGAGAG-3' (reverse); human *PDCD1* ITSM mutation site (Y248F): 5'-GCTAGGAAAGACAATGGTGGCAAACTCCGTCTGCTCAGGG-3' (forward), and 5'-CAGACGGAGTTTGCCACCATTGTCTTTCCTAGCGGAATGG-3' (reverse). Murine PD-1 (*Pdcd1*) ITIM mutation site (Y225F): 5'-CGTCCCTGGAAGTCCAGCTCCTCAAAGGCCACACTAGGGAC-3' (forward) and 5'-CCCTAGTGTGGCCTTTGAGGAGCTGGACTTCCAGGGACGAGAG-3' (reverse); murine *Pdcd1* ITSM mutation site (Y248F): 5'-CCTTCAGTGAAGACAATGGTGGCAAATTCTGTGTGCACACAGG-3' (forward) and 5'-GTGCACACAGAATTTGCCACCATTGTCTTCACTGAAGGGCTGG-3' (reverse). Fidelity of vectors was validated by bidirectional sequencing using the human *PDCD1* or murine *Pdcd1* cloning primers described above. Mutant human *PDCD1* or murine *Pdcd1* variants were

packaged into retroviral particles as described above and used to infect human A375 or C8161, or murine B16-F0 or B16-F10 melanoma cell lines. Transduced cell lines were selected as above and flow-sorted for > 95% purity of mutated human or murine PD-1 overexpressing populations. Wildtype or mutant *PDCD1* overexpression was confirmed by quantitative RT-PCR and flow cytometry for all cell lines prior to the *in vivo* tumorigenicity studies described below.

Three-dimensional melanoma culture. Melanoma tumor sphere cultures were established, as described previously (Aceto et al., 2012; Civenni et al., 2011), in standard culture medium, as above, without exogenous growth factors. Briefly, native, PD-1 (*PDCD1/Pdcd1*) KD, wildtype or mutant *PDCD1/Pdcd1*-OE murine B16, human A375, C8161, or G3361 melanoma cell line variants were plated in the presence or absence of anti-mouse PD-1, anti-human PD-1, or isotype control mAb (50μg/ml, respectively), recombinant mouse PD-L1 Ig, human PD-L1 Ig, or control Ig (5μg/ml, respectively) in 6-well ultra-low attachment plates (Corning) at a density of 1,000-10,000 viable cells per well in culture medium, as above, supplemented with 0.5% (wt/v) methyl cellulose (Sigma-Aldrich), and cultured for 7-14 days at 37 °C, 5% CO_2. To ensure plating of single, viable cells, melanoma cultures were harvested with 0.1% (v/v) versene solution (Gibco), passed through a 40 μm nylon mesh, followed by AO/PI (Nexcelom) counterstaining and automated live/dead nucleated cell counting on a Cellometer Auto 2000 cell viability counter (Nexcelom). Tumor spheroid cultures were fed every three days with 0.5 ml of fresh medium with or without anti-PD-1 or isotype control mAbs, recombinant PD-L1 or control Ig in concentrations as above. Spheres were stained with 180 μl of 0.4% (wt/v) p-iodonitrotetrazolium violet solution (Sigma-Aldrich) overnight at 37 °C, 5% CO_2 and photographed using a Canon T1i camera and Sigma 50mm/f2.8 macro lens. Subsequently, numbers of tumor spheres per well

from $n \geq 3$ independent experiments were quantified digitally using the ImageJ software (National Institutes of Health).

Animals. C57BL/6, Rag(-/-) knockout (KO) C57BL/6 (Rag) and nonobese diabetic/severe combined immunodeficiency (NOD/SCID) interleukin (IL)-2Rγ(-/-) KO (NSG) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). PD-1(-/-) KO C57BL/6 and PD-L1(-/-) KO Rag mice were generated and maintained in our animal facility (Francisco et al., 2009; Latchman et al., 2001). Age- and sex-matched mice that were at least 6 weeks of age were used for all experiments. All mice were used according to the Harvard Medical School Standing Committee on Animals and National Institutes of Animal Healthcare Guidelines. Animal protocols were approved by the Harvard Medical School Standing Committee on Animals.

