**Temporally Distinct PD-L1 Expression by Tumor and Host Cells Contributes to Immune Escape**


**Abstract**

Antibody blockade of programmed death-1 (PD-1) or its ligand, PD-L1, has led to unprecedented therapeutic responses in certain tumor-bearing individuals, but PD-L1 expression’s prognostic value in stratifying cancer patients for such treatment remains unclear. Reports conflict on the significance of correlations between PD-L1 on tumor cells and positive clinical outcomes to PD-1/PD-L1 blockade. We investigated this issue using genomically related, clonal subsets from the same methylcholanthrene-induced sarcoma: a highly immunogenic subset that is spontaneously eliminated in vivo by adaptive immunity and a less immunogenic subset that forms tumors in immunocompetent mice, but is sensitive to PD-1/PD-L1 blockade. Using CRISPR/Cas9-induced loss-of-function approaches and overexpression gain-of-function techniques, we confirmed that PD-L1 on tumor cells is key to promoting tumor escape. In addition, the capacity of PD-L1 to suppress antitumor responses was inversely proportional to tumor cell antigenicity. PD-L1 expression on host cells, particularly tumor-associated macrophages (TAM), was also important for tumor immune escape. We demonstrated that induction of PD-L1 on tumor cells was IFNγ-dependent and transient, but PD-L1 induction on TAMs was of greater magnitude, only partially IFNγ dependent, and was stable over time. Thus, PD-L1 expression on either tumor cells or host immune cells could lead to tumor escape from immune control, indicating that total PD-L1 expression in the immediate tumor microenvironment may represent a more accurate biomarker for predicting response to PD-1/PD-L1 blockade therapy, compared with monitoring PD-L1 expression on tumor cells alone.

**Introduction**

Monoclonal antibody (mAb) blockade of programmed death-1 (PD-1) or its major ligand PD-L1 can provoke durable antitumor responses in some cancer patients and tumor-bearing mice (1–5). Whereas expression of PD-1 is largely restricted to lymphocytes, PD-L1 has been observed on a wide variety of cells present in the tumor microenvironment, including tumor cells, lymphocytes, myeloid cells, and cells of epithelial and endothelial origin (6–8). Although high constitutive PD-L1 expression has been noted in a few tumors, it is more commonly induced in tumor and normal cells by cytokines, especially IFNγ (9). The complexity of PD-L1 expression has made it difficult to identify the specific PD-L1–expressing cells that contribute to a tumor’s escape from immune control. This issue has important mechanistic and clinical implications because PD-L1 expression may stratify patients for response to anti–PD-1/PD-L1 immunotherapy (3, 5, 10, 11). Past attempts to resolve this dilemma have been inconclusive (12–23). In addition, PD-L1 on immune cells is expressed more frequently than that on tumor cells in patients with non–small cell lung cancer, urothelial carcinoma, and esophageal squamous cell carcinoma (24–26), suggesting distinct extrinsic regulatory pathway(s) are involved with tumor versus immune cell PD-L1 induction. Here, using our well-characterized, methylcholanthrene (MCA)-induced sarcoma system (27–29), we investigated whether (i) PD-L1 expression on tumor cells known to be sensitive to anti–PD-1/anti–PD-L1 checkpoint blockade in vivo was required for tumor immune escape; (ii) the capacity of PD-L1 to inhibit immune elimination of a tumor was linked to the antigenicity of that tumor; (iii) PD-L1 expression on host cells participated in the process; and (iv) the extrinsic PD-L1 induction on tumor versus host immune cells was regulated in a distinct manner.

**Materials and Methods**

**Mice**

Male wild-type (WT) and Rag2−/− mice on a 129S6 background were purchased from Taconic Farms. Female WT mice on a C57Bl/6J background were purchased from The Jackson Laboratory. Mice used in the study were between 8 and 12 weeks of age and maintained in accordance with procedures approved by the
Association of Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited Animal Studies Committee of Washington University in St. Louis.

