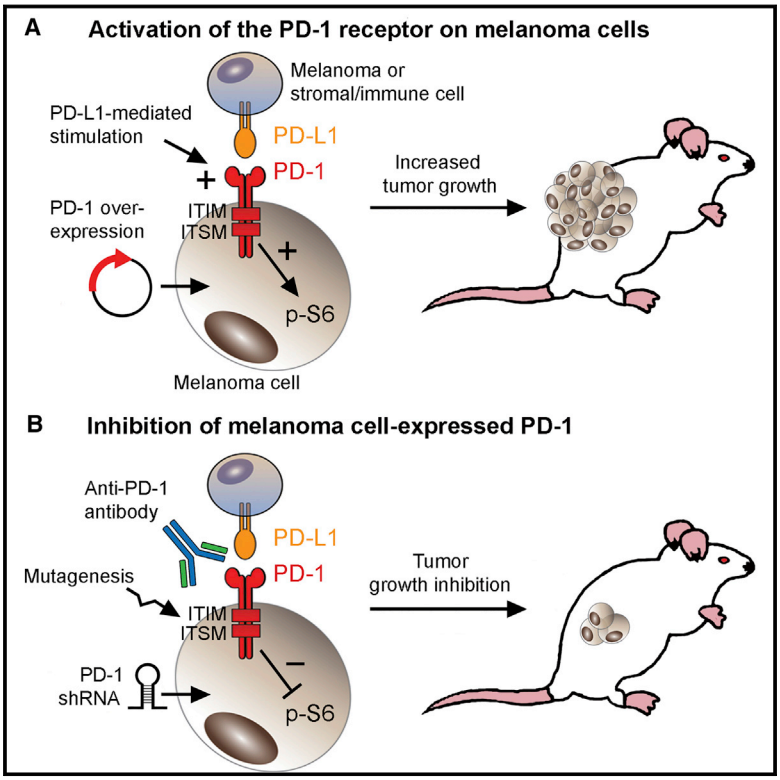
Article

Cell

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

Graphical Abstract



Highlights

- Human melanomas frequently contain PD-1-expressing cancer cell subpopulations
- Inhibition of melanoma-PD-1 reduces tumor growth, independently of adaptive immunity
- PD-1 overexpression and melanoma-PD-1:PD-L1 interactions promote tumor growth
- Activation of the melanoma-PD-1 receptor modulates downstream mTOR signaling

Authors

Sonja Kleffel, Christian Posch, Steven R. Barthel, ..., Arlene H. Sharpe, Thomas S. Kupper, Tobias Schatton

Correspondence

tschatton@bwh.harvard.edu

In Brief

PD-1/PD-L1 signaling has cell-intrinsic functions in certain types of mouse and human tumors, boosting cancer growth and promoting tumorigenesis. This suggests that immunotherapy with PD-1 blockers may produce an effect on tumor growth that is separate from their effect on the immune response.

Kleffel et al., 2015, Cell *162*, 1242–1256
September 10, 2015
http://dx.doi.org/10.1016/j.cell.2015.08.052

CellPress

Cell

Article

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

Sonja Kleffel,[1] Christian Posch,[1,2] Steven R. Barthel,[1] Hansgeorg Mueller,[1,3] Christoph Schlapbach,[4] Emmanuella Guenova,[5] Christopher P. Elco,[1,6] Nayoung Lee,[1] Vikram R. Juneja,[7] Qian Zhan,[6] Christine G. Lian,[6] Rahel Thomi,[4] Wolfram Hoetzenecker,[5] Antonio Cozzio,[5] Reinhard Dummer,[5] Martin C. Mihm, Jr.,[1] Keith T. Flaherty,[8] Markus H. Frank,[1,9,10] George F. Murphy,[6] Arlene H. Sharpe,[6,7,11] Thomas S. Kupper,[1] and Tobias Schatton[1,9,*]

[1]Harvard Skin Disease Research Center, Department of Dermatology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA

[2]Department of Dermatology, The Rudolfstiftung Hospital, 1030 Vienna, Austria

[3]Department of Dermatology, Innsbruck Medical University, 6020 Innsbruck, Austria

[4]Department of Dermatology, University of Bern, 3010 Bern, Switzerland

[5]Department of Dermatology, University Hospital Zurich, 8091 Zurich, Switzerland

[6]Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA

[7]Department of Microbiology and Immunobiology, Harvard Medical School, Boston, MA 02115, USA

[8]Division of Medical Oncology, Massachusetts General Hospital Cancer Center, Harvard Medical School, Boston, MA 02114, USA

[9]Department of Medicine, Children's Hospital Boston, Harvard Medical School, Boston, MA 02115, USA

[10]School of Medical Sciences, Edith Cowan University, Joondalup, WA 6027, Australia

[11]Evergrande Center for Immunologic Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA

*Correspondence: tschatton@bwh.harvard.edu

http://dx.doi.org/10.1016/j.cell.2015.08.052

SUMMARY

Therapeutic antibodies targeting programmed cell death 1 (PD-1) activate tumor-specific immunity and have shown remarkable efficacy in the treatment of melanoma. Yet, little is known about tumor cell-intrinsic PD-1 pathway effects. Here, we show that murine and human melanomas contain PD-1-expressing cancer subpopulations and demonstrate that melanoma cell-intrinsic PD-1 promotes tumorigenesis, even in mice lacking adaptive immunity. PD-1 inhibition on melanoma cells by RNAi, blocking antibodies, or mutagenesis of melanoma-PD-1 signaling motifs suppresses tumor growth in immunocompetent, immunocompromised, and PD-1-deficient tumor graft recipient mice. Conversely, melanoma-specific PD-1 overexpression enhances tumorigenicity, as does engagement of melanoma-PD-1 by its ligand, PD-L1, whereas melanoma-PD-L1 inhibition or knockout of host-PD-L1 attenuate growth of PD-1-positive melanomas. Mechanistically, the melanoma-PD-1 receptor modulates downstream effectors of mTOR signaling. Our results identify melanoma cell-intrinsic functions of the PD-1:PD-L1 axis in tumor growth and suggest that blocking melanoma-PD-1 might contribute to the striking clinical efficacy of anti-PD-1 therapy.

INTRODUCTION

Immune checkpoints are crucial regulatory pathways that maintain immune homeostasis by modulating the amplitude and quality of several adaptive and innate effector mechanisms in favor of immunogenic tolerance (Pardoll, 2012). Using various strategies, such as triggering functional exhaustion of tumor-reactive cytotoxic T-lymphocytes (CTLs), cancers exploit immune checkpoints to evade antitumor immunity. Programmed cell death 1 (PD-1) is a prominent checkpoint receptor that, upon engagement by its ligands, PD-L1 (also known as B7-H1) or PD-L2 (also known as B7-DC), dampens T effector functions by inhibiting signaling downstream of the T cell receptor (TCR) (Topalian et al., 2012a). Thus, expression of PD-1 ligands, and particularly PD-L1, in the tumor microenvironment (TME) protects cancers from immune-mediated rejection (Dong et al., 2002; Topalian et al., 2012a). Consequently, a number of antibody-based therapeutics targeting the PD-1:PD-L1 axis have entered clinical development or have been approved for melanoma therapy (Postow et al., 2015).

In phase I trials (Hamid et al., 2013; Herbst et al., 2014; Topalian et al., 2012b; Wolchok et al., 2013), PD-1 pathway blockade demonstrated unprecedented response rates and encouraging toxicity profiles in patients with advanced-stage cancers of various etiologies, including malignant melanoma. On the basis of recent phase III data demonstrating improved overall survival in melanoma patients receiving PD-1 inhibitors compared to those treated with chemotherapy, the FDA approved two anti-PD-1 antibodies, nivolumab and pembrolizumab, for the treatment of patients with advanced melanoma who are no longer responding to other drugs (Postow et al., 2015; Weber et al., 2015). PD-L1 expression by cancer cells and tumor-infiltrating lymphocytes (TILs) (Herbst et al., 2014; Topalian et al., 2012b; Tumeh et al., 2014), the presence of type 1 T-helper cell (Th1)-associated inflammatory mediators (Herbst et al., 2014; Tumeh et al., 2014), increased density and proliferation and decreased diversity in antigen specificity of $CD8^+$ T cells (Tumeh et al., 2014), and the frequency of tumor-associated neo-antigens within the TME (Gubin et al., 2014; Rizvi et al., 2015; Yadav et al., 2014) are associated with clinical response

to PD-1 pathway interference. These findings established that optimal anti-PD-1 cancer therapeutic efficacy requires the activation and expansion of tumor-specific T cell immunity.

However, in addition to benefiting patients afflicted with immunogenic cancers, such as malignant melanoma (Hamid et al., 2013; Herbst et al., 2014; Topalian et al., 2012b; Wolchok et al., 2013), PD-1 pathway blockade has also yielded meaningful clinical activity in patients with lesser immunogenic cancers that have hitherto not typically responded to immunotherapy (Herbst et al., 2014; Topalian et al., 2012b). Moreover, patients with advanced melanoma refractory to treatment with ipilimumab, an FDA-approved antibody targeting the immune checkpoint protein, cytotoxic T-lymphocyte antigen (CTLA)-4, showed marked clinical response to anti-PD-1 therapy (Hamid et al., 2013; Weber et al., 2015; Wolchok et al., 2013). While the presence of neoantigens and an immune-active TME are similarly associated with favorable outcome in melanoma patients treated with either PD-1- (Gubin et al., 2014; Rizvi et al., 2015; Yadav et al., 2014) or CTLA-4-directed checkpoint blockade (Snyder et al., 2014), current evidence suggests that PD-1 inhibitors produce greater anticancer activity and fewer immune-related adverse events than ipilimumab (Postow et al., 2015). Taken together, these observations raise the possibility that anti-PD-1 therapy, in addition to deregulating T-cell-specific immune checkpoint functions, may also inhibit complementary protumorigenic mechanisms, thereby contributing to its superior clinical efficacy compared to CTLA-4 blockade. Because PD-1 is not only expressed by immune cells, but also by melanoma subpopulations with enhanced tumorigenicity, even in highly immunocompromised tumor xenograft recipient mice (Schatton et al., 2010), we hypothesized that the growth-suppressive effects of PD-1 therapy might also partially result from the direct inhibition of this protein on melanoma cells.

Here, we report that established human and murine melanoma cell lines as well as clinical melanomas frequently contain PD-1-expressing cancer subpopulations and that enforced melanoma-PD-1 expression enhances melanoma growth, even in the absence of adaptive immunity. Conversely, antibody-mediated melanoma-PD-1 blockade and melanoma-specific PD-1 knockdown, as well as mutagenesis of melanoma-PD-1 signaling motifs inhibit tumor growth independently of adaptive immunity. Efficient melanoma-PD-1-driven tumorigenesis requires melanoma-PD-1 interactions with its predominant ligand, PD-L1, which activate effectors of the mTOR signaling pathway downstream of the melanoma-PD-1 receptor. Our results expand our current understanding of PD-1 pathway functions in melanoma and suggest that cancer cell-intrinsic PD-1 targeting might significantly contribute to the therapeutic efficacy of PD-1 antibodies, rendering PD-1 inhibition, in conjunction with its demonstrated effect on immune checkpoint blockade, superior to alternative therapies that target immune checkpoints alone.

