Targeting PD-L1 Initiates Effective Antitumor Immunity in a Murine Model of Cushing Disease


ABSTRACT

Purpose: Although pituitary adenoma is classified as benign, Cushing disease is associated with significant morbidity due to the numerous sequelae of elevated cortisol levels. Successful therapy for Cushing disease remains elusive due to high rates of treatment-refractory recurrence. The frequent emergence of lymphocytic hypophysitis following checkpoint blockade for other cancers, as well as the expression of PD-L1 on pituitary adenomas, suggest a role for immunotherapy.

Experimental Design: This study confirms PD-L1 expression on functioning pituitary adenomas and is the first to evaluate the efficacy of checkpoint blockade (anti-PD-L1) therapy in a preclinical model of Cushing disease.

Results: Herein, treatment with anti-PD-L1 was successful in reducing adrenocorticotropic hormone plasma levels, decreasing tumor growth, and increasing survival in our model. Furthermore, tumor-infiltrating T cells demonstrated a pattern of checkpoint expression similar to other checkpoint blockade–susceptible tumors.

Conclusions: This suggests that immunotherapy, particularly blockade of the PD1/PD-L1 axis, may be a novel therapeutic option for refractory Cushing disease. Clinical investigation is encouraged.

Introduction

Pituitary adenomas are among the most common intracranial tumors, occurring in up to 20% of the general population (1). While classified as benign, as many as 25%–55% of pituitary adenomas are invasive, exhibiting rapid growth patterns and posttreatment recurrence (2). Many of these tumors are associated with significant morbidity given their proximity to critical nerves and blood vessels (2). In addition, a variety of “functioning” pituitary adenomas secrete supraphysiologic levels of hormones, resulting in profound systemic effects that reflect the hormone(s) elaborated. One example is the adrenocorticotropic hormone (ACTH)-secreting pituitary adenoma, which stimulates the production and release of cortisol by the adrenal glands, resulting in Cushing disease. Cushing disease, in turn, is associated with various sequelae, including morbid weight gain, metabolic abnormalities such as diabetes and osteoporosis, immune-deficiency, reproductive dysfunction, and cardiovascular complications, among others (3–5).

Control of Cushing disease remains elusive. Trans-sphenoidal resection is the first-line intervention, but long-term follow-up reveals recurrence rates between 15% and 66% at 5–10 years (5–7). Upon recurrence, repeat surgical resection and medical therapy are variably effective, and radiation is a viable option, but can be limited by proximity to critical structures (7, 8). There is a significant need for additional, more effective adjuvant treatment options in this disease.

Immunotherapy, and checkpoint blockade in particular, has gained acceptance in various cancers, but remains untried in Cushing disease and other pituitary tumors (9–17). A common target of checkpoint blockade is the PD-1/PD-L1 axis, which restricts the effector phase of the T-cell response (9). The binding of PD-L1 (on tumor or other microenvironment cells) to the PD-1 receptor on activated T cells inhibits the cytotoxic antitumor function of T cells, (18–20) while blockade of this interaction permits a perpetuated T-cell response (9). Several therapies targeting this pathway have achieved FDA approval and have shown marked success in metastatic solid tumors such as melanoma and non–small cell lung cancer (17, 21). It is interesting to note that lymphocytic hypophysitis, T-cell–based inflammation within the pituitary gland, is a common side-effect of checkpoint blockade treatment (22, 23). This immune-related adverse event provides compelling evidence that checkpoint blockade can and does stimulate an immune response readily within the pituitary gland.

PD-L1 expression on pituitary adenomas has been previously characterized, with highest expression found on functional adenomas, although few data on expression by ACTH-secreting tumors are available (6, 24, 25). In addition, pituitary adenomas demonstrate a lymphocytic infiltrate (25), while T cells are otherwise rare in the normal pituitary (23). Given the presence of T-cell infiltrates within the tumor and expression of coinhibitory ligands in the tumor microenvironment, pituitary adenomas may be susceptible to an appropriate checkpoint blockade strategy. We therefore sought to better characterize PD-L1 expression on ACTH-secreting adenomas and determine whether blockade of the PD-1/PD-L1 axis could be utilized to target these tumors and improve outcomes in Cushing disease. In this study, we corroborate the expression of PD-L1 on human pituitary adenomas (including those secreting ACTH), as well as an ACTH-secreting murine adenoma cell line. We employ a novel in vivo murine model