Tumors
MCA-induced sarcoma cells used in this study were previously generated in male 129S6 WT and Rag2−/− mice (27). Resequencing of d42m1-T3 and d42m1-T9 cells confirmed their genomic stability over time. As variant calling algorithms have become significantly more accurate since our initial reporting of these cell lines (30), we reassessed the mutational landscapes of T3 and T9 cells using the original sequence data and data from tumor cell line resequencing and found that the number of expressed missense mutations in T3 and T9 were 827 and 815, respectively. This modification did not lead to an alteration of either the predicted or found dominant antigenic epitopes. Whereas the genomic landscapes of T3 and T9 were clearly similar to one another, they were completely distinct from that of F244, an independent MCA sarcoma derived from a different 129S6 WT mouse, expressing 943 other missense somatic mutations with the single exception that T3/T9 and F244 cells expressed an identical activating Kras G12C mutation.

Tumor cells were maintained in vitro in RPMI media (Hyclone) supplemented with 10% FCS (Hyclone) for less than 3 weeks prior to use in experiments. Note that 1.0 × 10⁷ tumor cells were injected subcutaneously unless otherwise indicated. Tumor growth was monitored at least 2 times a week using a digital caliper. The mean of long and short diameters was used for tumor growth curves. Mice were euthanized when tumors were > 2 cm or severely ulcerated. No statistical methods were used to predetermine sample size. However, adequate sample size was chosen based on extensive previous work with this animal model. No randomization or blinding was performed. Ex vivo analyses were performed as previously described (29). Murine glioma cell line GL261 with ectopic expression of murine PD-L2 (GL261-PD-L2) was kindly gifted from G.P. Dunn (Washington University School of Medicine). For detection of PD-L1 and MHC class I expression in vitro, tumor cells were treated with 300 U/mL murine IFNγ for 48 hours and PD-L1 cDNA amplification by PCR using a forward primer (5′-AGATCTATGAG-GATATTGCCTGGAATT-3′) and a reverse primer (5′-CTCGACTTACGTCTCCTGCAAATTGGTGTATC-3′). The PD-L1 cDNA was subsequently cloned into the pCR-TOPO-Blunt II vector (Invitrogen). The PD-L1 cDNA cloned from the MCA sarcoma cells showed an identical sequence to that from a spleen in a naïve 129S6 male mouse (data not shown).

Generation of expression-transduced tumor cells using the retroviral system
The retrovector with GFP (RV-GFP) was a gift of K. Murphy, Washington University. For generation of the retrovector without GFP (RV), RV-GFP was digested with SalI and self-ligated. Following digestion of the PD-L1-pCR-TOPO Blunt II vector with BglII and Xhol, PD-L1 cDNA was subcloned into the RV (RV-PD-L1). After 48 hours of retroviral production (28), the supernatant was subsequently used for transfection with tumor cells. Tumor clones such as T9-PD-L1GFP and T9-PD-L1GFP cells were obtained by limiting dilution.

Mutation-specific RT-PCR and qRT-PCR
The procedures for detection of mutant Spectrin-β2 by RT-PCR followed by restriction enzyme digestion were previously described in detail (28). For detection of mutant Lαm4 by qRT-PCR, a forward primer (5′-GGATGCCCGACGACACTCTC-TG-3′) and a reverse primer (5′-GTATGGTCTGAAATTTGAGGCTTA-3′) were used. For detection of mutant Alg8 by qRT-PCR, a forward primer (5′-TCCCGTTACCTTCCTCCAGG-3′) and a reverse primer (5′-AGCATACCGCCCTGCCAGGT-3′) were used.

