Differential Expression of Immune-Regulatory Genes Associated with PD-L1 Display in Melanoma: Implications for PD-1 Pathway Blockade

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Abstract

Purpose: Blocking the immunosuppressive PD-1/PD-L1 pathway has antitumor activity in multiple cancer types, and PD-L1 expression on tumor cells and infiltrating myeloid cells correlates with the likelihood of response. We previously found that IFNG (interferon-gamma) was overexpressed by tumor-infiltrating lymphocytes in PD-L1+ versus PD-L1(−) melanomas, creating adaptive immune resistance by promoting PD-L1 display. This study was undertaken to identify additional factors in the PD-L1 melanoma microenvironment coordinately contributing to immunosuppression.

Experimental Design: Archived, formalin-fixed paraffin-embedded melanoma specimens were assessed for PD-L1 protein expression at the tumor cell surface with IHC. Whole-genome expression analysis, quantitative (q)RT-PCR, IHC, and functional in vitro validation studies were used to assess factors differentially expressed in PD-L1+ versus PD-L1(−) melanomas.

Results: Functional annotation clustering based on whole-genome expression profiling revealed pathways upregulated in PD-L1+ melanomas, involving immune cell activation, inflammation, and antigen processing and presentation. Analysis by qRT-PCR demonstrated overexpression of functionally related genes in PD-L1+ melanomas, involved in CD8+ T-cell activation (CD8A, IFNG, PRF1, and CCL5), antigen presentation (CD163, TLR3, CXCL1, and IL1Z), and immunosuppression (PDCD1 (PD-1), CD274 (PD-L1), and LAG3, IL10). Functional studies demonstrated that some factors, including IL10 and IL32-gamma, induced PD-L1 expression on monocytes but not tumor cells.

Conclusions: These studies elucidate the complexity of immune checkpoint regulation in the tumor microenvironment, identifying multiple factors likely contributing to coordinated immunosuppression. These factors may provide tumor escape mechanisms from anti–PD-1/PD-L1 therapy, and should be considered for cotargeting in combinatorial immunomodulation treatment strategies.

Clin Cancer Res; 21(17); 3969–76. ©2015 AACR.

Introduction

Programmed death ligand 1 (PD-L1, or B7-H1) expression by antigen-presenting cells (APC) is a normal feedback mechanism for terminating immune responses appropriately and maintaining self-tolerance (1). Aberrant PD-L1 expression in cancers co-opt this mechanism, facilitating escape from immune attack. PD-1, the dominant receptor for PD-L1, is found on activated T, B, and NK cells in the tumor microenvironment (TME). Its ligation by PD-L1 downregulates antitumor immune effector functions. Antibodies (mAbs) interrupting the PD-1 pathway, blocking either PD-1 or PD-L1, have durable efficacy in patients with advanced melanoma and other cancers, further highlighting the key role of this pathway in local tumor immunosuppression (2, 3).

Multiple studies using different detection methods and analytic criteria have demonstrated that PD-L1 expression on tumor cells and/or leukocytes in the TME may predict response to PD-1 pathway blockade. In some cancers, such as MSI colon cancer, PD-L1 is expressed predominantly on tumor-infiltrating mononuclear cells rather than on tumor cells themselves (4), whereas we reported that PD-L1+ melanomas and head and neck cancers express PD-L1 on both tumor and mononuclear cells (5, 6). A recent study suggests that leukocyte expression of PD-L1 is most predictive of response to an anti–PD-L1 antibody (7). Therefore, understanding TME factors that coordinate immunosuppression of PD-L1 expression on tumor cells and/or leukocytes is essential to augmenting the clinical impact of anti–PD-1/PD-L1 therapies. Such factors may warrant further study as candidate...
Translational Relevance

Although drugs blocking the PD-1/PD-L1 pathway have shown efficacy in some patients with advanced cancers, a deeper knowledge of coordinated immunosuppression in the PD-L1+ tumor microenvironment (TME) is needed to improve upon these therapeutic results. Here, we show that PD-L1+ melanomas overexpress PD-1, LAG-3, IL10, and IL32, which may contribute to local immunosuppression and therefore are candidates for cotargeting in combination treatment regimens. This study further reveals factors that selectively induce PD-L1 on myeloid cells but not tumor cells, and thus begins to elucidate novel mechanisms for PD-L1 upregulation in the TME.