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Translational Relevance

This study confirms PD-L1 expression on functioning pituitary adenomas and is the first to evaluate the efficacy of checkpoint blockade (anti--PD-L1) therapy in a preclinical model of Cushing disease. Although pituitary adenoma is classified as benign, Cushing disease is associated with significant morbidity due to the numerous sequelae of elevated cortisol levels. Successful therapy for Cushing disease remains elusive due to high rates of treatment-refractory recurrence. The frequent emergence of lymphocytic hypophysitis following checkpoint blockade for other cancers, as well as the expression of PD-L1 on pituitary adenomas, suggest a role for immunotherapy. Herein, treatment with anti--PD-L1 was successful in reducing adrenocorticotropic hormone plasma levels, decreasing tumor growth, and increasing survival in our model. Furthermore, tumor-infiltrating T-cells demonstrated a pattern of checkpoint expression similar to other checkpoint blockade--susceptible tumors. This suggests that immunotherapy, particularly blockade of the PD1/PD-L1 axis, may be a novel therapeutic option for refractory Cushing disease. Clinical investigation is encouraged.

Materials and Methods

Clinical studies and specimen processing

Studies were conducted in accordance with the provisions of the Declaration of Helsinki and the Good Clinical Practice guidelines of the International Conference on Harmonisation. All studies were performed with approval by the Duke University Institutional Review Board. Patient specimens were collected following appropriate consent. A total of 67 human pituitary tumor tissue specimens were identified from the Duke University surgical pathology archives. These formalin-fixed paraffin-embedded (FFPE) samples were used for IHC staining. For human studies, antibodies to PD-L1 (790-4905, Gibco Thermo Fisher Scientific) with 2 mmol/L l-glutamine, 4.5 mg/mL glucose (Gibco Thermo Fisher Scientific) containing 10% FBS (Gemini Bio-Products) and 1% 200 mmol/L l-glutamine 100× (Gibco Thermo Fisher Scientific), SMA550, CT2A, B16, and LLC cells were grown in vitro in DMEM with 2 mmol/L l-glutamine and 4.5 mg/mL glucose (Gibco Thermo Fisher Scientific) containing 10% FBS (Gemini Bio-Products). E0771 cells were grown in vitro in RPMI1640 (Gibco Thermo Fisher Scientific) containing 10% FBS plus 1% HEPES (Gibco Thermo Fisher Scientific). Cells were harvested in the logarithmic growth phase. For subcutaneous implantation, 107 ATT-20/D16v.2 cells were delivered in a total volume of 200 μL per mouse into the subcutaneous tissues of the left flank of A/He) x C57L/J F1 (LAF1) mice. For intracranial (IC) implantation, ATT-20/D16v.2 cells in PBS were mixed 1:1 with 3% methylcellulose and loaded into a 250 μL Syringe (Hamilton). The needle was positioned 2 mm to the right of the bregma and 4 mm below the surface of the skull at the coronal suture using a stereotactic frame. A total of 5 × 104 ATT-20/D16v.2 cells were delivered in a total volume of 5 μL per mouse.

Tissue processing

For tumor growth models, area under the curve (AUC) was estimated using the trapezoidal rule based on data starting from the first day of treatment. AUC was then standardized (sAUC) by number of days followed for each animal to allow for animals that dropped out to be appropriately included in the analysis. For subcutaneous tumor-bearing mice, humane endpoints included tumor size greater than 20 mm in one dimension, 2,000 mm3 in total volume, or tumor ulceration or necrosis. Subcutaneous tumors were harvested, minced, and incubated in 100 U/mL collagenase IV (Sigma-Aldrich) and 0.1 mg/mL DNAse I (Roche Diagnostics) in RPMI supplemented with 10% FBS for 20–30 minutes in a Stomacher machine set at normal speed, and washed through 70 μm nylon Cell Strainers (Falcon; BD Biosciences) in PBS with 2% FBS. Cells were immediately stained and subsequently analyzed by flow cytometry. Murine pituitary glands were identified and removed, processed in RPMI, minced into single-cell suspensions, cell-strained, counted, stained with antibodies, and analyzed via flow cytometry. Blood samples were directly labeled with antibodies and red blood cells subsequently lysed using RBC Lysis Buffer (eBioscience) or BD Pharm Lyse (BD Biosciences).