In vitro cytotoxicity assay
The mutant Spectrin-β2–specific T-cell line (C3) was established as previously described (28). Following treatment with 300 U/mL IFNγ for 48 hours, tumor cells were labeled with eFluor 670 (eBioscience) at 0.5 μmol/L as a target. 10,000 tumor cells and different numbers of T cells at the indicated ratios were incubated in a well of a 96-well plate for 12 hours. Another 10,000 tumor cells labeled with eFluor 670 at 5 μmol/L were used to calculate numbers of tumor cells killed as a reference. Dead cells were stained with Po-Pro-1. Killing efficiency was calculated by the following formula: 100% × (1 – [(% tumor cells with 5 μmol/Lcontrol × (% tumor cells with 0.5 μmol/L)target) / (% tumor cells with 5 μmol/Lcontrol × (% tumor cells with 5 μmol/L)target)].
ELISA
Following treatment with 300 U/ml IFNγ for 48 hours, tumor cells were irradiated at 10,000 rads. T cells and tumor cells were cocultured in a well of a 96-well plate for 48 hours. IFNγ concentration in the supernatant was measured by mouse IFN gamma ELISA Ready-SET-Go! (eBioscience).

Generation of PD-L1 knockout tumor lines using CRISPR-Cas9
To generate T3 lines lacking PD-L1 expression (T3APDL1.1-7) and a F244 line lacking PD-L1 expression (F244APDL1.1), we designed the single-guide RNAs (sgRNA) at http://crispr.mit.edu in June 2014. The sgRNA targeting mouse PD-L1 (5'-GTATGGCAGCAACTGCTACGA-3') was subcloned into the pX330 (pX330-PD-L1; Addgene plasmid 42230). Tumor cells were transiently transfected with pX330-PD-L1 and pmaxGFP (Lonza) using FuGENE HD (Promega) according to the manufacturer’s instruction. GFP-positive cells were subsequently sorted 72 hours after transfection. Following generation of clones by limiting dilution, we performed targeting deep sequencing of the PD-L1 genomic locus and confirmed the presence of premature stop codons in all alleles. For detection of Cas9 cDNA, a forward primer (5'-CCGAAAGGGCAAAACCACA-3') and a reverse primer (5'-CCGAGCTCGTGAGAACG-3') were used. PD-L1 WT parental tumor cells treated with pX330 and pmaxGFP, and the PD-L1 knockout were subsequently transduced with either the RV or the RV-PD-L1 to generate T3WT-Mock, T3APDL1-Mock, and T3APDL1-APDL1. For the other F244 tumor lines lacking PD-L1 expression (F244APDL1.2 and F244APDL1.3), the extracellular domain of murine PD-L1 was genetically deleted by two-step CRISPR-Cas9. The sgRNAs targeting 5'-AAAGAAGGCACTGATATAGTATAGTACATA-3' (g7) and 5'-TTCTACTACAGCAGCCGG-3' (g43) were used for the first step. Subsequently, those targeting 5'-CAGCAGTGGCAGCTAGGGTA-3' (g2) and 5'-AGTCATTGAG-3' (g29) were used for the second. Gene deletion was confirmed by diagnostic PCR. For generation of MC38 lines lacking PD-L1 expression (MC38APDL1.1 and MC38APDL1.2), MC38 cells were transiently transfected with a vector encoding Cas9-2A-GFP and the guide oligo (5'-GCCAGGGCAAAACCACAG-3') derived from exon IV of the mouse PD-L1 gene.

cDNA-CapSeq and mutation calling
Following data generation and alignment of Illumina paired-end reads to the mouse reference genome sequence, somatic variant analysis was done comparing tumor cDNA-CapSeq data with matched normal exome data. We used a combination of three variant callers—Samtools (33, 34), Sniper (35), and VarScan (36, 37)—as previously described (30). Missense mutations were then translated into a 17-mer aminoadic Fasta sequence and analyzed through pVAC-Seq (30) to identify and shortlist potential high-affinity neoantigens. Briefly, to only target variants in the expressed genes, we restricted our subsequent analysis to genes with expression level (in fragments per kilobase of exon per million reads mapped) values of > 1, and wherein we could identify evidence that the mutant allele was expressed. Also, we filtered out any variants with normal coverage ≤ 5 × and normal VAF of ≥ 2%. In addition, only variants with tumor coverage of ≥ 10 × with a VAF of ≥ 25% were considered.