Materials and Methods

Melanoma specimens

Forty-nine formalin-fixed paraffin-embedded (FFPE) melanoma specimens were characterized for PD-L1 expression by IHC as described (5), including four primary and 45 metastatic lesions. “PD-L1+” was defined as ≥5% of tumor cells showing cell surface staining with the murine anti-human PD-L1 mAb 5H1 (Lieping Chen, Yale University). The geographic association of PD-L1 expression with the presence of tumor-infiltrating lymphocytes (TILs) was also noted, and TILs were scored as none (0), mild (1), moderate (2), or severe (3) in intensity, as previously described (5). Eleven specimens were subjected to laser capture microdissection (LCM) followed by cDNA-mediated Annealing, Selection, extension and Ligation (DASL) microarray to profile differential gene expression between 5 PD-L1+ and 6 PD-L1(−) melanomas. Another set of 11 specimens, including four specimens previously assessed with microarray and 7 new cases, was used to validate differential expression of candidate genes with quantitative (q)RT-PCR, including 6 PD-L1+ cases and 5 PD-L1(−) specimens with TIL intensities similar to the original set (detailed in Supplementary Methods). A separate cohort of 8 lymph node metastases was used to develop amplified in situ hybridization (ISH) detection methods for LAG3 expression, and 25 lymph node metastases were used to investigate geographic relationships between tumor cell PD-L1 expression and TIL LAG-3 expression with IHC. Six specimens were each included in two cohorts. Studies were approved by the Institutional Review Board at the Johns Hopkins University School of Medicine.

Laser capture microdissection and RNA isolation

Tumor cells and neighboring immune infiltrates (lymphocytes and macrophages) were excised from FFPE melanoma specimens with LCM, avoiding necrotic areas. RNA was isolated as previously described using the High Pure RNA Paraffin Kit (Roche Diagnostics; ref. 5). For PD-L1+ tumors, IHC on neighboring tissue sections was used to identify areas of PD-L1 expression for excision. For PD-L1(−) tumors, regions of tumor and associated infiltrating immune cells were sampled.

Whole-genome microarray analysis

Gene expression was detected by DASL assays arrayed on the Illumina Human HT-12 WG-DASL V4.0 R2 expression bead chip (GEO platform GPL14951), per the manufacturer’s specifications. This platform detects 29,377 annotated transcripts and is designed to detect partially degraded mRNAs such as typically found in FFPE tissue specimens (8). Briefly, mRNA isolated from melanoma specimens was reverse transcribed and amplified by PCR using universal primers. PCR products were denatured and hybridized to the Illumina array, washed and scanned to obtain gene-expression intensity data. A single intensity (expression) value for each Illumina probe on the DASL array was obtained using Illumina GenomeStudio software with standard settings and no background correction (the dataset is available at NCBI’s Gene Expression Omnibus under the GEO Series accession number GSE65041). On the basis of examining the histograms of the expression values for each sample, which were generally bimodal, a probe was considered to be Present in a given sample if its corresponding expression value was at or above 1,024 (210), and no normalization was performed. The expression values for all probes and samples were log (base 2) transformed before performing statistical analysis. Analysis for differential expression was carried out for each Illumina microarray probe. Lists of genes passing specified distinguishing criteria were examined for significant enrichment in gene annotation categories, and in functionally related categories, including KEGG pathways, using the DAVID web tool (http://david.abcc.ncl.ac.uk/; refs. 9, 10). Additional details are provided in Supplementary Methods.