Flow cytometry

Murine-specific antibodies were purchased from BD Biosciences (CD3: 145-2C11; PD-L1/PD-12C474; MHC: CD4; TCRαβ: 53-67-23; PD-1: 143.0; LAG-3: 15F5; Ly6G: 1A8) and from BioLegend (CD-45; 30F11; CD3: 2C11, B220: RA3-6B2, CD11b: M1/70, CD11c: HI3, NK1.1 PK136). Appropriate isotype controls were used when applicable. The LIVE/DEAD Fixable Yellow Dead Cell Stain Kit (Thermo Fisher Scientific) was used...
to exclude dead cells. Cells were analyzed with an LSRFortessa (BD Biosciences) and data were analyzed with FlowJo Software (TreeStar).

ELISA
In the subcutaneous tumor-bearing model, $10^7$ ATT-20/D16v.2 cells were injected subcutaneously into the left flank of LAF1 mice on day 0. At day 1, prior to tumor implantation, mice underwent 200 µL retro-orbital bleed EDTA tubes (MCTV200, Kent Scientific Corporation) with an added 20 µL Aprotinin (RK-APRO, Phoenix Pharmaceuticals, Inc.). This blood mixture was then centrifuged at 4°C at 2,000 x g for 15 minutes and aliquots of plasma were stored at −80°C. At day 38, mice underwent an additional 200 µL retro-orbital bleed, which was processed and stored as described previously. ACTH concentrations were measured by Enzyme Immunoassay Kit (Phoenix Pharmaceuticals Inc.). The manufacturer’s specifications were followed accordingly to determine plasma ACTH concentrations.

**In vivo antibody injection**
In the subcutaneous tumor-bearing model, $10^7$ ATT-20/D16v.2 cells were injected subcutaneously into the left flank of LAF1 mice on day 0. Following tumor implantation, mice were treated intraperitoneally (ip) with 200 µg of either anti–PD-L1 (clone 10F.9G2; BioXCell) or isotype control antibody (Rat IgG2b; BioXCell) every 3 days starting on day 3 for 12 total doses until day 36. In our intracranial tumor-bearing model, $5 \times 10^5$ D16v.2 cells were injected intracranially at day 0. Beginning on day 3, mice were treated intraperitoneally with either 200 µg of anti–PD-L1 (10F.9G2; BioXCell) or PBS (Gibco Thermo Fisher Scientific) control every 3 days starting on day 3 for 15 total doses until day 45.

**Results**

Human ACTH-secreting pituitary adenomas express PD-L1 and are infiltrated by T cells

Increased levels of PD-L1 by IHC on various types of human pituitary adenomas have been reported, with expression found to be highest among functional adenomas (6). Levels of PD-L1 on the ACTH-secreting tumors constituting Cushing disease, however, are not well characterized. To assess PD-L1 levels on the normal pituitary gland, we showed that expression of PD-L1 was observed on B16 melanoma and ATT/D16v.2 adenoma. The most prominent expression of PD-L1 was in GH- and prolactin-secreting samples was 45% (9/20) and 37% (7/19), respectively (Fig. 1D). The proportion of PD-L1–positive tumors trended higher among primary tumors (12/50—24%) than among recurrent adenomas (3/17—17.6%), although the difference was not significant (Fig. 1E).

After confirming the presence of CD3+ T cells, we showed that CD3+ counts per HPF were greater in functioning (6.76) compared with nonfunctioning adenomas (1.45; Fig. 1F). Immunoreactivity for CD3 was highest in GH-secreting samples (9.64/HPF), followed by prolactin-secreting (4.18/HPF) tumors (Fig. 1G). CD3+ infiltrates were more prevalent in recurrent tumors (9.84/HPF) compared with primary adenomas (3.98/HPF; Fig. 1H).

**PD-L1 expression is recapitulated on murine ACTH-secreting pituitary adenoma**

PD-L1 expression was assessed in vitro on the murine ACTH-secreting pituitary adenoma cell line (ATT/D16v.2) via flow cytometry (Fig. 2A), and compared with other murine tumor cell lines, including B16 melanoma, E0771 breast adenocarcinoma, Lewis Lung Carcinoma (LLC), GL261 glioma, CT2A glioma, SMA560 glioma, and KR158B glioma (Fig. 2B). The most prominent expression of PD-L1 was observed on B16 melanoma and ATT/D16v.2 adenoma.