RESULTS

Melanomas Frequently Contain PD-1-Expressing Cancer Subpopulations

We first examined PD-1 expression in a series of melanoma patient samples and established melanoma cell lines to further expand upon the potential clinical significance of our previous demonstration that melanoma cells can express PD-1 (Schatton et al., 2010). Flow cytometric analysis of single-cell suspensions derived from clinical tumor specimens (n = 8 patients) revealed PD-1 surface protein expression by melanoma subpopulations negative for the pan-lymphocyte marker, CD45, and the endothelial marker, CD31, in 8/8 melanoma specimens examined, with tumor cell frequencies ranging from 3.5% to 16.5% (cell frequency 8.7% ± 1.5%, mean ± SEM, Figure 1A and Figure S1A). Immunofluorescence double labeling of clinical melanoma biopsies (n = 50) for PD-1 and the melanoma antigen recognized by T cells (MART)-1 further confirmed PD-1 protein expression by subpopulations of MART-1$^+$ melanoma cells that were cytologically distinct from CD45$^+$ lymphocytes (Figure 1B), with n = 22/36 melanoma patients demonstrating melanoma-PD-1 positivity in at least one of their tumor lesions (Table S1).

Based on our intention to mechanistically dissect the role of melanoma-expressed PD-1 in experimental tumor growth, we next characterized PD-1 expression in established human and murine melanoma cell lines. RT-PCR amplification and sequencing of the full coding sequence (CDS) of the human PD-1 (*PDCD1*) gene revealed *PDCD1* mRNA expression (Figure 1C), and immunoblot analysis demonstrated PD-1 protein expression by human A375, C8161, and G3361 melanoma cells (Figure 1D). Flow cytometric analyses showed PD-1 surface protein expression by 8/8 melanoma lines tested, with PD-1$^+$ tumor cell frequencies ranging from 11.3% ± 1.2% to 29.5% ± 3.7% (mean ± SEM, Figure 1E), and revealed preferential PD-1 expression by melanoma cell subsets positive for the tumor-initiating cell determinant (Schatton et al., 2008), ABCB5 (Figures S2A–S2C), consistent with our previous demonstration of PD-1 expression by melanoma-initiating cells (Schatton et al., 2010). Human melanoma lines also demonstrated positivity for both PD-1 ligands, PD-L1 and PD-L2, ranging from 2.4% ± 0.1% to 99.2% ± 0.1% and 0.6% ± 0.1% to 88.9% ± 2.6% of cells (mean ± SEM), respectively (Figure S1B), and PD-1 co-expression with its ligands (not shown). Murine B16-F0 and B16-F10 cultures also expressed both PD-1 (*Pdcd1*) mRNA, as determined by amplification and sequencing of the full *Pdcd1* CDS (Figure 1F), and PD-1 protein as determined by immunoblotting (Figure 1G). Flow cytometric analysis revealed PD-1 (cell frequency 9.4% ± 2.5% and 6.6% ± 2.4%, mean ± SEM, Figure 1H) and PD-L1 (43.4% ± 9.4% and 37.5% ± 2.3%), but not PD-L2 surface protein expression by B16-F0 and B16-F10 melanoma cells (Figure S1C). B16 melanoma grafts grown in non-obese diabetic severely combined immunodeficient (NOD/SCID) interleukin-2 receptor (IL-2R) γ-chain(−/−) null (NSG) mice lacking adaptive immunity also demonstrated PD-1 expression by MART-1$^+$ melanoma cells (Figure 1I).

Melanoma-Expressed PD-1 Promotes Murine Tumor Growth

To functionally dissect the potential role of melanoma-expressed PD-1 in tumor growth, we generated stable *Pdcd1* knockdown (KD) and *Pdcd1*-overexpressing (OE) B16 melanoma lines. Transduction of B16-F0 and B16-F10 cells with two distinct short hairpin (sh) RNAs targeting *Pdcd1* inhibited murine PD-1 mRNA expression by ≥59% and significantly blocked PD-1 protein expression compared to controls (Figure 2A), but did not

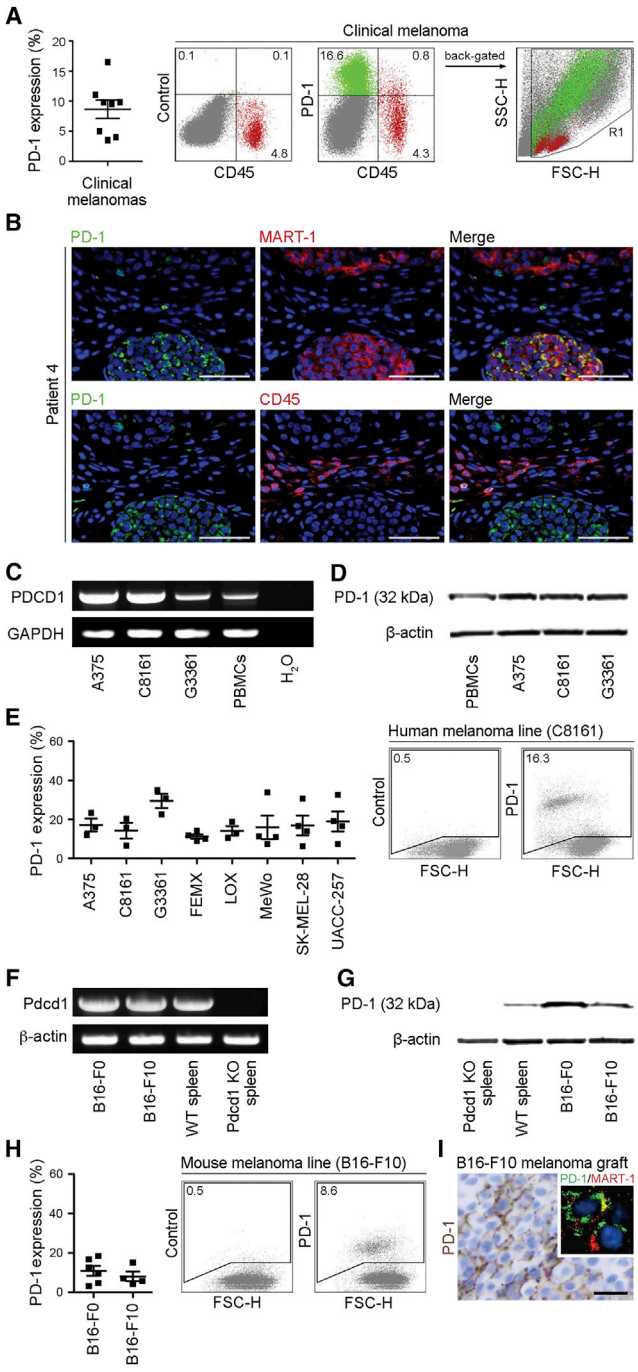


Figure 1. PD-1 Expression by Melanoma Cells

(A) Percentages (mean ± SEM) (left) and representative flow cytometry plots (right) of PD-1 surface protein expression by clinical tumor biopsy-derived melanoma cells (green) from n = 8 distinct melanoma patients. These cells are negative for the CD45 lymphocyte common antigen (red) and the CD31 endothelial marker (see also Figure S1A).

(B) Representative immunofluorescence double staining of a clinical melanoma biopsy for co-expression of PD-1 (green) and MART-1 (red) or of PD-1 (green) and CD45 (red) on a serial tissue section. Nuclei were counterstained with DAPI (blue). Size bars, 100 μm. Representative of n = 22/36 melanoma patients demonstrating melanoma-PD-1 positivity. A patient was considered melanoma-PD-1 positive if any tumor biopsy (total of n = 50) showed expression of PD-1 by MART-1$^+$ and/or CD45$^-$ cells. See also Table S1.

(C and D) RT-PCR expression analysis (C) of full-length PD-1 (*PDCD1*) mRNA and (D) immunoblot of PD-1 protein expression by human melanoma lines and PBMCs.

(E) Percentages (mean ± SEM, left) and representative flow cytometry plots (right) of PD-1 surface protein expression by human melanoma lines (n = 3–4 independent experiments, respectively).

(F and G) RT-PCR expression analysis (F) of full-length PD-1 (*Pdcd1*) mRNA and (G) immunoblot of PD-1 protein expression by murine B16-F0 and B16-F10 melanoma cells, wild-type (WT), and *Pdcd1* knockout (KO) C57BL/6-derived splenocytes.

(H) Percentages (mean ± SEM, left) and representative flow cytometry plots (right) of PD-1 surface protein expression by B16 cells (n = 4–6 independent experiments, respectively).

(I) Representative PD-1 immunohistochemistry and immunofluorescence double staining for co-expression of PD-1 (green) with MART-1 (red) (inset photomicrograph) of a B16-F10 melanoma graft grown in NSG mice (size bar, 50 μm).

See also Figures S1 and S2, and Table S1.

significantly alter expression of PD-L1 or PD-L2, respectively (not shown). Conversely, transduction of B16 cells with *Pdcd1*-encoding constructs resulted in upregulation of PD-1, both at the mRNA and protein level (Figure 2B). Melanoma-specific *Pdcd1*-KD resulted in decreased and *Pdcd1*-OE in increased B16-F0 and B16-F10 melanoma growth in immunocompetent C57BL/6 mice compared to that of vector controls (Figure 2C). *Pdcd1*-KD melanoma grafts demonstrated diminished (Figure S3A) and *Pdcd1*-OE melanomas significantly enhanced *Pdcd1* mRNA and PD-1 protein expression compared to control tumors at the experimental endpoint (Figure S3B). We next compared the tumorigenic ability of native PD-1$^+$- versus PD-1$^-$-sorted B16-F0 and B16-F10 melanoma cells and found that PD-1$^+$ subpopulations demonstrated significantly increased growth in C57BL/6 mice compared to PD-1$^-$ cells (Figure S3C). Together, these findings identify melanoma-expressed PD-1 as a protumorigenic mechanism.

PD-1 expressed by cells of the adaptive immune system has been established as a modulator of tumor-specific immunity (Topalian et al., 2012a). To determine whether the observed tumor growth-accelerating effects of melanoma-expressed PD-1 depend on melanoma-PD-1:lymphocyte interactions, we compared the abilities of *Pdcd1*-KD and *Pdcd1*-OE versus control B16 melanomas to initiate tumor growth in immunocompromised, T-, and B-cell-deficient NSG mice. We found that *Pdcd1*-KD inhibited and *Pdcd1* overexpression increased tumorigenicity of B16-F0 and B16-F10 melanomas in NSG mice compared to controls (Figure 2D), suggesting lymphocyte-independent roles of melanoma-PD-1 in tumorigenesis. Significant *Pdcd1*-KD (Figure S4A) and overexpression (Figure S4B) in B16 melanoma grafts was confirmed after in vivo growth. Consistent with our findings using *Pdccd1*-overexpressing melanoma variants, we found that PD-1$^+$ melanoma subpopulations purified from native B16-F0 and B16-F10 lines demonstrated increased tumorigenicity in NSG mice compared to PD-1$^-$ cell isolates (Figure S4C).