Multiplex qRT-PCR

Total RNA from each melanoma specimen was reverse-transcribed, premultiplied, and added to TaqMan Array Micro Fluidic cards per protocol (Applied Biosystems; see Supplementary Methods). These cards were custom-designed with 64 gene-specific primers/probes in triplicate, including internal controls (Supplementary Table S1). PCRs were run using a 7900 HT Fast Real Time PCR system, and data analysis and display were performed using the manufacturer’s software (Applied Biosystems). Analysis was based on tumor PD-L1 expression status (positive or negative by IHC), using the manufacturer’s software.

Analysis of lymphocyte activation gene 3 (LAG-3) expression in tissue sections

LAG-3 protein expression was analyzed in 5-μm thick FFPE tissue sections by IHC. Antigen retrieval was performed at 120°C for 10 minutes in citrate buffer, pH 6.0. Primary anti-LAG-3 mAb (murine anti-human clone 17B4; LS Bio) was used at a concentration of 1.0 μg/mL and incubated for 2 hours at room temperature. An anti-mouse Ig HRP polymer detection kit was used for visualization (ImmPRESS, Vector Laboratories). Cases where ≥5% of TILs expressed LAG-3 were considered positive.

LAG3 mRNA expression was detected by amplified ISH, performed by an automated stainer (Ventana Discovery Ultra, Ventana Medical Systems) using the RNAscope Kit (Advanced Cell Diagnostics Inc.) according to the manufacturer’s instructions. In brief, 5-μm thick FFPE tissue sections were deparaffinized and rehydrated before pretreatment with heat and protease. They were then hybridized with LAG3-specific probes, followed by the application of the preamplifier, amplifier, and horseradish peroxidase-labeled probes (Advanced Cell Diagnostics). Color
development was performed with dexamethasone. Probes for products of the bacterial gene adaP and the housekeeping gene PPIB (peptidylprolyl isomerase B, cyclophilin B) were used as negative and positive controls, respectively, for mRNA expression. Brown, punctate dots visualized in the cytoplasm by light microscopy were considered positive signals.

Cell cultures

Human monocyte and T-cell cultures were generated from leukapheresis specimens from consenting donors under IRB-approved protocols. Peripheral blood mononuclear cells (PBMC) were isolated following density gradient centrifugation (Ficoll-Hypaque, GE Healthcare) and cryopreserved. Short-term monocyte cultures were established from thawed PBMCs by plastic adherence for 2 hours at 37°C, in RPMI-1640 medium with 10% heat-inactivated AB serum (Life Technologies). Nonadherent cells were removed by washing to enrich for adherent monocytes. CD3+ T cells were isolated from PBMCs using the MACS Pan T Cell Isolation Kit II (Miltenyi Biotec) and cultured in RPMI-1640 medium with 10% heat-inactivated AB serum and cytokines as described above. The melanoma cell lines 537-mel, 1363-mel, and 1558-mel, established from metastatic melanoma specimens as previously described (11), were cultured in RPMI-1640 medium with 10% heat-inactivated FBS (Life Technologies; or Sigma-Aldrich). All cultures were maintained in a 37°C, 5% CO2 incubator.

Recombinant cytokines and chemokines

Commercially available recombinant human cytokines and chemokines were added to cell cultures at final concentrations of 100 or 250 ng/mL, except IFN-gamma (IFN-g), which was used at 100 or 250 IU/mL. These concentrations were selected as biologically active based on published literature. IL10, CCL5 (RANTES), and CXCL1 were purchased from Peprotech; IL18, IL10, CCL5, and 1558-mel, established from metastatic melanoma specimens as previously described (11), were cultured in RPMI-1640 medium with 10% heat-inactivated FBS (Life Technologies; or Sigma-Aldrich). All cultures were maintained in a 37°C, 5% CO2 incubator.