To examine whether PD-L1 levels on ATT/D16v.2 were maintained in vivo, $10^7$ cells were implanted into the left flank of syngeneic LAF1 mice. Subcutaneous (sc) implantation was performed initially to permit sufficient tumor growth for analysis. Tumors were allowed to progress for up to eight weeks and then were harvested for profiling. Hematoxylin and eosin (H&E) staining confirmed histology consistent with pituitary adenoma (Fig. 2C). PD-L1 expression was confirmed on the harvested in vivo tumor by IHC (representative positive staining shown in Fig. 2D). Likewise, PD-L1 levels on harvested tumors were compared by flow cytometry to levels on normal pituitary harvested from host LAF1 mice. PD-L1 expression was present on the harvested adenoma, but absent on the normal pituitary gland (Fig. 2E).

**Establishment of a murine model of Cushing disease**

To establish a heterotopic murine model of Cushing disease, $10^7$ ACTH-secreting ATT/D16v.2 cells were implanted into the flank of LAF1 mice. The first objective was to identify and confirm ACTH production by the ATT/D16v.2 cell line in vivo.
subcutaneous growth for approximately 4 weeks, tumor was harvested, and ACTH expression was demonstrated via IHC (Fig. 3A). Serum levels of ACTH were measured by ELISA and compared at baseline and 8 weeks posttumor implantation, with anticipated rises in serum ACTH levels accompanying tumor growth (Fig. 3B). Expected weight gain also accompanied increases in tumor volume. An approximate 30% increase in weight from baseline was observed by week 5 in these mice (Fig. 3C). Gross phenotypic changes related to Cushing syndrome after subcutaneous implantation of the ATT/D16v.2 cell line are depicted in Fig. 3D.

Characterization of T-cell influxes within ACTH-secreting adenoma

The presence of intratumoral, as opposed to peritumoral, T cells was confirmed via IHC (Fig. 4A). We then focused specifically on T cells and further characterized phenotypic markers of exhaustion. TIL exhaustion, marked by the upregulation of costimulatory molecules such as LAG-3, TIM-3, and PD-1, has proven particularly limiting for checkpoint blockade strategies in cancer, as we and others have shown (26, 27). More specifically, an increased expression of a combination of PD-1, Tim-3, and Lag-3 on TIL has been shown to...
correlate inversely with tumor response to checkpoint blockade (26, 28). We therefore examined the presence of all three markers on TILs and in blood harvested from tumor-bearing and naive mice. A representative flow cytometry plot is shown in Fig. 4B. The combination of PD-1, TIM-3, and LAG-3 were present to a greater extent in TIL than in the blood of either tumor-bearing or naive mice (Fig. 4C). PD-1, TIM-3, and LAG-3 were present in modest levels on TILs in ATT/D16v2 and resemble levels of immunogenic tumors (B16 melanoma, LLC). A heatmap comparing levels of each exhaustion marker against TIL from several tumor models is shown in Fig. 4D.

Treatment with anti–PD-L1 restricts tumor growth and ACTH production in a model of Cushing disease

Encouraged by our TIL characterization, we used the heterotopic murine model of Cushing disease established above to evaluate the antitumor efficacy of treatment with the anti–PD-L1 antibody (10F.9G2; BioXCell). Initial use of a heterotopic flank tumor model permitted assessment of treatment effect on tumor volumes, specifically. Following subcutaneous tumor implantation, mice were treated intraperitoneally with either anti–PD-L1 or isotype control antibody (Rat IgG2b; BioXCell) every 3 days starting on day 3 for 12 total doses (through day 36). Treatment with anti–PD-L1 resulted in significant inhibition of tumor growth (Fig. 5A), and likewise precipitated a decrease in levels of serum ACTH at 8 weeks posttreatment compared with untreated tumor-bearing mice (Fig. 5B). A subset of mice achieved complete tumor regressions.

To evaluate whether this effect was a T-cell–dependent phenomenon, subcutaneous tumors were also implanted into nude (athymic) mice. Mice were then treated intraperitoneally with either anti–PD-L1 or isotype control antibody over the same time course. Tumor-bearing nude mice likewise developed a Cushingoid phenotype, marked by weight gain and increased fat deposits (Fig. 5C). Treatment with anti–PD-L1 still produced a modest restriction to tumor growth (Fig. 5D) suggesting that although present and contributory, T cells were not necessary for efficacy.