Murine melanoma induction and human melanoma xenotransplantation. Stable PD-1 (*Pdcd1*)- or PD-L1 (*Cd274*, also known as *Pdcd1lg1*) KD, sorted PD-1^+ or PD-1^-, wildtype *Pdcd1*-overexpressing (OE) with or without concurrent *Pdcd1lg1* KD, ITIM-, ITSM-mutant, or ITIM/ITSM double mutant *Pdcd1*-OE, murine B16-F0 or B16-F10 or their respective control cell line variants were injected subcutaneously (2×10^5 cells/inoculum) into the flanks of recipient wildtype C57BL/6, NSG, Rag, PD-1(-/-) C57BL/6, and/or PD-L1(-/-) KO Rag mice. Stable *PDCD1*-KD, sorted PD-1^+ or PD-1^-, wildtype *PDCD1*-OE, ITIM- or ITSM-mutant, or ITIM/ITSM double mutant *PDCD1*-OE human A375, C8161, and/or G3361 or their respective control cell line variants were injected subcutaneously (1×10^6 cells/ inoculum) into the flanks of recipient NSG mice, as described (Schatton et al., 2008). Age- and sex-matched recipient mice were randomly assigned to experimental groups. Tumor formation/growth was assessed every 2-4 days as a time course until the experimental endpoint, and tumor volume was calculated as described (Schatton et al., 2008). Tumors were harvested in their entirety 3 weeks (murine B16

melanomas) or 4-5 weeks (human melanoma xenografts) after tumor cell inoculation for histologic and/or qPCR analysis, unless excessive tumor size or disease state required protocol-stipulated euthanasia earlier. Mice that required euthanasia before the experimental endpoint were excluded from tumor growth analysis. These exclusion criteria were pre-established. While the investigators assessing tumor growth were not blinded, all pathologists and laboratory personnel who performed histologic or qPCR characterizations of tumor specimens were blinded to the group allocation. Sample sizes were chosen to ensure statistical power of detection based on projected outcomes. Differences in tumor volume were statistically assessed using the unpaired Student's *t*-test, the nonparametric Mann-Whitney test, or repeated measures two-way ANOVA followed by the Bonferroni correction with two-tailed *P* values <0.05 considered significant (Schatton et al., 2008).

Tumorigenicity experiments with anti-PD-1 and anti-PD-L1 blocking antibodies. For PD-1 and PD-L1 targeting experiments, murine B16–F10 melanoma cells or *Pdcd1*-OE vs. control B16-F10 melanoma variants were grafted subcutaneously into wildtype vs. PD-1(-/-) KO C57BL/6, wildtype vs. PD-L1(-/-) KO Rag, wildtype and innate immune cell-depleted NSG mice, as described above. Human A375, C8161 or G3361 melanoma cells, *PDCD1*-OE vs. control C8161 melanoma variants, or single cell suspensions of clinical melanoma metastases derived from $n=3$ distinct melanoma patients were grafted subcutaneously into the flanks of recipient NSG mice, as described above. Mice were randomly assigned to experimental treatment groups and sample sizes chosen to ensure statistical power of detection based on projected outcomes. Animals were injected intraperitoneally (200μg per injection, respectively) with anti-mouse PD-1, PD-L1, or anti-human PD-1 mAb vs. respective isotype control mAb every other day starting 1 day before melanoma cell inoculation for the duration of 3 weeks

(murine melanomas) or 4-5 weeks (human melanoma xenografts). Tumor formation/growth was assayed as described above, until the experimental endpoint of 3-5 weeks, or when excessive tumor burden or disease state required protocol-stipulated euthanasia earlier. Mice that required euthanasia before the experimental endpoint were excluded from tumor growth analysis. These exclusion criteria were pre-established. While the investigators assessing tumor growth were not blinded, all pathologists and laboratory personel who performed histologic or qPCR characterizations of tumor specimens were blinded to the group allocation. Differences in tumor volume were statistically assessed using the unpaired Student's *t*-test test or repeated measures two-way ANOVA followed by the Bonferroni correction, with two-tailed *P* values <0.05 considered significant.

Assessment of PD-1 antibody titer by ELISA. To determine the concentration of PD-1 antibody in the serum of wildtype C57BL/6, PD-1(-/-) KO C57BL/6, and NSG mice, animals were grafted with B16-F10 melanoma cells and treated with 200μg of anti-PD-1 antibody per 20g of body weight. Two weeks post melanoma cell inoculation and 12 hours post final administration of the PD-1 antibody, mouse blood was collected by cardiac puncture and serum prepared using serum separator tubes (BD Biosciences). Serum obtained from C57BL/6, PD-1(-/-) KO C57BL/6, and NSG mice that had not been treated with anti-PD-1 antibody was used as a negative control. Subsequently, the serum concentration of rat anti-mouse PD-1 antibody was measured using a rat IgG2a-specific enzyme-linked immunosorbent assay (ELISA) kit (Bethyl Laboratories), per the manufacturers instructions.