Effects of cytokines and chemokines on PD-L1 expression by cultured melanomas

Cultured melanoma cells were seeded into 24-well plates at 50% confluence and allowed to adhere for at least 24 hours. Then they were cultured under three conditions: without cytokines, with individual cytokines or chemokines, or with a combination of IFN-g plus each cytokine or chemokine. Cells were harvested after 1, 2, or 3 days and costained for CD14 and the following markers: PD-1, PD-L1, and CD69. Monocytes in these cultures were evaluated by gating on CD3(–)FSC(hi)/SSC(hi) events.

To determine the effects of IL32-g on T cells in the absence of monocytes, CD3+ cells were isolated (>98% purity) and cultured with or without anti-CD3/CD28 stimulation in the presence or absence of IL32-g (100 ng/mL) for 1 or 3 days. Cells were then costained for CD8 or CD4, and the following markers: PD-1, PD-L1, and CD69.

To investigate the effects of IL10 and IL32-g on monocytes in the absence of lymphocytes, monocytes were enriched by plastic adherence and cultured under the following conditions: (i) no cytokines; (ii) IFN-g alone (100 or 250 IU/mL); (iii) IL10 alone or IL32-g alone (100 ng/mL); and (iv) IFN-g plus either IL10 or IL32-g. After 2 days, cells were collected and costained for CD14 and the following markers: PD-1, PD-L1, PD-L2 (clone MH118), CD66, and HLA-DR.

Flow cytometric analysis

Nonspecific mouse IgG (Life Technologies) was used to block Fc receptors on PBMCs and enriched monocytes. All fluorochrome-conjugated specific mAbs and their isotype-matched controls were purchased from BD Biosciences or eBioscience. Samples were acquired on the BD FACSCalibur and data were analyzed with FlowJo Software (TreeStar).

Results

Whole-genome microarray and functional clustering analysis

To identify genes differentially expressed between PD-L1+ and (−) melanomas, LCM was used to precisely capture boundary areas containing tumor cells and TILs (“immune fronts”) from 5 PD-L1+ melanoma specimens and 6 PD-L1−melanomas. Although there is a general association between the presence of TILs and PD-L1 expression, a significant number of TIL-infiltrated tumors do not express PD-L1 (5); this subset of tumors represented the PD-L1(−) subset. Cases with PD-L1 expression had moderate to severe TILs (grade 2–3), whereas PD-L1(−) cases had more modest immune infiltrates (grade 1). High-throughput whole-genome microarray analysis was performed. The list of 1,660 Illumina probes upregulated at least 2-fold in PD-L1+ specimens (Supplementary Table S2) was submitted to the NIH DAVID database for functional annotation clustering. Twelve resulting categories containing ≥10 distinct genes from this list and having a Benjamini–Hochberg adjusted P value (FDR) ≤0.015 were obtained (12). As shown in Fig. 1 and Supplementary Tables S3 and S4, genes overexpressed in PD-L1+ melanomas were functionally related in pathways involving immune cell activation, inflammation, and antigen processing and presentation, among others. These results demonstrate an enhanced immune-reactive microenvironment in PD-L1+ compared with PD-L1(−) melanomas.

Gene-expression profiling with multiplex qRT-PCR

A custom qRT-PCR array was designed to validate differential expression of genes revealed by whole-genome microarray analysis, and to test additional candidate genes potentially associated with tumor PD-L1 expression. A new cohort of 11 melanoma specimens (6 PD-L1+ and 5 PD-L1(−)) was subjected to LCM and RNA isolation. Sixty genes were analyzed, including 45 candidate genes and 15 genes selected from the microarray analysis based on their degree of upregulation in PD-L1+ melanomas and functional relevance to immunoregulatory pathways (Supplementary...
Impact of cytokines and chemokines on melanoma cell expression of PD-L1