Anti–PD-L1 retains efficacy in the intracranial environment

We next examined whether anti–PD-L1’s capacity to restrict tumor growth would be maintained when tumors were implanted intracranially. For reasons of practicality, tumors were placed in the right frontal lobe rather than within the sella. Stereotactic introduction of $5 \times 10^4$ ATT/D16v.2 cells to the base of the right frontal lobe led to
unincumbered intracranial tumor growth, resulting in 100% mortality after approximately 3 weeks. This proved a fairly stringent, aggressive model, as it was more lethal than the human condition. Mice with intracranial ATT/D16v.2 were then administered anti–PD-L1 or control antibody every 3 days, beginning on day 3 following tumor implantation. The median overall survival among the treatment cohort was extended to 29.5 days (95% CI: 15, not estimable), compared with a median survival of 21.5 days (95% CI, 15.0–24.0) in the control group. In addition, treatment with anti–PD-L1 enhanced long-term survival, as the 30-day survival estimate in the treated group was 50% (95% CI, 18.4%–75.3%) and 60-day survival estimate was 40% (95% CI, 12.3%–67%) while in the control group, survival was 0% at both time points (Fig. 6A).

Given our earlier observation in nude mice that T cells were dispensable to treatment efficacy (Fig. 5D), we opted to characterize the immune infiltrates within the more relevant intracranial tumor microenvironment, to better determine which population(s) might be mediating the therapeutic effect. The vast majority of tumor-infiltrating CD45+ cells proved to be myeloid cells expressing the CD11b marker, although T-cell infiltrates were also present as expected (Fig. 6B). Furthermore, when examined by flow cytometry, myeloid cells demonstrated particularly high levels of PD-L1 expression, even when compared with tumor cells themselves. (Fig. 6C, representative flow cytometry plot depicted in Fig. 6D).

Discussion
Over the past decade, the emerging success of immunotherapeutic strategies has altered the approach to treating various cancers. Among such strategies, immune checkpoint blockade has perhaps made the greatest gains, with targeting of the PD-1/PD-L1 axis evolving as the frequently preferred approach. Clinical trials with anti–PD-1/PD-L1 have shown promising results against a variety of malignancies, including non–small cell lung cancer, renal cell, melanoma, ovarian, and bladder cancer (17, 21, 29–32). While checkpoint blockade is now FDA-approved for various tumors, this study is the first to systematically explore its employment against pituitary adenomas.

Prior studies have demonstrated PD-L1 expression on human pituitary adenomas (6, 24). Mei and colleagues identified PD-L1 at both the transcript and protein level across 48 human pituitary...
adenomas. They found that PD-L1 expression was higher in functioning tumors, in line with our own results. Wang and colleagues assessed 191 human pituitary adenomas by IHC, and found that PD-L1 stained positively among 36.6% of samples. They also found that positivity was more frequent among functioning adenomas, with positive staining in 58.8% of functioning tumors compared with 34.3% of nonfunctioning adenomas. This group observed that PD-L1 expression was significantly correlated with expression of GH and prolactin, but not ACTH, although 17 of 28 ACTH-producing tumors did express PD-L1 (25).

In concordance with these studies, we found PD-L1 expression to be highest among functional pituitary adenomas, with the highest expression in GH-producing adenomas, followed by those producing prolactin and ACTH. Subtler differences regarding the proportions staining positively in each study might be explained by technical discrepancies, sample sizes employed, and defined criteria for positivity.

PD-L1 expression is often viewed as a potential biomarker for response to checkpoint blockade. In some cancers, it has been shown to correlate positively with treatment response to the PD-1 blockade (i.e., nivolumab; refs. 29–33), while other studies have not found the same correlation (31). Given the PD-L1 expression we observed on ACTH-secreting adenomas, however, we chose to begin with direct targeting of PD-L1, avoiding some of this controversy and simplifying our approach. We were further encouraged by the detection of a lymphocytic infiltrate in pituitary adenomas (including our own), suggesting the presence of an antitumor effector arm that might be licensed with anti–PD-L1 therapy (6, 25). Given the mechanism of action for checkpoint blockade therapy, the minimum requirement is the presence of both ligand and receptor for whichever inhibitory axis is targeted (e.g., PD-1 and PD-L1 present in high levels when treating with nivolumab), as well as T-cell infiltrates within the tumor. Therefore, immunogenic “hot” tumors with a high degree of T-cell infiltration and expression of PD-L1 on the tumor cell surface are most capable of responding to checkpoint blockade therapy. This is juxtaposed with immunologically “cold” tumors that are resistant to immunotherapy based on their dearth of immune infiltrates. Our findings suggest that ACTH-secreting pituitary adenomas may respond to checkpoint blockade therapy targeting the PD-1/PD-L1 axis given the presence of CD3+ TILs and expression of PD-L1 on the tumor.