We next examined whether melanoma-specific *Pdcd1* silencing or overexpression affects melanoma cell growth in vitro, in the complete absence of immune cells, using an

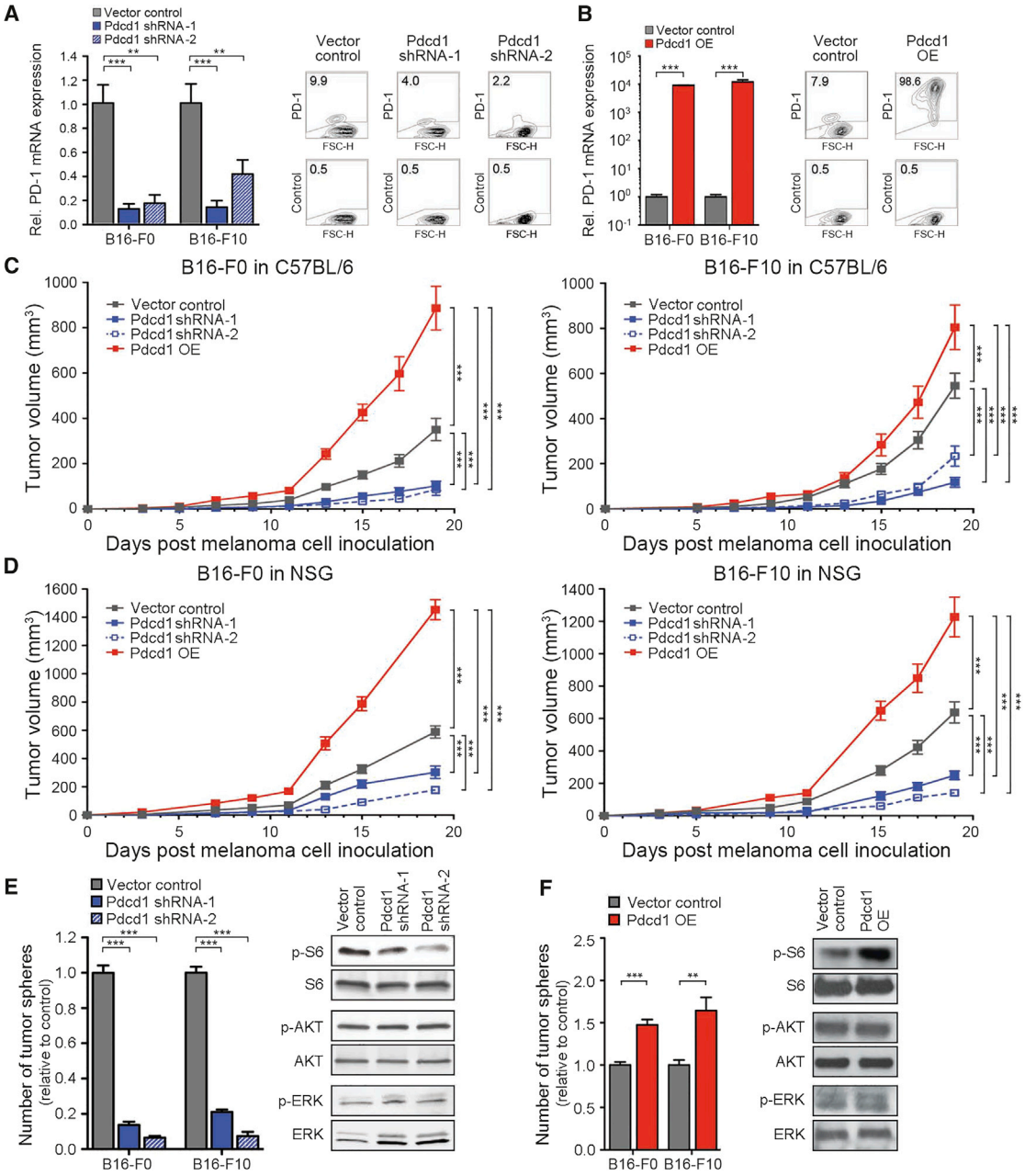


Figure 2. Melanoma-Expressed PD-1 Promotes Tumorigenicity in Murine Melanoma Models

(A and B) PD-1 mRNA and protein expression by *Pdcd1*-shRNA-1 and *Pdcd1*-shRNA-2 versus vector control (A) and by *Pdcd1*-overexpressing (OE) versus vector-control B16-F0 or B16-F10 melanoma cells (B). Representative flow cytometry plots show PD-1 expression in B16-F10 melanoma variants.

(C and D) Tumor growth kinetics (mean ± SD) of *Pdcd1*-shRNA-1/-2 versus *Pdcd1*-OE versus vector control B16-F0 or B16-F10 melanomas in C57BL/6 mice (n = 10–30 each) (C) or NSG mice (n = 10–20 each) (D).

(E and F) Mean number of tumor spheres ± SEM (left) and immunoblot analysis of phosphorylated (p) and total S6, AKT, and ERK (right) in *Pdcd1*-shRNA-1 and *Pdcd1*-shRNA-2 versus control (E) and *Pdcd1*-OE versus vector-control B16 melanoma variants (F). Results are representative of n = 2–3 independent experiments (**p < 0.01, ***p < 0.001).

See also Figures S3 and S4.

established culture system designed for the study of tumorigenic minority populations (Aceto et al., 2012; Civenni et al., 2011). Consistent with our in vivo findings, *Pdcd1*-KD impaired (Figure 2E) and *Pdcd1*-OE promoted in vitro three-dimensional B16-F0 and B16-F10 culture growth compared to respective controls (Figure 2F). Because PD-1 receptor signaling in T cells modulates several downstream pathways (Riley, 2009) that also serve critical roles in melanomagenesis (Flaherty et al.,

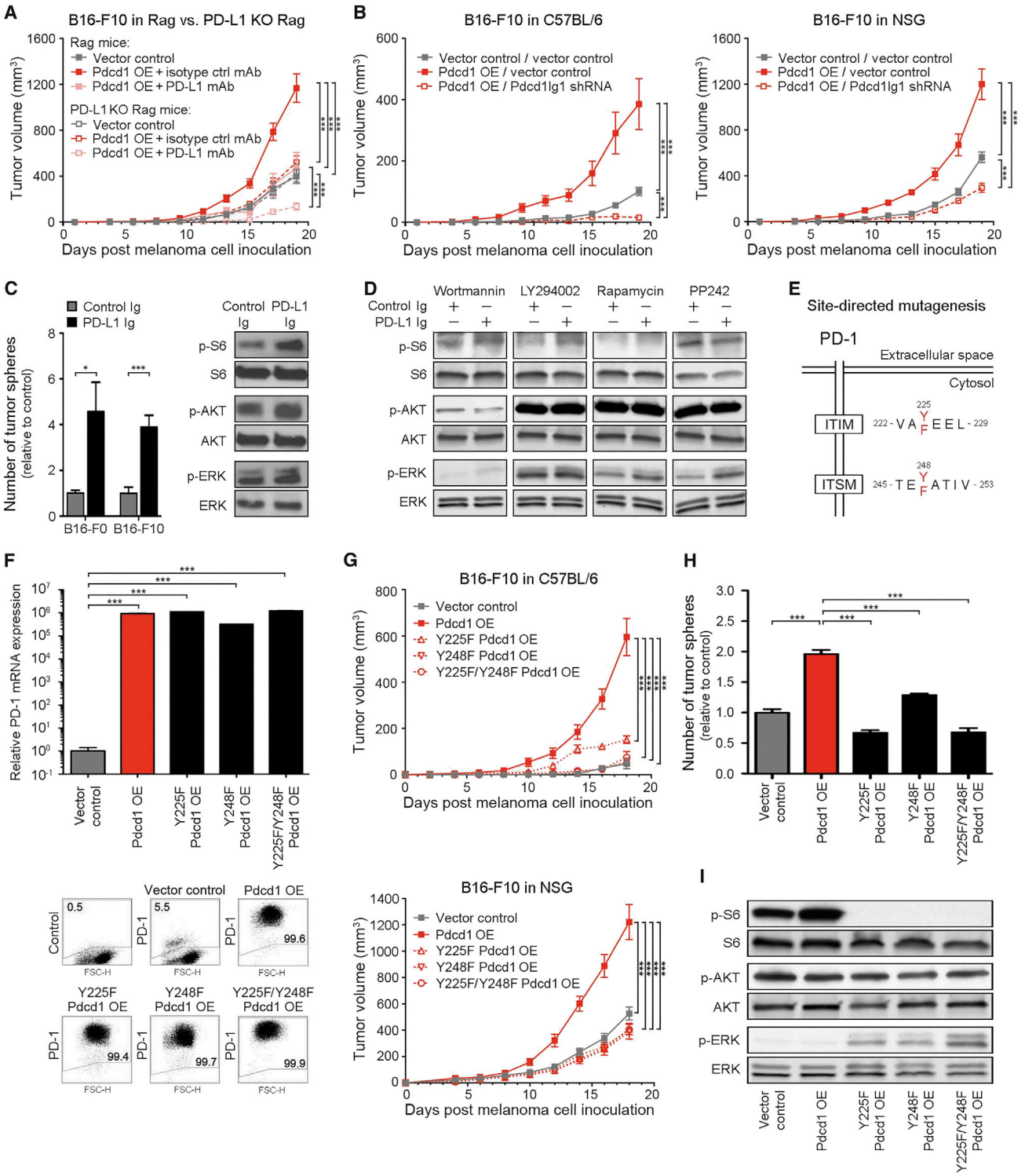


Figure 3. Tumor Cell-Intrinsic PD-1 Signaling Promotes Murine Melanoma Growth

(A) Growth kinetics (mean ± SD) of *Pdcd1*-OE versus vector control B16-F10 melanomas in PD-L1(−/−) KO Rag(−/−) KO (n = 14 versus 20 versus 10) versus wild-type Rag(−/−) KO recipients (n = 14 versus 14 versus 8) treated with anti-PD-L1- versus isotype control monoclonal antibody (mAb).

(B) Growth kinetics (mean ± SD) in C57BL/6 (left) and NSG mice (right) of *Pdcd1*-OE B16-F10 cells co-transduced with PD-L1 (*Cd274*, also known as *Pdcd1lg1*)-shRNA versus control-shRNA compared to vector controls (n = 10 each).

(legend continued on next page)

2012), such as MAPK/ERK, PI3K/AKT, and mTOR signaling, we next examined melanoma-*Pdcd1*-specific changes in phospho (p)-ERK1/2, p-AKT, and p-S6 ribosomal protein levels. *Pdcd1*-KD reduced (Figure 2E) and *Pdcd1*-OE increased phosphorylation of the mTOR effector molecule, S6, compared to control B16 melanoma cells (Figure 2F), indicating melanoma cell-intrinsic, PD-1-mediated induction of protumorigenic mTOR pathway activity. Together, these in vitro findings suggest lymphocyte-independent, cancer cell-intrinsic functions of melanoma-expressed PD-1 in tumor growth.

Melanoma-PD-1:PD-L1 Interactions Promote Murine Melanoma Growth

We next examined whether ligation of melanoma-PD-1 to its predominant ligand, PD-L1, is required for PD-1-driven tumorigenesis. To test whether melanoma-PD-1:host-PD-L1 interactions promote tumor growth in the absence of adaptive immunity, we grafted *Pdcd1*-OE versus control B16-F10 cells to wild-type Rag(−/−) versus PD-L1(−/−) KO Rag(−/−) mice (Francisco et al., 2009). We found that the growth of *Pdcd1*-OE melanomas was attenuated in PD-L1(−/−) KO Rag(−/−) compared to PD-L1(+/+) Rag(−/−) recipients (Figure S5A). To examine if PD-L1 expressed by melanoma cells (Figure S1) also contributes to melanoma-PD-1-dependent tumorigenesis, we treated PD-L1(−/−) KO Rag(−/−) versus wild-type Rag(−/−) mice grafted with *Pdcd1*-OE melanomas with a PD-L1 blocking antibody. We found that PD-L1 blockade inhibited *Pdcd1*-OE B16-F10 melanoma growth compared to isotype control antibody treatment in PD-L1(−/−) KO mice (Figure 3A). Additionally, PD-L1 antibody treatment resulted in significantly reduced tumor growth of *Pdcd1*-OE melanomas in PD-L1(−/−) KO Rag(−/−) compared to wild-type Rag(−/−) mice (Figure 3A). These findings suggest growth-accelerating functions not only of host-PD-L1:melanoma-PD-1, but also of melanoma-PD-L1:melanoma-PD-1 interactions.