Statistical analysis
Prism 6 (GraphPad Software, Inc.) was used for statistical analysis. No samples or animals were excluded from the analysis. Comparison between samples was performed using an unpaired, two-tailed Student t test or one-way ANOVA followed by multiple comparison test. Welch corrections were used when variances among groups were unequal. P < 0.05 was considered as statistically significant.

Results
We previously reported that the d42m1 sarcoma line, derived from an MCA-treated immunodeficient 129S6 strain Rag2<−/− mouse, comprises two genomically related tumor cell subsets that display distinct immunogenicities (refs. 28, 29; Fig. 1). The
major subset, comprising approximately 80% of d42m1 cells (exemplified by d42m1-T9 (T9) cells), represents the highly immunogenic, unedited (38) variant because it expresses an R913L somatic point mutation in Spectrin-B2 (mSb2) that functions as a strong rejection neoantigen responsible, at least in part, for the spontaneous elimination of T9 cells when transplanted into naïve syngeneic WT recipients. The minor subset, comprising approximately 20% of d42m1 cells (exemplified by d42m1-T3 (T3) cells), represents edited variants of d42m1 that emerge following T-cell–dependent immunoselection of parental d42m1 sarcoma cells. T3 cells do not express mutant mSb2, are capable of forming progressively growing tumors in WT mice, but can be immunologically eliminated when tumor-bearing mice are treated with mAbs that block the PD-1/PD-L1 axis (Fig. 1) or CTLA-4 (29). Such checkpoint blockade–induced immune rejection of T3 tumors is the result of reinvigoration of T cells with specificities against two dominant neoantigens specifically expressed in T3 cells derived from somatic point mutations in Laminin α subunit 4 (mLama4) and Asparaginyl-linked glycosylation 8 (α-lgC-glucosyltransferase; mAlg8; ref. 29).

We began this study by asking whether PD-L1 expression on T3 sarcoma cells plays an important role in preventing their immune elimination in vivo. Using a PD-1–guided CRISPR-Cas9 gene editing approach, we generated seven T3-based sarcoma lines lacking PD-L1 (T3APDL1.1–T3APDL1.7). Deep sequencing of the PD-L1 genomic locus of each line showed the presence of premature stop codons in all alleles (Supplementary Fig. S1A). Expression of the Cas9 protein should be transient, but since the mature stop codons in all alleles (Supplementary Fig. S1A).

PD-L1 genomic locus of each line showed the presence of pre-
demonstrating the induction of tumor-specific T3WT, but not challenge with unrelated F244 MCA sarcoma cells, whereas WT T3 cells (T3WT) constitutively expressed low
titative (q)RT-PCR (Supplementary Fig. S1C and S1D). Function-
retained expression of mLama4 and mAlg8 as detected by quan-
To examine the quantitative requirements of PD-L1 expression on the immunogenicity of unedited sarcomas, we isolated T9-PDL1-PDL1 cells, either constitutively or following exposure to IFNγ, into WT recipients. Most PD-L1–transduced T9 cells (T9-PDL1 cells) in the bulk population expressed much more PD-L1 than either untreated or IFNγ-treated parental T9 cells (T9WT; Supplementary Fig. S4B). T9-PDL1 cells, but not mock-transduced T9 cells, formed progressively growing tumors in WT mice (Supplementary Fig. S4C). All progressively growing T9-PDL1 tumors retained expression of mSb2 (Supplementary Fig. S4D and S4E), thereby ruling out the possibility that tumor cell outgrowth was due to loss of the major rejection antigen.

To further assess the functional consequences of differences in PD-L1 expression on T9-PDL1 cells, we compared their relative sensitivities to in vitro killing by the C3 mSb2-specific CTL clone. T9-PDL1mSb2 cells were poorly killed by C3 CTL compared with T9-PDL1mSb0 cells or parental T9 sarcoma cells (Supplementary Fig. S3A and S3B). However, when anti-PD-1 or anti-PD-L1 was added into the in vitro culture, killing efficiency against T9-PDL1mSb2 cells was restored to levels similar to those against T9-PDL1mSb0 or parental T9 sarcoma cells (Fig. 5E). Similar results were obtained when IFNγ secretion from C3 CTL was used as the read-out (Fig. 5F).