Several cytokines and chemokines that were overexpressed in the PD-L1+ melanoma microenvironment were tested in vitro for their potential effects on PD-L1 expression by three melanoma cell lines. These recombinant proteins included CCL5 (RANTES), CXCL1, IL10, IL18, and IL21 (Supplementary Table S5). In addition, the alpha and gamma isoforms of IL32 were assessed in vitro. IL32 mRNA, detected by one of two probes in the whole-genome microarray recognizing a shared sequence in all IL32 isoforms, was upregulated 15-fold in PD-L1+ melanomas (P = 0.014; Supplementary Table S2). Although this was not validated by qRT-PCR, the available commercial probe detected a different region of IL32. The melanomas 537-mel, 1363-mel, and 1558-mel, known to upregulate cell surface PD-L1 and HLA-DR protein expression in response to IFN-γ exposure in vitro (5), were incubated with each recombinant protein at 100 or 250 ng/ml, with or without IFN-γ at 100 IU/ml. Notably, none of these individual factors or combinations affected the intensity of melanoma cell surface PD-L1 or HLA-DR expression after 1, 2, or 3 days of culture (not shown). In addition, at 6 days, no effects were observed on melanoma proliferation. Therefore, these factors did not appear to directly affect melanoma cell expression of PD-L1 in vitro.

Impact of cytokines and chemokines on immune cell expression of PD-1 and its ligands

We next investigated the potential effects of factors overexpressed in PD-L1+ melanomas, on immune cell expression of PD-1 and its ligands. PBMCs were cultured for 1 to 3 days in the absence or presence of recombinant CCL5, CXCL1, IL10, IL18, IL21, IL32-a, or IL32-g. Cultures were conducted with or without T-cell stimulation with anti-CD3 alone (suboptimal) or in combination with anti-CD28 (optimal). Changes in cell surface expression of PD-1, PD-L1, and the activation markers HLA-DR and CD69 on CD3+ cells, or on CD3+(−)FSC<hi>SSC<hi> cells (monocyte population), were examined.

When IL10 was present during T-cell stimulation with anti-CD3 or anti-CD3/CD28, decreased activation was observed, evidenced by decreased expression of PD-1, PD-L1, CD69, and HLA-DR on CD3+ cells in 2 of 2 donors tested (not shown). At the same time, increased expression of PD-L1 and decreased expression of HLA-DR on CD3+(−)FSC<hi>SSC<hi> cells (monocyte population) were seen. To determine whether IL10 directly affected monocytes in the absence of T cells, monocytes were enriched from PBMCs by plastic adherence and were exposed to IL10 for 48 hours in the presence or absence of IFN-γ. IL10 alone selectively increased expression of the coinhibitory ligands PD-L1 and PD-L2 on CD14+ cells, while
decreasing the costimulatory molecules CD86 and HLA-DR. As expected, IFN-g alone upregulated expression of all four molecules. Combining IL10 with IFN-g further increased PD-L1 expression, compared with either cytokine alone. However, this cytokine combination reduced PD-L2, CD86, and HLA-DR expression below levels achieved with IFN-g alone, and in some cases below baseline levels in the absence of cytokines (Fig. 4A). Thus, IL10 appeared to dampen T-cell activation while shifting the balance of monocyte expression of coregulatory molecules toward an immunosuppressive profile.