To further demonstrate protumorigenic melanoma-PD-L1 effects in the absence of adaptive immunity, we generated PD-L1 gene (*Cd274*, also known as *Pdcd1lg1*)-KD B16-F10 melanoma cells (Figure S5B) and tested their ability to maintain culture growth and form tumors. Compared to vector controls, *Pdcd1lg1* silencing impaired three-dimensional B16 melanoma growth in vitro (Figure S5C) and in vivo tumorigenesis in both immunocompetent C57BL/6 and immunocompromised NSG mice (Figure S5D). Moreover, *Pdcd1lg1*-KD reversed the significant increase in tumorigenicity of *Pdcd1*-OE versus vector control B16-F10 melanoma cells in C57BL/6 and NSG mice (Figure 3B). To further demonstrate that PD-L1 interactions with melanoma-PD-1 promote melanoma growth, we treated native B16-F0 and B16-F10 cultures with a recombinant PD-L1 Fc-fusion protein (PD-L1 Ig), known to elicit changes in PD-1 receptor signaling in T cells (Francisco et al., 2009). Compared to control Ig treatment, addition of PD-L1 Ig to B16 cultures significantly augmented three-dimensional growth and phosphorylation of S6 ribosomal protein (Figure 3C). Because both PI3K/AKT and mTOR signaling are known to feed into downstream S6 phosphorylation, we examined whether pharmacologic inhibition of either pathway can reverse the observed increase in p-S6 expression. We found that mTOR pathway blockade (via rapamycin or PP242) but not PI3K inhibition (via wortmannin or LY294002) suppressed the PD-L1 Ig-dependent phosphorylation of S6 in murine B16-F10 melanoma cells (Figure 3D). Together, these findings demonstrate that interactions between melanoma-expressed PD-1 with its ligand, PD-L1, promote tumor growth and activate mTOR signaling.

Tumor Cell-Intrinsic PD-1 Signaling Is Required for Efficient Murine Melanoma Growth

To determine whether melanoma cell-intrinsic PD-1 signaling is required for efficient tumor growth, we generated *Pdcd1*-OE B16 variants containing tyrosine to phenylalanine single-point mutations of two PD-1 signaling motifs, the immunoreceptor tyrosine-based inhibitory motif (ITIM, disrupted by Y225F mutation) and the immunoreceptor tyrosine-based switch motif (ITSM, disrupted by Y248F mutation), within the cytoplasmic tail of melanoma-PD-1 (Figure 3E). A construct containing point mutations of both tyrosines (Y225F/Y248F) was also created. In immune cells, ITIM and ITSM play pivotal roles in PD-1 signaling (Riley, 2009). Transduction of wild-type versus mutant *Pdcd1* constructs into B16-F0 or B16-F10 melanoma cells resulted in similarly high expression levels of PD-1 (Figure 3F and Figure S5E), permitting a direct comparison between wild-type *Pdcd1*-OE and each of the mutant variants. Strikingly, mutation of either one (Y225F or Y248F) or both (Y225F/Y248F) melanoma-PD-1 signaling motifs significantly abrogated the increased tumor growth observed in both C57BL/6 and NSG mice grafted with wild-type *Pdcd1*-OE versus vector-control B16 melanoma variants (Figure 3G and Figure S5F), suppressed three-dimensional tumor growth in vitro (Figure 3H and Figure S5G), and phosphorylation of S6 ribosomal protein (Figure 3I) compared to enforced expression of wild-type *Pdcd1*, respectively.

Melanoma Cell-Intrinsic PD-1 Enhances Human Tumor Xenograft Growth

We next analyzed the effects of melanoma-specific PD-1 knockdown versus PD-1 overexpression on human melanoma xenograft growth. Transduction of human A375, C8161, or G3361

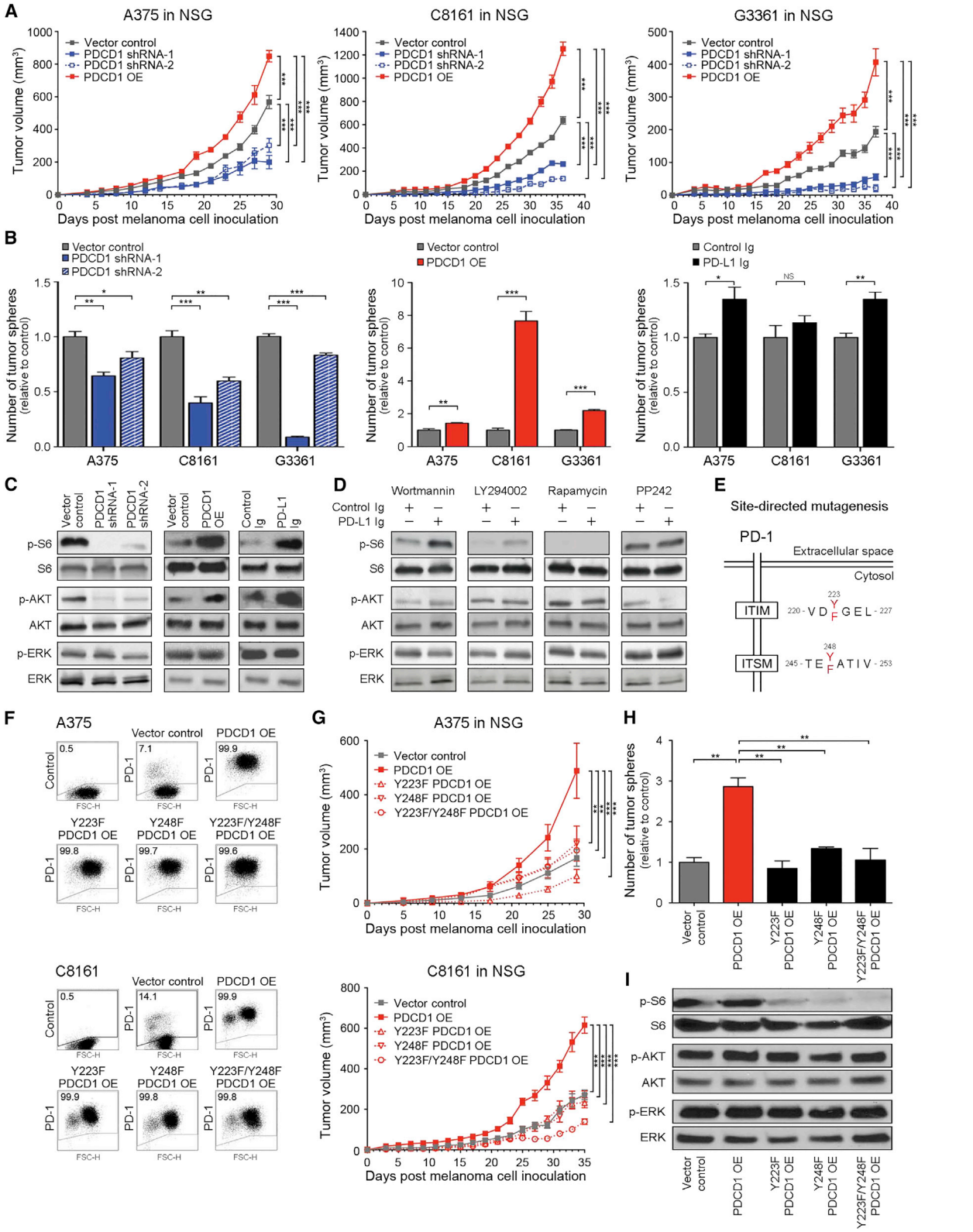
(C) Mean number of tumor spheres ± SEM (left), and immunoblot analysis of p- and total S6, AKT, and ERK in PD-L1 Ig versus control Ig-treated B16 cultures (right).

(D) Immunoblot analysis of p- and total S6, AKT, and ERK in PD-L1 Ig versus control Ig-treated B16-F10 melanoma cells cultured in the presence of the pharmacologic PI3K inhibitors, wortmannin or LY294002, or the mTOR pathway inhibitors, rapamycin or PP242.

(E) Schematic diagram illustrating the introduction of tyrosine to phenylalanine mutations to murine PD-1 signaling motifs via site-directed mutagenesis.

(F) Relative *Pdcd1* mRNA expression (top, mean ± SEM) and representative flow cytometry plots of PD-1 surface protein expression (bottom) by wild-type *Pdcd1*-OE versus Y225F-*Pdcd1*-OE, Y248F-*Pdcd1*-OE, Y225F/Y248F-*Pdcd1*-OE, and vector-control B16-F10 variants.

(G–I) Tumor growth kinetics (G) in C57BL/6 (top, n = 10–14 each) and NSG mice (bottom, n = 8–10 each), (H) mean number of tumor spheres ± SEM, and (I) immunoblot analysis of p- and total S6, AKT, and ERK in B16-F10 melanoma variants as in (F). Immunoblot results are representative of n = 2 independent experiments, respectively (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). See also Figure S5.



(legend on next page)

melanoma cells with two distinct *PDCD1*-shRNAs significantly inhibited PD-1 mRNA expression and blocked PD-1 protein expression between 53%–75% (Figure S6A), and infection with *PDCD1*-OE constructs resulted in marked upregulation of PD-1, both at the mRNA and protein level (>90% positivity), respectively (Figure S6B). *PDCD1*-KD significantly inhibited and *PDCD1*-OE markedly increased human melanoma xenograft growth in NSG mice compared to vector-control-transduced A375, C8161, or G3361 tumors (Figure 4A). Preservation of *PDCD1* silencing and overexpression were confirmed for all melanoma xenografts at the experimental endpoint, respectively (Figures S6C and S6D). Moreover, PD-1^{+} cancer cell subsets purified from native C8161 cultures showed significantly increased tumorigenicity in NSG mice, compared to PD-1^{-} C8161 cells (Figure S6E). Consistent with our in vivo findings, *PDCD1*-KD impaired and *PDCD1*-OE promoted three-dimensional A375, C8161, and G3361 culture growth compared to controls (Figure 4B). Furthermore, relative to control Ig treatment, addition of human PD-L1 Ig augmented tumor sphere formation of A375 and G3361, but not C8161 melanoma cultures (Figure 4B), the latter of which express greater than 3-fold higher endogenous PD-L1 levels than A375 and G3361 cells (Figure S1B). Similar to our findings in murine B16 cells, human *PDCD1*-KD lines showed a reduction and *PDCD1*-OE and PD-L1 Ig-treated human G3361 melanoma cells an increase in p-S6 levels compared to respective controls (Figure 4C). Additionally, pharmacologic inhibition of mTOR but not PI3K signaling blocked the increase in p-S6 expression in PD-L1 Ig compared to control Ig-treated human G3361 melanoma cells (Figure 4D), indicating mTOR pathway dependence of S6 phosphorylation downstream of the melanoma-PD-1 receptor, consistent with our findings in murine B16 melanoma cells (Figure 3D).

Furthermore, generation of human *PDCD1*-OE ITIM (Y223F) and/or ITSM (Y248F) mutant A375 and C8161 cell lines (Figure 4E) with similarly high expression levels of human PD-1 (Figure 4F) revealed that mutation of either one (Y223F or Y248F) or both (Y223F/Y248F) signaling motifs within the melanoma-PD-1 cytoplasmic tail abrogated the increased tumor growth observed in mice grafted with wild-type *PDCD1*-OE versus vector-control A375 or C8161 variants (Figure 4G). Additionally, Y223F-, Y248F-, and Y223F/Y248F mutant *PDCD1*-OE C8161 melanoma cells demonstrated significantly impaired three-dimensional culture growth (Figure 4H) and reduced p-S6 levels compared to wild-type *PDCD1*-OE C8161 cells (Figure 4I). Together, these findings identify PD-1 expressed by human melanoma cells as a lymphocyte-independent tumor growth-accelerating mechanism.