Innate immune cell depletion. NK cell depletion was achieved by i.p. injection of 50 μL undiluted anti-asialo GM1 antibodies every 5 days, as described (Civenni et al., 2011), starting 3 days before melanoma cell engraftment, which resulted in >80% depletion of $CD3^-$

$CD45.1^+CD49b(DX5)^+$ NK cells in the circulation of NSG mice for the duration of the experiment, as determined by triple-color flow cytometry. Macrophages were depleted by treating NSG mice with clodronate (dichloromethylene bisphosphonate) liposomes (200 µL of undiluted stock solution every 5 days, starting 3 days before melanoma cell engraftment) (Huang et al., 2009), resulting in >85% depletion of $CD45.1^+CD11b^+F4/80^+$ macrophages compared to untreated control mice. NSG mice were rendered neutropenic, as described (Jaeger et al., 2012), by i.p. injection of 100µg anti-Ly-6G mAb on days -3, -1, 4, 9, 14, and 19 post tumor cell inoculation, resulting in >90% depletion of $CD45.1^+CD11b^+CD11c^-$ neutrophils in NSG mice. NK cell-, macrophage- and neutrophil depletion regimens, as above, were also used in combination to generate NSG mice lacking all three innate immune cell subtypes.

Cell viability measurements. To assess the effects of melanoma-specific *Pdcd1/PDCD1*-KD, *Pdcd1/PDCD1* overexpression, or anti-PD-1 mAb blockade on cell viability *in vitro*, melanoma cells were cultured in the presence of anti-PD-1 mAb or isotype control mAb, as above, for 48 hours. Cell death was quantified by annexin V-Alexa Fluor 647 (Biolegend) / 7-amino-actinomycin D (7-AAD) (BD Biosciences) staining and subsequent flow cytometric analysis as described (Schatton et al., 2010), and/or by AO/PI (Nexcelom) staining and automated live/dead nucleated cell counting on a Cellometer Auto 2000 cell viability counter (Nexcelom). No significant differences in cell viability were found between anti-PD-1 antibody vs. isotype control antibody, *PDCD1*/*Pdcd1*-KD vs. shRNA control-, or *PDCD1*/*Pdcd1*-OE vs. vector control-transduced melanoma cells.

Statistical analysis. Statistical differences between gene and protein expression levels, tumor spheroid and *in vivo* melanoma growth were compared statistically using the unpaired Student's *t* test or the nonparametric Mann-Whitney test (comparison of two experimental groups) or

repeated measures two-way ANOVA followed by the Bonferroni correction (comparison of three or more experimental groups). Kaplan-Meier estimates and the log-rank test were used to analyze statistical differences in progression-free and overall survival between melanoma patients treated systemic anti-PD-1 antibody-based therapy whose pre-treatment tumor biopsies showed low (<25%) vs. high (>25%) melanoma cell expression of p-S6. The corresponding hazard ratio was estimated using the Cox proportional hazards model. Progression-free survival was defined as the time from the first administration of anti-PD1-based therapy to the first documented radiographic evidence of progressive disease. Overall survival was defined as the time from the first administration of anti-PD1-based therapy to the date of death, regardless of cause. Differences in p-S6 expression in patient-matched tumor biospecimens obtained before and after PD-1 therapy were statistically compared using the paired Student's *t* test. Data was tested for normal distribution using the D'Agostino and Pearson omnibus normality test. Statistical analyses were performed using the R programming language and Origin Pro 9.1G software (OriginLab) or the Prism software (GraphPad Software, Inc.). A two-sided value of $P<0.05$ was considered statistically significant.

Supplemental Tables

Table S1. Melanoma patient biopsies analyzed for melanoma-PD-1 expression. Related to Figure 1.