Prior groups have also created Cushing disease murine models (34, 35). For our use, we employed an ACTH-secreting pituitary adenoma cell line (ATT-20/D16v2), which prior studies have
confirmed maintains properties of anterior pituitary cells (36, 37). We passaged this line in vivo to select for a penetrant subclone and confirmed surface expression of PD-L1 both in vitro and in vivo. Four weeks after subcutaneous tumor implantation, average body weight had increased by more than 20%, and mice had developed a Cushingoid phenotype that included increased subcutaneous fat deposits and marked obesity. These changes were accompanied by a 3-fold increase in ACTH concentration in the plasma. Treating these mice with a PD-L1–blocking antibody significantly slowed tumor growth and led to a decrease in serum ACTH levels. Importantly, our findings also demonstrated an antitumor effect of PD-L1-blockade that extended intracranially, providing context for translation.

Our study is the first to demonstrate efficacy for checkpoint blockade against pituitary adenoma in a preclinical model. Clinical use for adenoma has also not been reported. There has been a single case report in which a patient with resection- and chemotherapy-refractory pituitary carcinoma was treated with a combination of ipilimumab and nivolumab. This patient achieved a radiographic response in both her sellar tumor as well as among extracranial metastases, suggesting the intracranial compartment and pituitary gland are not barriers to effective checkpoint blockade (38).

The use of checkpoint inhibitors against intracranially situated lesions continues to be an area of investigation. While therapies targeting PD-1 have failed in clinical trials for glioblastoma, (39) there have been reported successes against intracranial metastases (40–43). Recently, a multicenter phase II study of ipilimumab + nivolumab for intracranial metastatic melanoma demonstrated intracranial response rates of 57%, comparable with the response rate seen extracranially (21). This suggests that the primary obstacle to checkpoint blockade is unlikely to be access to the intracranial compartment. There is indeed evidence that checkpoint blockade antibodies penetrate the blood–brain barrier (BBB; ref. 44), although it is less understood that BBB and tumor penetration are requisites for success. Certainly, activated T cells cross the BBB, and this may or may not be the more crucial component to therapeutic response. Regardless, portions of the pituitary are situated outside the BBB, perhaps obviating the issue of drug access for these tumors specifically.

It is salient to note that lymphocytic hypophysitis, T-cell–based inflammation within the pituitary gland, emerges as a common side effect of treatment with checkpoint blockade in clinical trials for other cancers (22, 23). It is most common with anti–CTLA-4 (ipilimumab), but has also been shown with anti–PD-1 treatment (nivolumab; ref. 45). While problematic, this provides compelling evidence that checkpoint blockade can and does stimulate an immune response readily within the pituitary gland, perhaps obviating concerns to the contrary. Our own data showing 40% long-term survival in an intracranial Cushing disease model treated with anti–PD-L1 provide further optimism for this approach despite the intracranial locale.

Figure 5. Treatment with anti–PD-L1 prevents tumor growth and ACTH production in a heterotopic Cushing disease model. A, Following subcutaneous (SC) implantation of AtT20/D16v2 adenomas, treatment with anti–PD-L1 (n = 8) significantly restricted tumor growth compared with treatment with isotype control (n = 16; P = 0.0002; exact Wilcoxon of sAUC). B, Serum ACTH levels measured by ELISA demonstrate decreased ACTH levels following anti–PD-L1 treatment (P < 0.0001; one-way ANOVA; Naive n = 20; TB + control n = 14; TB + anti–PD-L1 n = 7). Results are pooled across multiple experiments. C, Nude (athymic) mice implanted SC with AtT20/D16v2 tumors also develop a Cushingoid phenotype. D, When treated with anti-PDL1 antibody, nude tumor-bearing mice (n = 7) demonstrate a more modest restriction of tumor growth compared with controls (n = 8; P = 0.0401; exact Wilcoxon of sAUC).
The efficacy of checkpoint blockade against intracranial tumors, and cancer more generally, has been variably restricted by tumor-imposed T-cell dysfunction among TIL. Exhaustion, marked frequently by the upregulation of alternative immune checkpoints, has proved particularly limiting (26–28, 46). Our group has shown, for instance, that gliomas exhibit particularly severe patterns of TIL exhaustion, whether in orthotopic or heterotopic models, and likewise prove resistant to checkpoint monoblockade therapy. Conversely, checkpoint-sensitive tumors such as melanoma and lung cancers demonstrate milder TIL exhaustion signatures, as expression of PD-1, TIM3, and LAG3 function to modulate T-cell activation at homeostasis, and activated T cells are necessary for an antitumor response (26).