To confirm the generality of these findings, we enforced PD-L1 expression in a second, unedited highly immunogenic MCA sarcoma line (H311m1; refs. 28, 43, 44; Fig. 6A). As was the case for T9-PDL1 cells, levels of ectopically expressed PD-L1 on H311m1-PDL1 were considerably higher than those on the
Figure 2.
Ablation of PD-L1 in edited T3 sarcoma cells leads to augmented growth inhibition in WT mice. A, *In vitro* PD-L1 and MHC class I (H2-Kb) expressions on cells treated with IFNγ were analyzed by flow cytometry. Black, isotype control; blue, untreated; red, IFNγ treated. Data are shown from at least three independent experiments. B, *In vivo* tumor growth of T3WT and T3ΔPDL1 lines in syngeneic WT (black) or Rag2−/− mice (red). T3WT are parental T3 sarcoma cells. T3ΔPDL1.1-T3ΔPDL1.7 are T3 lines treated with CRISPR-Cas9 þ sgRNA that lack PD-L1 expression (T3ΔPDL1). Each panel represents data from two to three independent experiments. Numbers in parentheses show tumor-free WT mice/total WT mice on day 50 after transplantation. C, Mice rejecting T3ΔPDL1 cells mount a memory response to parental T3 cells. Seven naive syngeneic WT mice were challenged with T3ΔPDL1.1 cells on day 0. After *in vivo* rejection, mice were rested for 45 days and then challenged with T3 (n = 4) or F244 (n = 3) sarcoma cells. Data are shown from at least two independent experiments. D, *In vitro* IFNγ secretion from mutant Lama4-specific T cells (CTL74.17) against T3WT, T3ΔPDL1.2, and T3ΔPDL1.6 cells. Data are shown by mean ± SEM of technical duplicates from two independent experiments. Samples were compared using an unpaired, two-tailed Student t test. *, P < 0.05; **, P < 0.01.
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In vitro experiments (n = 4H2). Data are shown by mean ± SEM from at least two independent experiments (n = 5).

parental H31m1 cells treated with IFNγ (Fig. 6B). Progressively growing H31m1-PDL1 tumors were observed in 17 of 20 challenged WT mice compared with 0 of 20 WT mice injected with control H31m1 tumor cells (Fig. 6C). Together, these results show that highly immunogenic unedited sarcoma cells require abnormally high expression of PD-L1 to escape immune control and form progressively growing tumors in immunocompetent mice. Thus, levels of PD-L1 expression that can be induced on tumor cells is transient, and PD-L1 expression on tumor cells is tightly linked to IFNγ exposure in vivo, because it was completely inhibited when mice were treated with IFNγ-neutralizing mAb (Fig. 7C). The IFNγ dependency of PD-L1 induction on tumor cells was also observed in vitro (Supplementary Fig. S6A). Similarly, PD-L1 expression on host immune cells in the tumor microenvironment reached maximal values at 9 days and declined thereafter although at a slower rate than seen on tumor cells (Fig. 1; Supplementary Fig. S6A).