Patient number	Gender	Age	Tumor site	Melanoma-PD-1 expression[a]
1	F	68	Cutaneous primary	++
2	F	31	Cutaneous primary	n.d.
3	F	91	Cutaneous metastasis	++
4	F	79	Cutaneous primary	+
5a	M	35	Cutaneous metastasis	n.d.
5b			Brain metastasis	n.d.
6a	M	55	Subcutaneous metastasis	+
6b			Cutaneous metastasis	n.d.
7	M	77	Cutaneous metastasis	n.d.
8	F	67	Cutaneous metastasis	+
9	M	49	Cutaneous metastasis	+
10a	M	47	Lymph node metastasis	n.d.
10b			Lymph node metastasis	+
11	M	71	Brain metastasis	+
12a	M	56	Cutaneous metastasis	n.d.
12b			Lymph node metastasis	n.d.
13	F	59	Cutaneous metastasis	n.d.
14	M	74	Pulmonary metastasis	n.d.
15	F	52	Subcutaneous metastasis	+
16	M	68	Cutaneous metastasis	+++
17	F	54	Lymph node metastasis	+
18	M	57	Subcutaneous metastasis	+
19a	M	59	Cutaneous metastasis	+
19b			Cutaneous metastasis	n.d.
20	F	52	Subcutaneous metastasis	+
21a	M	82	Cutaneous metastasis	+
21b			Cutaneous metastasis	n.d.
22	F	75	Cutaneous metastasis	+
23	F	78	Cutaneous metastasis	n.d.
24	M	42	Subcutaneous metastasis	n.d.
25a	M	77	Subcutaneous metastasis	n.d.
25b			Lymph node metastasis	n.d.
26	M	65	Abdominal metastasis	+
27a	F	75	Subcutaneous metastasis	n.d.
27b			Subcutaneous metastasis	+
28	M	88	Subcutaneous metastasis	+
29a	M	55	Subcutaneous metastasis	n.d.
29b			Subcutaneous metastasis	n.d.
29c			Subcutaneous metastasis	n.d.
30	F	56	Subcutaneous metastasis	+++
31	M	49	Subcutaneous metastasis	n.d.
32a	M	72	Subcutaneous metastasis	n.d.
32b			Subcutaneous metastasis	+
33	M	51	Lymph node metastasis	n.d.
34a	F	40	Subcutaneous metastasis	n.d.
34b			Subcutaneous metastasis	n.d.
35	F	36	Subcutaneous metastasis	n.d.
36a	F	69	Subcutaneous metastasis	n.d.
36b			Subcutaneous metastasis	++
36c			Adrenal metastasis	n.d.

[a]Melanoma-PD-1 expression was determined by immunofluorescence double labeling and defined as PD-1(+)CD45(-) and/or PD-1(+)MART-1(+); (+, 1-10%; ++, 10-25%; +++, >25%; n.d., not detected).

Table S2. Correlation of progression-free and overall survival with p-S6 expression in tumor biospecimens obtained from melanoma patients before initiation of anti-PD-1 antibody therapy. Related to Figure 7.

	Group	Cases	Events[a]	Mean time to progression or death [months (95% CI[b])]	Log-rank test statistic	Log-rank test *P*-value
Time to progression (PFS)[c]	p-S6 <25%	14	13	4.5 (2-7)	10.74, df[e]=1	0.00105
	p-S6 >25%	20	11	17.0 (11-23)		
Time to death (OS)[d]	p-S6 <25%	14	8	13.0 (8-18)	3.99, df=1	0.04586
	p-S6 >25%	20	5	25.1 (20-30)		

[a]Disease progression or death; [b]confidence interval; [c]progression-free survival; [d]overall survival; [e]degree of freedom.

Supplemental References

Huang, X., Venet, F., Wang, Y.L., Lepape, A., Yuan, Z., Chen, Y., Swan, R., Kherouf, H., Monneret, G., Chung, C.S., *et al.* (2009). PD-1 expression by macrophages plays a pathologic role in altering microbial clearance and the innate inflammatory response to sepsis. Proc. Natl. Acad. Sci. *106*, 6303-6308.

Jaeger, B.N., Donadieu, J., Cognet, C., Bernat, C., Ordonez-Rueda, D., Barlogis, V., Mahlaoui, N., Fenis, A., Narni-Mancinelli, E., Beaupain, B., *et al.* (2012). Neutrophil depletion impairs natural killer cell maturation, function, and homeostasis. J. Exp. Med. *209*, 565-580.

Latchman, Y., Wood, C.R., Chernova, T., Chaudhary, D., Borde, M., Chernova, I., Iwai, Y., Long, A.J., Brown, J.A., Nunes, R., *et al.* (2001). PD-L2 is a second ligand for PD-1 and inhibits T cell activation. Nat. Immunol. *2*, 261-268.