Antibody-Mediated Blockade of PD-1 on Melanoma Cells Inhibits Murine Melanoma Growth

We next examined whether antibody-mediated melanoma-PD-1 blockade significantly inhibits tumor growth, even in immunocompromised NSG hosts, as would be expected based upon the herein demonstrated melanoma cell-intrinsic, protumorigenic PD-1 receptor functions. First, administration of a PD-1 blocking antibody to immunocompetent, C57BL/6 recipients starting one day before inoculation with B16-F10 cells resulted in modest inhibition of melanoma growth between days 5 and 11 post inoculation ($p < 0.01$), but showed no significant differences in tumorigenicity compared to isotype control antibody-treatment at later time points (Figure 5A), consistent with previous studies (Peng et al., 2012; Woo et al., 2012). However, we found that antibody-mediated PD-1 blockade significantly ($p < 0.05$) inhibited B16-F10 melanoma growth in PD-1(−/−) KO C57BL/6 mice compared to controls, for the entire duration of the experiment (Figure 5B). Immunohistochemical examination of melanoma grafts harvested at the experimental endpoint revealed >5-fold increased binding ($p < 0.05$) of in vivo-administered anti-PD-1 antibody to B16 melanoma target tissue in PD-1(−/−) KO (Figure 5B) compared to wild-type C57BL/6 hosts (Figure 5A), supporting the notion of a more pronounced PD-1 antibody effect on melanoma cells in PD-1(−/−) KO mice. A >20% increase in PD-1 antibody titer in the serum of PD-1(−/−) KO versus PD-1(+/+) C57BL/6 hosts, as determined by rat-IgG2a-specific ELISA (Figure S7A), further indicated that increased PD-1 antibody availability might, at least in part, contribute to the growth-inhibitory effect of PD-1 blockade in PD-1(−/−) KO hosts. Anti-PD-1 antibody administration to NSG mice also significantly ($p < 0.001$) diminished B16-F10 melanoma growth compared to isotype control antibody treatment (Figure 5C). Interestingly, while compared to B16 melanoma grafts grown in C57BL/6 mice, B16 melanomas grafted to NSG mice tended to show increased anti-PD-1 antibody binding, antibody titers were not increased in NSG mouse serum (Figure S7A), suggesting strain-specific differences in PD-1 antibody kinetics. To control for the possibility that the observed growth-inhibitory

Figure 4. PD-1 Expression by Human Melanoma Cells Promotes Experimental Tumor Growth

(A) Tumor growth kinetics (mean ± SD) of *PDCD1*-shRNA-1, *PDCD1*-shRNA-2, and *PDCD1*-OE versus vector control human A375 (left), C8161 (center), and G3361 melanoma cells (right) grafted to NSG mice (n = 8–20 each).

(B) Mean number of tumor spheres ± SEM and (C) immunoblot analysis (G3361) of phosphorylated (p) and total ribosomal protein S6, AKT, and ERK in *PDCD1*-shRNA-1/-2 versus vector control, *PDCD1*-OE versus vector-control, and PD-L1 Ig- versus control Ig-treated human A375, C8161, and G3361 melanoma cultures.

(D) Immunoblot analysis of p- and total S6, AKT, and ERK in PD-L1 Ig versus control Ig-treated G3361 melanoma cells cultured in the presence of the pharmacologic PI3K inhibitors, wortmannin or LY294002, or the mTOR pathway inhibitors, rapamycin or PP242.

(E) Schematic diagram illustrating the introduction of tyrosine to phenylalanine mutations to human PD-1 signaling motifs via site-directed mutagenesis.

(F and G) Representative flow cytometry plots (F) of PD-1 surface protein expression and (G) tumor growth kinetics (mean ± SD) of *PDCD1*-OE versus Y223F-*PDCD1*-OE, Y248F-*PDCD1*-OE, Y223F/Y248F-*PDCD1*-OE, and vector-control human A375 (top, n = 10–24) and C8161 melanomas (bottom, n = 10–12) in NSG mice, respectively.

(H and I) Mean number of tumor spheres (H) ± SEM and (I) immunoblot analysis of p- and total S6, AKT, and ERK in C8161 melanoma variants as in (D). Immunoblot results are representative of n = 2–3 independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

See also Figure S6.

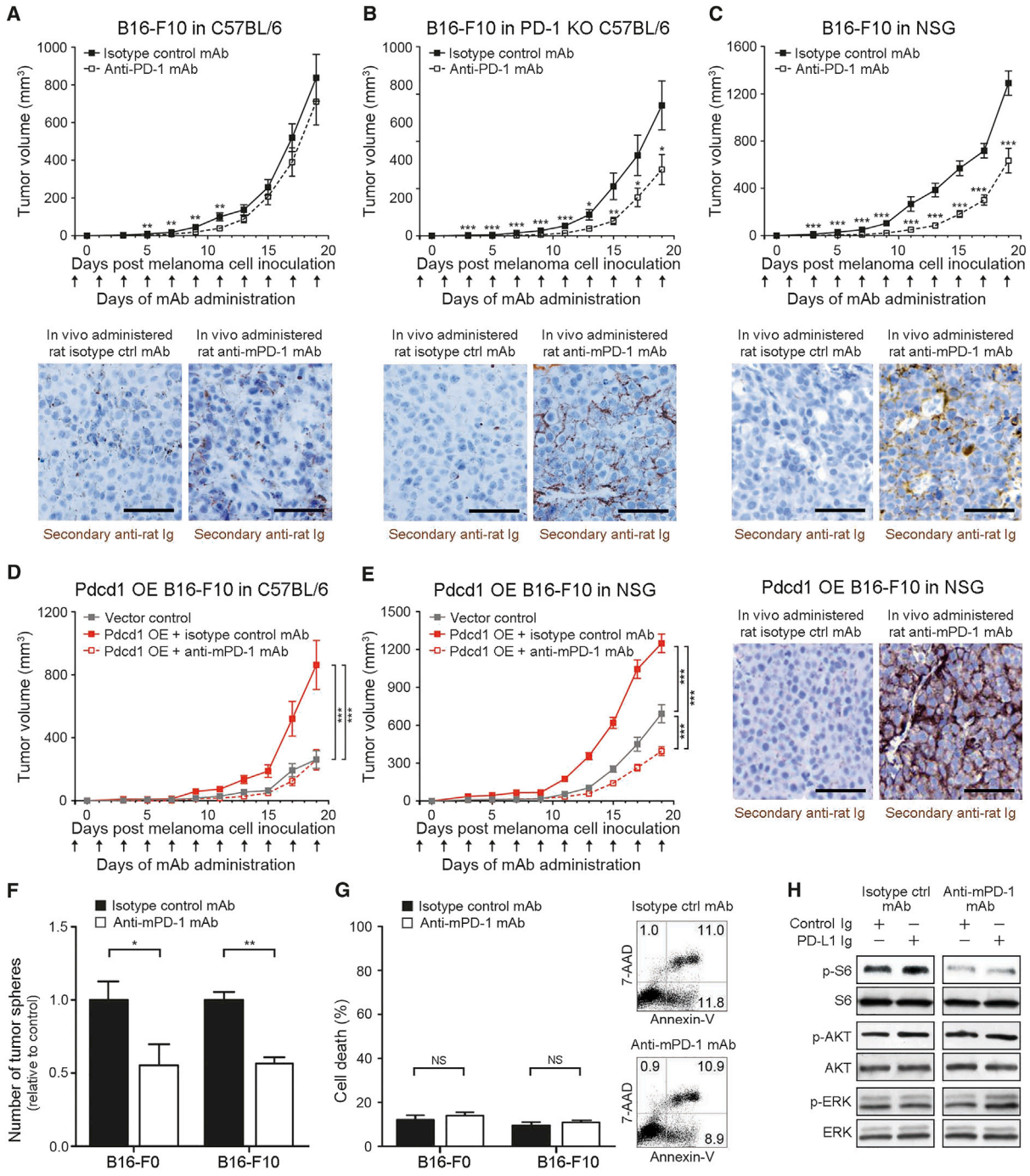


Figure 5. Anti-PD-1 Blocking Antibody Inhibits Murine Melanoma Growth in Immunocompetent, Immunocompromised and PD-1-Deficient Tumor Graft Recipient Mice

(A–E) Tumor growth kinetics (A) (mean ± SD) of B16-F10 melanomas in wild-type C57BL/6 (n = 32 versus 34), (B) PD-1(−/−) knockout (KO) C57BL/6 (n = 20 versus 16), and (C) NSG (n = 20 versus 18), and of (D) *Pdcd1*-overexpressing (OE) versus vector control B16-F10 melanomas in C57BL/6 (n = 10 each) or (E) NSG mice (n = 10 each) treated with anti-PD-1- versus isotype control antibody. Representative immunohistochemical images illustrate binding of in vivo-administered rat anti-mouse PD-1 blocking but not isotype control antibody to the respective B16-F10 melanoma grafts (size bars, 50 μm).

(F and G) Mean number of tumor spheres ± SEM (F) and (G) flow cytometric assessment of cell death (percent AnnexinV$^+$/7AAD$^+$ cells, mean ± SEM (left) and representative flow cytometry plots (right) of anti-PD-1- versus isotype control mAb-treated murine B16-F0 and B16-F10 melanoma cultures.

(H) Immunoblot analysis (representative of n = 2 independent experiments) of phosphorylated (p) and total ribosomal protein S6, AKT, and ERK in B16 cultures concurrently treated with PD-L1 Ig versus control Ig and/or anti-PD-1- versus isotype control mAb (NS: not significant, *p < 0.05, **p < 0.01, ***p < 0.001).

See also Figure S7.

effects might result from antibody-mediated blockade of PD-1-expressing innate immune cell subtypes present in NSG mice, we administered anti-PD-1 antibody to NK cell-, macrophage-, and neutrophil-depleted NSG recipients of B16-F10 melanoma cells (Figure S7B). We also generated NSG mice depleted of all three innate immune effector subsets. Anti-PD-1 antibody treatment inhibited B16 melanoma growth compared to isotype control antibody in all innate immune cell-depleted NSG hosts (Figure S7C).

To further confirm melanoma-specific PD-1 inhibition of the PD-1 blocking antibody, we administered anti-PD-1 antibody to C57BL/6 and NSG mice grafted with *Pdcd1*-OE versus vector control B16-F10 melanoma cells. Anti-PD-1 antibody treatment reversed the increase in tumor growth of isotype control-treated *Pdcd1*-OE compared to vector-control B16 melanomas in both C57BL/6 (Figure 5D) and NSG mice, concomitant with binding of in vivo-administered anti-PD-1 antibody to B16 melanomas (Figure 5E), thereby confirming recognition of melanoma-PD-1 by the PD-1 blocking antibody. Antibody-mediated PD-1 blockade also reduced three-dimensional B16-F0 and B16-F10 melanoma growth in vitro (Figure 5F), but did not induce significant cell death compared to isotype control antibody-treatment (Figure 5G). Moreover, treatment of B16 melanoma cultures with anti-PD-1 but not isotype control antibody inhibited phosphorylation of S6 ribosomal protein (Figure 5H). Together, these findings show that antibody-mediated PD-1 blockade directly on melanoma cells inhibits tumor cell-intrinsic, protumorigenic PD-1 functions, including in the absence of adaptive immunity.