This slow decay of PD-L1 expression on host immune cells was particularly noteworthy. We therefore defined three distinctive features about host cell–expressed PD-L1 expression. First, a detailed analysis of the cellular composition of the tumor microenvironment revealed that tumor-associated macrophages were not only the major host cell population in the tumor microenvironment, but they also expressed the vast majority of PD-L1 in the tumor (72% of total PD-L1 on tumor and host cells was expressed on TAMs at day 9; Fig. 7D; Supplementary Figs. S7A and S7B and S8A–S8D). Second, PD-L1 expression on TAMs was retained for long period of time, well after PD-L1 on tumor cells was completely extinguished (Fig. 7C). Third, PD-L1 expression on TAMs was increased in tumor-bearing mice treated with either saturating doses of neutralizing anti-IFNγ (Fig. 7C) or even with amounts that were 8-fold higher (Fig. 7E), although the magnitude of increased PD-L1 expression was less than when IFNγ was present. This observation was not due to changes in the tumor microenvironment by the early mAb injection protocol, because PD-L1 expression on TAMs at day 12 remained high even when IFNγ mAb was injected at day 9 (Fig. 7E). Similar results were obtained when the experiments were repeated with the unrelated F244 sarcoma cell line (Figs. 4A and 7F). Finally, when CD4+ or CD8+ cells were depleted from WT mice, we found that the IFNγ-independent induction/retention of PD-L1 on TAMs required the presence of CD4+, but not CD8+, T cells (Fig. 7F). Thus, PD-L1 on TAMs is induced by two alternative cell-extrinsic pathways involving CD4+ T cells, one that is IFNγ-dependent and one that is IFNγ-independent. Taken together, these results show that TAMs are the major host cell type that contributes PD-L1 in our sarcoma tumor model both quantitatively and temporally.

Discussion

This study provides novel functional and fundamental insights into the roles of PD-L1 in facilitating tumor escape from immune control. On edited tumor cells, whose antigenicity has been tempered by the tumor-sculpting power of immunity, IFNγ-dependent induction of PD-L1 expression initiates the establishment of the immunosuppressive force that facilitates tumor outgrowth. However, PD-L1 expression on tumor cells is transient, and expression is rapidly extinguished. This downregulation of PD-L1 most likely occurs, at least in part, as a consequence of PD-L1’s ability to inhibit IFNγ production by tumor-infiltrating lymphocytes. TAMs are the major cellular sources that maintain expression of PD-L1, long after PD-L1 on tumor cells is extinguished. The temporal dichotomy between PD-L1 expression on tumor cells...