Among the other factors tested, IL32-g had a reproducible effect on the expression of PD-1 ligands by cultured PBMCs. When unseparated PBMCs were cultured for 1 to 3 days in the presence of IL-32-g, both the proportion and intensity of PD-L1 and CD69 expression on CD3$^+$ T cells (CD8$^+$ and CD8(-)) increased (Supplementary Fig. S3). No effects were observed on anti-CD3/CD28 stimulated cells, which abundantly expressed PD-L1 and CD69. Interestingly, purified CD3$^+$ T cells were not affected by IL32-g (3 of 3 donors), implying that effects on T cells in unseparated PBMCs were mediated by factors produced by non-T cells. Because PD-L1 expression...
was also increased on CD3(−) FSChighSSC hi cells in unseparated PBMCs cultured with IL32-g, we next exposed enriched monocyte cultures to IL32-g. Similar to the effects observed with IL10, IL32-g alone was found to increase the expression of PD-L1 and PD-L2 on CD14+ cells, and in combination with IFN-g it mitigated IFN-induced enhancement of PD-L2, CD86 and HLA-DR expression (Fig. 4B). These effects were specific to IL32-g and were not observed with IL-32-a. Furthermore, when supernatants from IL32-g–exposed monocytes were added to purified CD3+ T cells, a modest increase in PD-L1 expression was observed on both CD8+ and CD4+ T cells, suggesting complex mechanisms by which IL32-g may modulate the expression of immune-regulatory molecules intratumorally.
Coexpression of Immune-Regulatory Genes in PD-L1 Melanomas

**Discussion**

Many human cancers contain tumor and/or stromal cells expressing the immunosuppressive ligand PD-L1. Tumor cell PD-L1 expression may result from dysregulated signaling pathways or somatic gene alterations including amplifications and translocations (14). However, its expression in melanoma is characterized by intratumoral immune infiltrates, a phenomenon termed "adaptive immune resistance" (5). IFN-g, a major inducer of human melanoma-specific TILs, is a major inducer of PD-L1 expression on tumor cells in vitro (15) and is selectively overexpressed in the milieu of PD-L1+ solid tumors, including melanomas (5, 6). This study confirms an association of IFN-g with PD-L1+ melanomas and goes beyond this to explore other factors coordinately expressed in the PD-L1+ melanoma microenvironment. It reveals a complex landscape of interacting receptors, ligands, and soluble factors, which may be exploited to therapeutic advantage.

Several of the molecules that were found to be significantly overexpressed in PD-L1+ versus PD-L1− melanomas are associated with activated CD8+ T cells (CD8A, PRF1, IL18, and IL21). The observed coexpression of mRNAs encoding CD274 and CD8A is consistent with published reports detecting the colocalization of PD-L1+ melanoma cells with infiltrating CD8+ T cells by IHC (16, 17). The presence of CD8+ TILs has recently been proposed as a biomarker of response to PD-1 blockade in melanoma (16). In addition to markers of T-cell activation, we also found evidence for overexpression of molecules associated with activated proinflammatory APCs, including CXCL1, TLR3, and LY2. This constellation of factors characterizes an immune-reactive microenvironment poised to eliminate cancer cells, if not for dominant inhibition exerted by the PD-L1 checkpoint.

Importantly, additional checkpoints were found to be overexpressed in the PD-L1+ melanoma microenvironment, including PDCD1, LAG3, and IL10. Although LAG-3 and IL10 appear to be subdominant in the hierarchy of intratumoral immunosuppression, they may nevertheless provide bypass mechanisms for melanoma to evade anti–PD-1/PD-L1 therapies. These findings suggest opportunities for cotargeted combination treatment regimens. For instance, coexpression of PD-1 and LAG-3, a distinct inhibitory receptor expressed on activated T cells, has been demonstrated in murine and human TILs (4, 18). Blocking LAG-3 is marginally effective as monotherapy but synergizes with anti–PD-1 in murine tumor models, providing a rationale for an ongoing clinical trial of anti–LAG-3 plus anti–PD-1 in patients with advanced solid tumors (NCT019568109). IL10 is an anti-inflammatory cytokine produced by T helper cells, regulatory cells, monocytes, and some human cancers (19). IL10 secretion by T cells has been shown to be stimulated by PD-L1 (20). In this study, IL10 exposure inhibited human T-cell activation and promoted monocyte PD-L1 expression in vitro, consistent with published reports (21), supporting a checkpoint role for this cytokine. The potential for IL10 to mediate nonantigen driven LAG-3 expression on human tumor-specific T cells has also been reported (22), further expanding the immunosuppressive profile of this cytokine. Finally, our study also revealed overexpression of the proinflammatory cytokine IL32 in the PD-L1+ melanoma microenvironment, although the cellular source of this cytokine was not determined. IL32 has been associated with progression and metastasis in some human cancers (23). Its most active isoform, IL32-g, has been shown to activate monocytes and promote their secretion of IFN-g and TNF-α (24, 25). However, the specific effects of IL32-g on lymphocyte and monocyte PD-L1 expression demonstrated here have not been reported previously to our knowledge, and suggest a complex functional profile for this cytokine which may contribute to local tumor immunosuppression. Notably, neither IL10 nor IL32-g affected PD-L1 expression by melanoma cells, either with or without IFN-g, which could reflect lack of expression of the corresponding cytokine receptors by tumor cells or differences in downstream signaling between melanoma cells and monocytes.