Antibody-Mediated PD-1 Blockade Inhibits Human Melanoma Xenograft Growth in Immunodeficient Mice

We next examined whether antibody-mediated PD-1 blockade can also inhibit human melanoma growth in NSG mice. To assess the translational relevance of targeting melanoma cell-intrinsic PD-1 to impede tumor growth, we first administered anti-PD-1 antibody to NSG mice grafted with patient-derived melanoma cells. Consistent with our findings in murine B16 models (Figure 5C), in vivo anti-PD-1 antibody administration to NSG mice significantly inhibited mean tumor volumes of clinical melanoma xenografts derived from three distinct melanoma patients (Figure 6A). Anti-PD-1 antibody treatment also significantly inhibited the growth of human A375, C8161, and G3361 melanoma xenografts in NSG mice compared to that of the respective control antibody-treated melanomas (Figure 6B). Immunohistochemical analysis revealed binding of in vivo-administered anti-human PD-1 antibody to melanoma xenografts (Figure 6C). Administration of anti-PD-1 antibody to NSG mice also abrogated the increased melanoma xenograft growth of isotype control-treated human *PDCD1*-OE compared to vector-control C8161 xenografts (Figure 6D). Marked melanoma binding of in vivo-administered anti-PD-1 antibody to *PDCD1*-OE C8161 melanomas (Figure 6D) confirmed melanoma-PD-1 reactivity of the human anti-PD-1 blocking antibody. Compared to isotype control antibody-treatment, PD-1 blockade also decreased three-dimensional melanoma growth in vitro (Figure 6E), but did not significantly induce apoptosis in human A375, C8161, or G3361 melanoma cultures (Figure 6F). Finally, treatment of G3361 melanoma cells with anti-PD-1 but not isotype control antibody inhibited PD-L1 Ig-dependent phosphorylation of S6 ribosomal protein (Figure 6G). Together, our findings in NSG mice indicate that anti-PD-1-mediated melanoma growth inhibition results from direct interference with melanoma-expressed PD-1 and is not necessarily dependent on adaptive immunity.

Melanoma Cell Expression of the PD-1 Effector Molecule, p-S6, Correlates with Response to PD-1 Therapy in Cancer Patients

To further assess the translational relevance of melanoma cell-intrinsic PD-1 receptor signaling, we performed p-S6 staining and quantitatively assessed melanoma-p-S6 positivity in pre-treatment versus post-treatment tumor biopsies obtained from n = 11 melanoma patients undergoing anti-PD-1 therapy. We found that melanoma biospecimens sampled post PD-1 therapy demonstrated significantly ($p = 0.005$) decreased p-S6 expression compared to patient-matched pre-treatment biopsies (Figure 7A), consistent with our findings in PD-1 antibody-treated melanoma cell lines (Figures 5H and 6G). Additionally, in a cohort of n = 34 melanoma patients where pre-treatment tumor tissue was available for analysis, we found that patients with high p-S6 expression (>25% of melanoma cells, Figure 7B) prior to treatment showed a >3-fold increase in progression-free survival (mean progression-free survival: 17.0 versus 4.5 months, $p = 0.001$, Figure 7C) and significantly ($p < 0.05$) enhanced overall survival (mean overall survival: 25.1 versus 13.0 months, Figure 7D) compared to melanoma patients with low p-S6 levels (<25% of melanoma cells, Figure 7B) in pre-treatment tumor biospecimens (Table S2). These findings suggest a relationship between p-S6 and response to PD-1 pathway blockade, thereby indicating the potential translational relevance of melanoma cell-intrinsic PD-1 receptor functions.

DISCUSSION

Our study provides several insights into PD-1 pathway functions in melanoma. First, we have conducted a comprehensive characterization of PD-1 transcript and protein expression by cancer cells in clinical tumor biopsies and established melanoma lines. Until now, PD-1 expression has been mainly reported in immune-competent cells of the hematopoietic lineage (Topalian et al., 2012a). We found that both melanoma cell lines and surgical specimens frequently harbor PD-1-expressing cancer cells. However, PD-1 is not uniformly present on all melanoma cells among heterogeneous tumor samples. Rather, it is restricted to small melanoma subpopulations that are nonetheless critically important for tumor growth, consistent with our previous findings demonstrating preferential PD-1 expression by melanoma-initiating cells (Schatton et al., 2010). In our current study, RT-PCR, immunoblot, and flow cytometric analyses revealed PD-1 on melanoma cells in all cell lines and clinical tumor samples examined. Furthermore, immunofluorescence double labeling similarly showed PD-1 expression by melanoma subpopulations in clinical biopsy specimens obtained from >60% of melanoma patients. Thus, using various independent methods, our work clearly establishes that melanomas frequently contain PD-1$^+$ tumor cell fractions. Comparably, melanoma cell expression of the

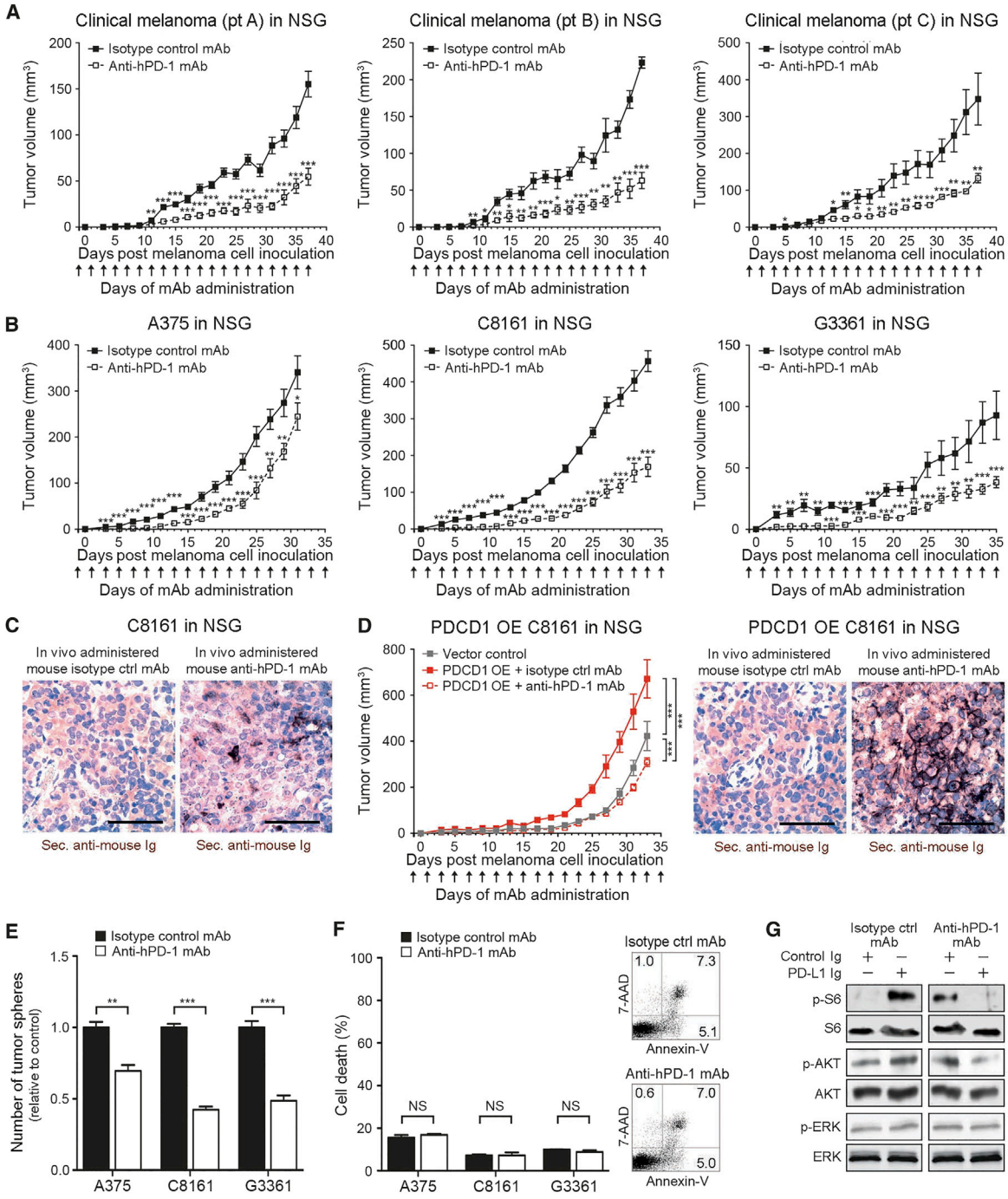


Figure 6. Anti-PD-1 Blocking Antibody Inhibits Human Melanoma Xenograft Growth in Immunocompromised Mice

(A) Kinetics (mean ± SD) of clinical melanoma xenograft growth in NSG mice treated with anti-human PD-1 or isotype control antibody (patient A, n = 7 each; patient B, n = 5 versus 4; patient C: n = 10 each).

(B–D) Tumor growth kinetics (B) (mean ± SD) and (C) representative secondary antibody staining (size bars, 50 μm) of mouse anti-human PD-1 versus isotype control antibody-treated human A375 (n = 14 each), C8161 (n = 14 each), or G3361 melanoma xenografts (n = 16 versus 12) or of (D) human *PDCD1*-OE versus vector control-transduced C8161 xenografts in NSG mice (n = 10 each). Immunohistochemical images illustrate binding of in vivo-administered mouse anti-human PD-1 blocking but not isotype control antibody to the respective human melanoma xenograft (size bars, 50 μm).

(E and F) Mean number of tumor spheres (E) ± SEM and (F) flow cytometric assessment of cell death (percent $AnnexinV^+/7AAD^+$ cells, mean ± SEM (left) and representative flow cytometry plots (right) of anti-PD-1- versus isotype control mAb-treated human A375, C8161, and G3361 melanoma cultures.

(H) Immunoblot analysis (representative of n = 3 independent experiments) of phosphorylated (p) and total ribosomal protein S6, AKT, and ERK in G3361 cultures concurrently treated with PD-L1 Ig versus control Ig and/or anti-PD-1- versus isotype control mAb (NS: not significant, *p < 0.05, **p < 0.01, ***p < 0.001).

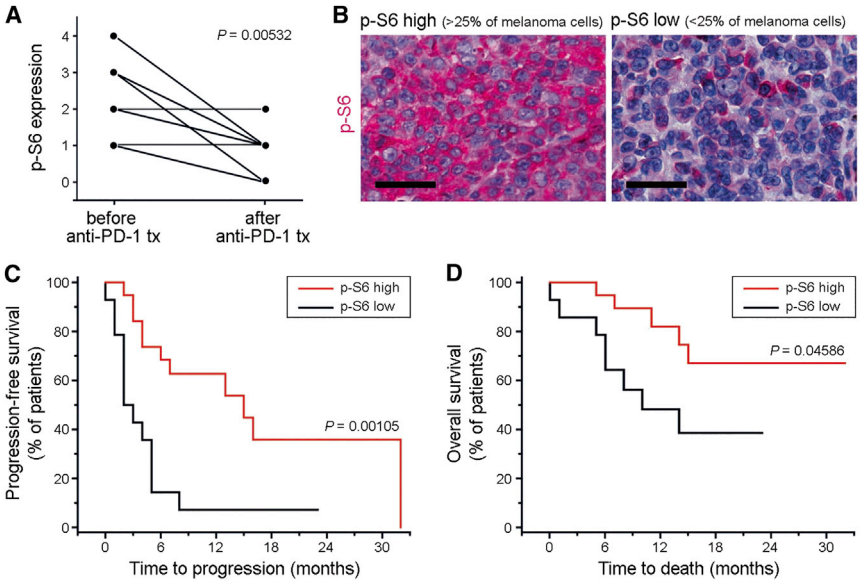


Figure 7. Analysis of p-S6 Expression in Tumor Biospecimens Obtained from Patients with Advanced-Stage Melanoma Undergoing anti-PD-1 Antibody Therapy

(A) Expression of phospho (p)-S6 ribosomal protein by melanoma cells in tumor biospecimens obtained from n = 11 patients with stage IV melanoma before treatment start compared to that in patient-matched progressive lesions sampled after initiation of anti-PD-1 antibody therapy. p-S6 expression by melanoma cells was determined by immunohistochemical analysis and 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).