Figure 4. Ablation of PD-L1 in the edited F244 MCA sarcoma and MC38 colorectal carcinoma leads to augmented growth inhibition in WT mice. A, Top, In vivo tumor growth of edited F244 MCA sarcoma cells in WT mice. Tumor-bearing mice were left untreated or treated with anti-PD-1 (RMP1-14) or anti-PD-L1 mAb (10F.9G2). Data are shown by mean ± SEM from at least two independent experiments (n = 5). Bottom, Numbers of PD-L1 molecules on tumor (CD45−) and immune cells (CD45+) in vivo. Data are shown by mean ± SEM from two independent experiments (n = 4). B, In vitro PD-L1 and MHC class I (H2-Kb) expression on F244 tumor lines lacking PD-L1 expression (F244ΔPDL1.1–F244ΔPDL1.3) treated with IFNγ were analyzed by flow cytometry. Black, isotype control; blue, untreated; red, IFNγ treated. Data are shown from at least two independent experiments. C, In vivo tumor growth of F244WT and F244ΔPDL1 lines in syngeneic 129S6 WT (black) or Rag2−/− mice (red). Each panel represents data from two to three independent experiments. Numbers in parentheses show tumor-free WT mice/total WT mice on day 50 after transplantation. D, In vivo tumor growth of MC38WT and MC38ΔPDL1 lines in C57BL/6J WT mice. Tumor cells (0.5 × 10^6) were injected subcutaneously on day 0 in the mice. Mice injected with MC38WT cells were treated on day 7 after implantation with control or anti-PD-1 mAb (4H2). Numbers in parentheses show tumor-free WT mice/total WT mice on day 45 after transplantation. MC38WT, but not MC38ΔPDL1, cells upregulated PD-L1 expression in vitro after IFNγ stimulation as evidenced by flow cytometry. Data shown in this figure are representative of at least two independent experiments (n = 10).
Figure 5.
Physiologic levels of PD-L1 are not sufficient to prevent immune elimination of highly immunogenic unedited T9 sarcoma cells. A, In vitro PD-L1 expression with or without IFNγ on T9 sarcoma cells constitutively expressing ectopic PD-L1 either at physiologic levels comparable with that induced by IFNγ on parental T9 cells (T9-PDL1phy) or those at high level overexpression (T9-PDL1ovr). Data are shown from at least three independent experiments. B, Numbers of PD-L1 molecules expressed on cells after IFNγ treatment in vitro. Data are shown by mean ± SEM of technical triplicates from at least three independent experiments. C, In vivo growth of three clones of T9-PDL1phy or T9-PDL1ovr cells in WT mice. Data are shown by mean ± SEM from at least two independent experiments (n = 5). D, Anti–PD-1 (4H2) or anti–PD-L1 (14D8) leads to tumor rejection in T9-PDL1™–bearing mice. Data are shown by mean ± SEM from two independent experiments (n = 5). E, In vitro cytotoxicity assay of mutant Spectrin–β2–specific CTL (C3) against tumor cells with anti–PD-1 (4H2)/anti–PD-L1 (14D8) blockade. Data are shown by mean ± SEM of technical triplicates from at least two independent experiments. F, In vitro IFNγ secretion from mutant Spectrin–β2–specific CTL (C3) against tumor cells with or without anti–PD-L1 (14D8). Data are shown by mean ± SEM of technical triplicates from at least two independent experiments. Samples in E and F were compared using an unpaired, two-tailed Student t test. *, P < 0.05; **, P < 0.01.
versus TAMs results in the establishment of the immunosuppressive tumor microenvironment in which the majority of PD-L1 is contributed by TAMs as opposed to tumor cells. The prolonged PD-L1 expression on TAMs is cell extrinsic, IFNγ-independent, but requires CD4+ T cells. The eventual discovery of the molecule(s) responsible for the chronic PD-L1 expression by TAMs may well provide new opportunities for cancer immunotherapy.