In summary, although IFN-g appears to be a dominant factor mediating adaptive immune resistance in melanoma and other human tumors, this study reveals additional interacting factors contributing to this phenomenon. Some of these factors may selectively enhance PD-L1 expression on tumor infiltrating myeloid cells rather than tumor cells. It is only by identifying and targeting such coordinated immunosuppressive molecular networks that the impact of anti–PD-1/PD-L1 immunotherapies can be fully realized.

**Disclosure of Potential Conflicts of Interest**

J.M. Taube reports receiving a commercial research grant from and is a consultant/advisory board member for Bristol-Myers Squibb. D.M. Pardoll has ownership interest (including patents) in Bristol-Myers Squibb. S.L. Topalian reports receiving a commercial research grant from and holds ownership interest (including patents) in Bristol-Myers Squibb, and is a consultant/advisory board member for Bristol-Myers Squibb, Five Prime Therapeutics, GlaxoSmithKline, and Joune Therapeutics. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

Conception and design: J.M. Taube, G.D. Young, S.L. Topalian


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.M. Taube, G.D. Young, T.L. McMiller, S. Chen, J.T. Salas, T.S. Pritchard, A.E. Berger, D.M. Pardoll, S.L. Topalian

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.M. Taube, G.D. Young, T.L. McMiller, S. Chen, J.T. Salas, T.S. Pritchard, C. Cheadle, A. E. Berger, D. M. Pardoll, S. L. Topalian

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**Acknowledgments**

The authors thank Dr. Lieping Chen (Yale University) for providing the anti–PD-L1 monoclonal antibody SH1; Drs. Chris Umbricht and Mariana Friat (Johns Hopkins University) for technical advice; Dr. Maria Asciento (Johns Hopkins University) for helpful discussions; and Jung H. Kim for technical assistance.

**Grant Support**

This work was supported by the Melanoma Research Alliance (to J.M. Taube, D.M. Pardoll, and S.L. Topalian), the Dermatology Foundation (to J.M. Taube), the National Cancer Institute (1R01CA142779; to D.M. Pardoll and S.L. Topalian), the Barney Foundation (to J.M. Taube, D.M. Pardoll, and S.L. Topalian), the Laverna Hahn Charitable Trust (to J.M. Taube and S.L. Topalian), the Commonwealth Foundation (to J.M. Taube and D.M. Pardoll), Moving for Melanoma of Delaware (to J.M. Taube, D.M. Pardoll, and S.L. Topalian), and a Stand Up To Cancer—Cancer Research Institute for helpful discussions; and Jung H. Kim for technical assistance.
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Cancer Immunology Translational Cancer Research Grant (S12CA-AACR-DT1012) to J.M. Taube, D.M. Pardoll, and S.L. Topalian. Stand Up To Cancer is a program of the Entertainment Industry Foundation administered by the American Association for Cancer Research.

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Received January 29, 2015; revised March 31, 2015; accepted April 16, 2015; published OnlineFirst May 5, 2015.

References


Clinical Cancer Research

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