(B) Representative p-S6 immunohistochemistry of tumor biospecimens obtained from melanoma patients before initiation with systemic anti-PD-1 antibody therapy showing low (<25%) versus high (>25%) melanoma cell expression of p-S6. Size bars, 50 μm.

(C and D) Kaplan-Meier estimates (C) of progression-free survival and (D) of overall survival probability in stage IV melanoma patients (n = 34) demonstrating low (<25%, n = 14 patients) versus high melanoma cell-expression of p-S6 (>25%, n = 20 patients) in tumor biospecimens obtained before initiation of systemic anti-PD-1 antibody treatment.

See also Table S2.

PD-1 ligand, PD-L1, is often confined to small subsets of cancer cells within clinical tumor specimens (Herbst et al., 2014; Topalian et al., 2012b).

Second, this study demonstrates PD-1 receptor signaling in a non-immune cell type, i.e., melanoma cells. To date, PD-1 immunobiology has been mainly studied in T cells (Topalian et al., 2012a). Binding of T-cell-expressed PD-1 to its ligands mediates inhibitory signals that downmodulate T effector functions. For example, in the cancer context, PD-1 expression by tumor-reactive CTLs results in their exhaustion or functional impairment (Fourcade et al., 2010; Sakuishi et al., 2010), which represents a key mechanism underlying tumor immune evasion (Pardoll, 2012). Similar to the protumorigenic effects of T-cell-expressed PD-1, our findings establish PD-1 expressed by melanoma cells as a tumor-growth-promoting mechanism in multiple independent experimental in vitro and in vivo systems. However, while T cell-PD-1 promotes cancer progression by dampening antitumor immune responses, melanoma-PD-1 promotes tumor growth, even in the absence of a functional adaptive immune system.

Our results further indicate that efficient, PD-1-driven tumorigenesis requires melanoma-PD-1 interactions with host- and/or melanoma-expressed PD-L1, because both PD-L1-deficient and PD-L1 antibody-treated mice grafted with *Pdcd1*-OE melanomas demonstrate decreased tumor growth compared to respective controls. Additionally, PD-L1 (*Pdcd1lg1*) silencing reversed melanoma-PD-1-driven tumorigenesis and recombinant PD-L1 Ig treatment promoted melanoma spheroid growth. PD-L1 expression by melanoma cells has established roles in tumor immune evasion (Dong et al., 2002). Beyond promoting cancer progression by engaging with TIL-expressed PD-1, our study indicates that melanoma-expressed PD-L1 may also promote tumor growth via paracrine or autocrine interactions with the melanoma-PD-1 receptor.

In T cells, PD-1 engagement by its ligands modulates signaling networks downstream of the TCR, including mTOR and PI3K/AKT (Riley, 2009). Consistent with the interrelationship of PD-1 and PI3K/AKT/mTOR signaling in T cells, the important role of these pathways in melanoma proliferation (Flaherty et al., 2012), and the herein described protumorigenic effects of melanoma-PD-1, we found that *PDCD1*-KD, antibody-mediated PD-1 blockade, and mutagenesis of melanoma-PD-1 signaling motifs decreased, while *PDCD1*-OE and PD-L1 Ig-treatment increased phosphorylation of the mTOR effector molecule (Corcoran et al., 2013), ribosomal protein S6. Melanoma-PD-1-dependent S6 phosphorylation was reversed via pharmacologic inhibition of mTOR but not PI3K, suggesting that the PD-1 receptor on melanoma cells activates downstream mTOR signaling through a PI3K/AKT-independent pathway. However, whereas PD-1 activation augments p-S6 levels in melanoma cells and enhances tumor growth, it dampens mTOR signaling in T cells, leading to diminished proliferation (Riley, 2009). Because S6 phosphorylation represents a point of convergence that integrates multiple upstream signaling networks (Corcoran et al., 2013; Flaherty et al., 2012), it is possible that melanoma-PD-1 might also modulate several alternative signaling networks, in addition to the mTOR pathway.

PD-1 ligation in T-lymphocytes is known to recruit phosphatases SHP-1 and SHP-2 to its ITIM and ITSM cytosolic loci, which induces dephosphorylation of proximal TCR signaling intermediaries and subsequent suppression of several pathways downstream of the TCR, including mTOR (Riley, 2009). SHP-2 is also expressed by melanoma cells (Ostman et al., 2006) and tumor-initiating cell subsets in other cancers (Aceto et al.,

2012; Liu et al., 2011), paralleling our previous findings of preferential PD-1 expression by melanoma-initiating cells (Schatton et al., 2010). In cancer cells, SHP-2-dependent signaling promotes activation of protumorigenic pathways, including mTOR (Liu et al., 2011; Ostman et al., 2006). The divergent effects of PD-1 ligation on mTOR signaling in melanoma cells versus T cells are thus entirely consistent with the opposing, protumorigenic versus growth-inhibitory roles of SHP-2 in the respective tissues.

Finally, our work reveals that PD-1 pathway interference exerts tumor growth-inhibitory effects, not only in mice with fully intact immunity, but also in melanoma cultures devoid of immune cells and in severely immunocompromised, T cell-, B cell-, and innate-immune-cell-deficient hosts. Together, these results show that antibody-mediated blockade of PD-1 at the level of the melanoma cell inhibits tumor growth. Because PD-1 pathway inhibitors have produced unprecedented response rates in otherwise treatment-refractory patients with advanced cancers, including malignant melanoma (Hamid et al., 2013; Herbst et al., 2014; Topalian et al., 2012b; Weber et al., 2015; Wolchok et al., 2013), our findings are of potential translational importance. It has been well established that PD-1 blockade reverses cancer-antigen-specific T cell exhaustion, thereby restoring antitumor immunity (Fourcade et al., 2010; Sakuishi et al., 2010). Nevertheless, our data suggest that blockade of PD-1 directly on melanoma cells might represent an important additional, tumor cell-intrinsic mechanism that could contribute to the clinical effectiveness of PD-1 cancer therapy. In support of this possibility, our data obtained in a small cohort of patients with stage IV melanoma suggests that tumoral expression of the PD-1 receptor signaling mediator, p-S6, appears to correlate with response to anti-PD-1 antibodies. However, the possible utility of p-S6 as a potential biomarker of PD-1 inhibitor sensitivity will require independent validation in larger patient cohorts, including prospective cohort studies.

Consistent with our findings of protumorigenic effects of the melanoma-PD-1:PD-L1 axis, clinical trial data suggest a correlation between melanoma-PD-L1 and TIL-PD-L1 expression and objective response to PD-1 checkpoint blockade (Herbst et al., 2014; Topalian et al., 2012b; Tumeh et al., 2014). Interestingly, both elevated PD-L1 (Jiang et al., 2013) and p-S6 expression levels (Corcoran et al., 2013) have evolved as potential biomarkers of resistance to melanoma therapies targeting oncogenic BRAF mutations. Therefore, our data suggest that combination of therapies targeting the MAPK pathway (Flaherty et al., 2012) with PD-1 inhibitors may be effective, not only because they activate tumor-specific immunity while concurrently blocking the MAPK oncogenic pathway, but also because PD-1/PD-L1 blockade might additionally suppress mTOR-associated protumorigenic signals. Furthermore, in light of our findings, the superior clinical activity and safety profile of anti-PD-1- compared to anti-CTLA-4 therapy (Hamid et al., 2013; Pardoll, 2012; Postow et al., 2015; Weber et al., 2015) might, at least in part, relate to the fact that the latter merely interferes with T cell function, whereas PD-1 antibody treatment may also directly target other PD-1-expressing immune cell types (Topalian et al., 2012a) or the tumor itself, as suggested by our data. Finally, robust clinical response to anti-PD-1 therapy in patients with cancers that have hitherto not typically responded to immunotherapy (Herbst et al., 2014; Topalian et al., 2012b) could at least be partially explained by direct PD-1 inhibition on tumor cells in the respective malignancies.

In summary, our findings identify PD-1 expressed by melanoma cells as a tumor growth receptor and molecular mediator of melanoma cell-intrinsic mTOR signaling, serving to promote tumorigenesis in addition to its protumorigenic role when expressed by immune cells. Recognition of melanoma-PD-1 receptor-driven tumorigenesis critically enhances our understanding of the mechanisms underlying melanoma progression and could contribute to the further refinement of PD-1-targeted therapies, for improved outcomes in patients with advanced stage cancer.

EXPERIMENTAL PROCEDURES

Melanoma Cell Lines, Culture Methods, and Clinical Specimens

Melanoma cell lines were cultured as described (Schatton et al., 2008). Human PBMCs were obtained from healthy volunteers and clinical tumor biospecimens were obtained from melanoma patients in accordance with protocols approved by the IRBs of Partners Health Care Management, the Dana-Farber Cancer Institute, the University of Zurich, Switzerland, and the University of Bern, Switzerland. Informed consent was obtained from all subjects and all studies were conducted in accordance with the Declaration of Helsinki. PD-1^{+} and PD-1^{-} melanoma subpopulations were generated as described (Schatton et al., 2008; Schatton et al., 2010).

RT-PCR, Real-Time qPCR, and Flow Cytometry

Full-length *PDCD1* was amplified and sequenced following reverse transcription of total mRNA using *PDCD1*-specific primer pairs. Relative *PDCD1*, *PDCD1LG1* (*CD274*), and *PDCD1LG2* (*CD273*) transcript levels were determined by real-time qRT-PCR and calculated using the $2^{(-\Delta\Delta Ct)}$ method (Schatton et al., 2008; Schatton et al., 2010). PD-1 surface protein expression by established melanoma lines and patient-derived melanoma single-cell suspensions was analyzed by flow cytometry (Schatton et al., 2008; Schatton et al., 2010).

Western Blot Analysis

Cells were lysed, total protein separated by SDS/PAGE and transferred to a PVDF membrane by electroblotting (Posch et al., 2013). Expression levels of human and murine PD-1 and of phosphorylated versus total ERK1/2, AKT, and S6 proteins were determined using enhanced chemiluminescence (Posch et al., 2013) or the Odyssey CLx imaging system (LI-COR Biosciences).

Immunohistochemistry and Immunofluorescence Staining

Immunofluorescence double labeling for PD-1, MART-1, and/or CD45, and immunohistochemical analysis of PD-1 expression in experimental tumors and of p-S6 expression in tumor biospecimens obtained from melanoma patients undergoing anti-PD-1 antibody therapy were carried out as described (Schatton et al., 2008; Schatton et al., 2010). 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).

Generation of Stable PD-1 or PD-L1 Knockdown and PD-1-Overexpressing Melanoma Cell Line Variants

Stable PD-1 or PD-L1 knockdown melanoma lines were generated using lentiviral transduction particles containing shRNAs against human *PDCD1*, murine *Pdcd1*, or murine *Pdcd1lg1* (*Cd274*), and PD-1-overexpressing melanoma lines by infection with viral particles containing the full-length murine *Pdcd1* or human *PDCD1* CDS. *PDCD1*-OE melanoma variants containing tyrosine to phenylalanine single-point mutations within PD-1 signaling motifs were

generated by site-directed mutagenesis followed by enforced expression, as above.

Three-Dimensional Melanoma Culture

Melanoma tumor sphere cultures of native or melanoma-PD-1 variant lines were maintained, as described (Aceto et al., 2012; Civenni et al., 2011), in standard culture medium, as above, in the presence or absence of anti-PD-1 or isotype control mAb, recombinant PD-L1 Ig or control Ig.