Our findings also are consistent with two mechanisms by which TAMs could exert their immunosuppressive activity in progressively growing tumors. It is likely that PD-L1–expressing TAMs in tumors are recognized by tumor-specific T cells and deliver their PD-L1–dependent inhibitory signal to these T cells just as if they were tumor cells themselves. This scenario suggests that PD-L1–expressing TAMs function in cis to prolong the immunosuppressive state in the microenvironment of a progressively growing tumor. This mechanism is consistent with that observed in vitro with antigen-presenting cells (APCs) that also bear high amounts of PD-L1 (7, 45). The second scenario is one in which TAMs, or even other host cell types, repress T-cell function by supplying the high levels of PD-L1 in trans. The latter mechanism is consistent with data from in vitro studies in which T-cell activation was assessed following coculture of PD-L1–expressing monocytes with T cells stimulated with anti-CD3/anti-CD28 (46, 47). It is certainly possible that

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**Figure 6.**

H31m1-PDL1 cells form progressively growing tumors in WT mice. **A,** Left: in vivo tumor growth of unedited H31m1 MCA sarcoma cells in WT mice. Mice bearing H31m1 cells were left untreated. Data are shown by mean ± SEM from at least two independent experiments (n = 5). Right: Numbers of PD-L1 molecules on tumor (CD45–) and immune cells (CD45+) in vivo. Data are shown by mean ± SEM from two independent experiments (n = 4). **B,** in vitro PD-L1 expression on cells treated with or without IFNγ (100 ng/mL) for 24 hours. Data are shown from at least two independent experiments. Red, unstained; blue, isotype control; orange, anti–PD-L1. **C,** in vivo tumor growth of H31m1 parental and H31m1-PDL1 tumor cells in WT mice. Tumor cells (10 × 10^6) were injected on day 0. Data shown in this figure are representative of at least two independent experiments (n = 20).
Figure 7.
Host PD-L1 participates in inhibiting immune elimination of T3 sarcoma cells through distinct regulatory machineries. A, Percentage of progressively growing T3ΔPD-L1 tumors in WT mice treated either with control mAb or with anti–PD-L1. Numbers represent mice with progressively growing tumors/all mice injected with the indicated number of tumor cells on day 0. Data are shown from at least two independent experiments. B, Treatment of WT mice with anti–PD-L1 (10F.9G2) leads to rejection of 10^6 T3ΔPD-L1 cells. Data are shown by mean ± SEM from two independent experiments (n = 5). C, PD-L1 expression on tumor cells and TAMs in T3 tumors on days 9 and 12. Red, isotype; blue, untreated mice; orange, mice treated with anti–IFNγ. Data are shown from three independent experiments. D, Absolute numbers of PD-L1 molecules expressed on cell types in pooled three T3 tumors on days 9 and 12. Data are shown by mean ± SEM from three independent experiments (n = 3). E, A large proportion of PD-L1 molecules on TAMs are IFNγ independent. Mice were treated with anti–IFNγ-neutralizing mAb (2.0 mg/mouse) either on days -1 and 6, or on day 9, and injected with T3 sarcoma cells on day 0. PD-L1 expression on tumor cells and TAMs was analyzed on day 12. Data are shown by mean ± SEM from three independent experiments (n = 6). F, CD4^+ T cells contribute to PD-L1 expression on TAMs in the absence of IFNγ. F244 sarcoma cells were injected into either WT or Rag2^-/- mice. WT mice were left untreated or treated with anti–IFNγ-neutralizing mAb, anti–CD4 mAb, anti–CD8 mAb, or the combination. PD-L1 expression on tumor cells and macrophages in the tumors was analyzed on day 8. Data are shown by mean ± SEM from at least two independent experiments (n = 3). Data in D, E, and F were compared using one-way ANOVA followed by multiple comparison test. N.S., not significant; *, P < 0.05; **, P < 0.01; †††, P < 0.0001.
different types of tumors could display distinct distributions of PD-L1 between cancer cells and host cells, or that differences in the nature, quantity, or kinetics of tumor-infiltrating hematopoietic cells determine the ultimate distribution of tumor-versus host cell–expressed PD-L1. Future studies will need to explore these two scenarios in more depth to determine the conditions where one or the other predominates. Therefore, the results of this study argue strongly that PD-L1 expression on either tumor cells or host cells should be used as the biomarker for determining whether a patient is a good candidate for PD-1/PD-L1 blockade therapy, presumably when tumors retain high mutation burden (18, 48).

Finally, this study also provides the fundamental insight that PD-L1 expression on edited tumor cells (such as T3), whose antigenicities have undergone immunologic sculpting, versus highly antigenic unedited tumor cells, such as those that have not undergone cancer immunoediting (ref. 38; e.g., T9), results in very different outcomes. This report thus demonstrates the inverse relationship between tumor antigenicity and the capacity of PD-L1 to promote tumor escape. This observation leads to the logical conclusion that adaptive immune resistance (49), the process wherein immune attack on a tumor results in the upregulation of immunosuppressive moieties that inhibit immune control of the tumor, is relevant predominantly to tumors of either inherently low antigenicity or to tumors that have gone through the cancer immunooediting process, resulting in generation of antigen-loss tumor variants with reduced antigenicities (38, 50). The important implication, then, is that cancer immunooediting and adaptive immune resistance are not separate processes but rather part of the same continuum of immune system–tumor interactions.

Disclosure of Potential Conflicts of Interest
A. Korman, M. Selby, and R. Graziano are employees of Bristol-Myers Squibb and have ownership interest in Bristol-Myers Squibb. R.D. Schreiber reports receiving commercial research grant from Bristol Myers Squibb; has ownership interest (including patents) in BioLegend, Igenica Biopharmaceuticals, Jounce Therapeutics, and Neon Therapeutics; and is a consultant/advisory board member for Novartis and NGM Therapeutics. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Noguchi, M.M. Gubin, M.J. Selby, R.F. Graziano, E.R. Mardis
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Noguchi, C.D. Arthur, A.J. Korman
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References
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