Murine Melanoma Induction and Human Melanoma Xenotransplantation

C57BL/6, PD-1(−/−) KO C57BL/6, NSG, Rag(−/−), and PD-L1(−/−) KO Rag(−/−) mice (Francisco et al., 2009) were maintained and experiments performed in accordance with IACUC approved experimental protocols. For tumorigenicity studies, melanoma cells were injected subcutaneously into flanks of recipient mice (Schatton et al., 2008). For PD-1 and PD-L1 targeting experiments melanoma cells were grafted, mice intraperitoneally injected with anti-PD-1, anti-PD-L1, or isotype control mAbs (200 μg, respectively) every other day starting one day before melanoma inoculation and tumor formation/growth assessed as described (Schatton et al., 2008).

Statistical Analysis

Gene and protein expression levels, tumor spheroid, and in vivo melanoma growth were compared statistically using the unpaired Student's t test, 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 with anti-PD-1 antibody therapy, whose pre-treatment tumor biopsies showed low versus high melanoma cell expression of p-S6. 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 were tested for normal distribution using the D'Agostino and Pearson omnibus normality test. A two-sided value of $p < 0.05$ was considered statistically significant.

See also the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.08.052.

AUTHOR CONTRIBUTIONS

S.K. and T.S. planned the project. S.K., C.P., S.R.B., H.M., C.S., E.G., C.P.E., N.L., V.R.J., Q.Z., R.T., W.H., and T.S. carried out experimental work. S.K., C.P., S.R.B., H.M., C.S., E.G., C.P.E., N.L., V.R.J., Q.Z., W.H., A.C., R.D., M.C.M., K.T.F., M.H.F., G.F.M., A.H.S., T.S.K., and T.S. analyzed data. S.K. and T.S. wrote the paper. All authors discussed the results and commented on the manuscript.

ACKNOWLEDGMENTS

We thank M. Joubert, I. Portugal, C. Correia, and C. Lee for technical assistance. This work was supported by an Innovative Research Grant from the Melanoma International Foundation, a Fund to Sustain Research Excellence from the Brigham Research Institute, Brigham and Women's Hospital, Department of Dermatology funding for new investigators (to T.S.), a Swiss National Science Foundation grant PMDPP3_151326 (to E.G.), and NIH/NCI grants 1R01CA158467 (to M.H.F. and G.F.M.), and U54 CA163125 (to A.H.S.). T.S. is the recipient of a Research Career Development award from the Dermatology Foundation. C.P. and H.M. are recipients of a Klaus Wolff Fellowship by the Austrian Society of Dermatology and Venereology and received salary support from the Fondation René Touraine. C.P. was awarded a Melanoma Research Scholar Award from the Outrun the Sun Melanoma Foundation. N.L. is the recipient of a Medical Student Grant from the American Skin Association. V.R.J. is supported by the Department of Defense through the National Defense Science and Engineering Graduate Fellowship (NDSEG) Program. The authors declare that there are no conflicts of interest.

Received: April 10, 2015
Revised: August 18, 2015
Accepted: August 25, 2015
Published: September 10, 2015

REFERENCES

Aceto, N., Sausgruber, N., Brinkhaus, H., Gaidatzis, D., Martiny-Baron, G., Mazzarol, G., Confalonieri, S., Quarto, M., Hu, G., Balwierz, P.J., et al. (2012). Tyrosine phosphatase SHP2 promotes breast cancer progression and maintains tumor-initiating cells via activation of key transcription factors and a positive feedback signaling loop. Nat. Med. *18*, 529–537.

Civenni, G., Walter, A., Kobert, N., Mihic-Probst, D., Zipser, M., Belloni, B., Seifert, B., Moch, H., Dummer, R., van den Broek, M., and Sommer, L. (2011). Human CD271-positive melanoma stem cells associated with metastasis establish tumor heterogeneity and long-term growth. Cancer Res. *71*, 3098–3109.

Corcoran, R.B., Rothenberg, S.M., Hata, A.N., Faber, A.C., Piris, A., Nazarian, R.M., Brown, R.D., Godfrey, J.T., Winokur, D., Walsh, J., et al. (2013). TORC1 suppression predicts responsiveness to RAF and MEK inhibition in BRAF-mutant melanoma. Sci. Transl. Med. *5*, 196ra98.

Dong, H., Strome, S.E., Salomao, D.R., Tamura, H., Hirano, F., Flies, D.B., Roche, P.C., Lu, J., Zhu, G., Tamada, K., et al. (2002). Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. Nat. Med. *8*, 793–800.

Flaherty, K.T., Hodi, F.S., and Fisher, D.E. (2012). From genes to drugs: targeted strategies for melanoma. Nat. Rev. Cancer *12*, 349–361.

Fourcade, J., Sun, Z., Benallaoua, M., Guillaume, P., Luescher, I.F., Sander, C., Kirkwood, J.M., Kuchroo, V., and Zarour, H.M. (2010). Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. J. Exp. Med. *207*, 2175–2186.

Francisco, L.M., Salinas, V.H., Brown, K.E., Vanguri, V.K., Freeman, G.J., Kuchroo, V.K., and Sharpe, A.H. (2009). PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. J. Exp. Med. *206*, 3015–3029.

Gubin, M.M., Zhang, X., Schuster, H., Caron, E., Ward, J.P., Noguchi, T., Ivanova, Y., Hundal, J., Arthur, C.D., Krebber, W.J., et al. (2014). Checkpoint blockade cancer immunotherapy targets tumour-specific mutant antigens. Nature *515*, 577–581.

Hamid, O., Robert, C., Daud, A., Hodi, F.S., Hwu, W.J., Kefford, R., Wolchok, J.D., Hersey, P., Joseph, R.W., Weber, J.S., et al. (2013). Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. N. Engl. J. Med. *369*, 134–144.

Herbst, R.S., Soria, J.C., Kowanetz, M., Fine, G.D., Hamid, O., Gordon, M.S., Sosman, J.A., McDermott, D.F., Powderly, J.D., Gettinger, S.N., et al. (2014). Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. Nature *515*, 563–567.

Jiang, X., Zhou, J., Giobbie-Hurder, A., Wargo, J., and Hodi, F.S. (2013). The activation of MAPK in melanoma cells resistant to BRAF inhibition promotes PD-L1 expression that is reversible by MEK and PI3K inhibition. Clin. Cancer Res. *19*, 598–609.

Liu, K.W., Feng, H., Bachoo, R., Kazlauskas, A., Smith, E.M., Symes, K., Hamilton, R.L., Nagane, M., Nishikawa, R., Hu, B., and Cheng, S.Y. (2011). SHP-2/PTPN11 mediates gliomagenesis driven by PDGFRA and INK4A/ARF aberrations in mice and humans. J. Clin. Invest. *121*, 905–917.

Ostman, A., Hellberg, C., and Böhmer, F.D. (2006). Protein-tyrosine phosphatases and cancer. Nat. Rev. Cancer *6*, 307–320.

Pardoll, D.M. (2012). The blockade of immune checkpoints in cancer immunotherapy. Nat. Rev. Cancer *12*, 252–264.

Peng, W., Liu, C., Xu, C., Lou, Y., Chen, J., Yang, Y., Yagita, H., Overwijk, W.W., Lizée, G., Radvanyi, L., and Hwu, P. (2012). PD-1 blockade enhances T-cell migration to tumors by elevating IFN-γ inducible chemokines. Cancer Res. *72*, 5209–5218.

Posch, C., Moslehi, H., Feeney, L., Green, G.A., Ebaee, A., Feichtenschlager, V., Chong, K., Peng, L., Dimon, M.T., Phillips, T., et al. (2013). Combined targeting of MEK and PI3K/mTOR effector pathways is necessary to effectively inhibit NRAS mutant melanoma in vitro and in vivo. Proc. Natl. Acad. Sci. USA *110*, 4015–4020.

Postow, M.A., Callahan, M.K., and Wolchok, J.D. (2015). Immune Checkpoint Blockade in Cancer Therapy. J. Clin. Oncol. *33*, 1974–1982.

Riley, J.L. (2009). PD-1 signaling in primary T cells. Immunol. Rev. *229*, 114–125.

Rizvi, N.A., Hellmann, M.D., Snyder, A., Kvistborg, P., Makarov, V., Havel, J.J., Lee, W., Yuan, J., Wong, P., Ho, T.S., et al. (2015). Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. Science *348*, 124–128.

Sakuishi, K., Apetoh, L., Sullivan, J.M., Blazar, B.R., Kuchroo, V.K., and Anderson, A.C. (2010). Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. J. Exp. Med. *207*, 2187–2194.

Schatton, T., Murphy, G.F., Frank, N.Y., Yamaura, K., Waaga-Gasser, A.M., Gasser, M., Zhan, Q., Jordan, S., Duncan, L.M., Weishaupt, C., et al. (2008). Identification of cells initiating human melanomas. Nature *451*, 345–349.

Schatton, T., Schütte, U., Frank, N.Y., Zhan, Q., Hoerning, A., Robles, S.C., Zhou, J., Hodi, F.S., Spagnoli, G.C., Murphy, G.F., and Frank, M.H. (2010). Modulation of T-cell activation by malignant melanoma initiating cells. Cancer Res. *70*, 697–708.

Snyder, A., Makarov, V., Merghoub, T., Yuan, J., Zaretsky, J.M., Desrichard, A., Walsh, L.A., Postow, M.A., Wong, P., Ho, T.S., et al. (2014). Genetic basis for clinical response to CTLA-4 blockade in melanoma. N. Engl. J. Med. *371*, 2189–2199.

Topalian, S.L., Drake, C.G., and Pardoll, D.M. (2012a). Targeting the PD-1/B7-H1(PD-L1) pathway to activate anti-tumor immunity. Curr. Opin. Immunol. *24*, 207–212.

Topalian, S.L., Hodi, F.S., Brahmer, J.R., Gettinger, S.N., Smith, D.C., McDermott, D.F., Powderly, J.D., Carvajal, R.D., Sosman, J.A., Atkins, M.B., et al. (2012b). Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N. Engl. J. Med. *366*, 2443–2454.

Tumeh, P.C., Harview, C.L., Yearley, J.H., Shintaku, I.P., Taylor, E.J., Robert, L., Chmielowski, B., Spasic, M., Henry, G., Ciobanu, V., et al. (2014). PD-1 blockade induces responses by inhibiting adaptive immune resistance. Nature *515*, 568–571.

Weber, J.S., D'Angelo, S.P., Minor, D., Hodi, F.S., Gutzmer, R., Neyns, B., Hoeller, C., Khushalani, N.I., Miller, W.H., Jr., Lao, C.D., et al. (2015). Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment: a randomised, controlled, open-label, phase 3 trial. Lancet Oncol. *16*, 375–384.

Wolchok, J.D., Kluger, H., Callahan, M.K., Postow, M.A., Rizvi, N.A., Lesokhin, A.M., Segal, N.H., Ariyan, C.E., Gordon, R.A., Reed, K., et al. (2013). Nivolumab plus ipilimumab in advanced melanoma. N. Engl. J. Med. *369*, 122–133.

Woo, S.R., Turnis, M.E., Goldberg, M.V., Bankoti, J., Selby, M., Nirschl, C.J., Bettini, M.L., Gravano, D.M., Vogel, P., Liu, C.L., et al. (2012). Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. Cancer Res. *72*, 917–927.

Yadav, M., Jhunjhunwala, S., Phung, Q.T., Lupardus, P., Tanguay, J., Bumbaca, S., Franci, C., Cheung, T.K., Fritsche, J., Weinschenk, T., et al. (2014). Predicting immunogenic tumour mutations by combining mass spectrometry and exome sequencing. Nature *515*, 572–576.