A specific mode of T-cell dysfunction arising in the setting of chronic, suboptimal antigen exposure, exhaustion is a transcriptional program often manifested by the expression of multiple coinhibitory molecules such as PD-1, TIM3, and LAG3 on the T-cell surface (26, 47).

In our study, pituitary adenoma TILs expressed these molecules, but at modest levels typical of immunogenic tumors, such as melanoma and lung carcinoma, which are responsive to checkpoint blockade therapy. Very few TILs expressed multiple coinhibitory molecules, which would have indicated more severe exhaustion and typified therapy-resistant tumors such as glioma (26).

The responsiveness to PD-L1 therapy in our studies, however, may be mediated through multiple immune cell types, as modest treatment responses were still observed in athymic mice lacking T cells. In keeping with a study by Lu and colleagues, we observed that the myeloid compartment constituted the largest fraction of the pituitary adenoma microenvironment (48). Furthermore, myeloid cells demonstrated high PD-L1 expression. This likewise raises the question as to whether PD-L1 expression in the tumor or its microenvironment is more relevant to therapeutic success. Such questions are likely to be best answered by clinical studies in patients, as the single cell line figure 6.

**Figure 6.**
PD-L1 is expressed in the tumor microenvironment and treatment with anti–PD-L1 promotes survival in an IC model of pituitary adenoma. **A,** Survival following IC implantation of ATT20/D16v2 adenomas into immunocompetent mice; treatment with anti–PD-L1 or isotype control. **B,** Characterization of CD45+ immune cells in the tumor microenvironment. **C,** PD-L1 MFI on tumor cells and myeloid cells in the IC tumor microenvironment. **D,** Representative flow cytometry of PD-L1 expression on tumor cells (top: CD45+/forward-scatter+) and myeloid cells (bottom: CD45+/CD11b+).
employed here creates little variability in tumor PD-L1 expression across mice.

One limitation of our study is the implantation of the murine ATT20/D16v.2 cell line in the right hemisphere given the technical restraints of direct sellar implantation. This method is imperfect as a true anatomical recapitulation of Cushing disease. Furthermore, as space in the intracranial compartment is limited, tumor growth is likewise limited. Mice succumb to tumor mass effect and systemic cachexia before tumors become large enough to secrete sufficient hormone to cause the Cushingoid phenotype, making it an imperfect model for patients with Cushing disease. However, the intracranial positioning allowed for demonstration of therapeutic efficacy against a tumor situated behind the BBB. Furthermore, these tumors in the IC compartment proved 100% lethal, which although not representative of their human counterparts, provided a seemingly more aggressive, difficult-to-treat model. Coupled with the experiments showing efficacy against subcutaneous tumors (which are not size-limited) and decreases to the associated ACTH levels, these experiments provide proof of concept for using anti–PD-L1 to treat the ACTH-driven morbidity that occurs in patients with Cushing disease from a pituitary tumor.

Future directions, then, include initiation of clinical trials targeting the PD-1/PD-L1 axis in patients with recurrent or treatment-refractory functional pituitary adenomas. Although we model Cushing disease in these studies, high PD-L1 expressors such as GH-secreting tumors may also be viable targets for therapy. Likewise, as the importance of PD-L1 for dictating responses to treatment remains indeterminate, clinical studies may ultimately reveal a role in nonfunctioning tumors where complete eradication is less a goal than mitigating mass effect. While all recurrent/refractory pituitary adenomas present challenges, however, the greatest area of need does perhaps surround ACTH-producing tumors. Because of the endocrinopathies associated with Cushing disease, patients are at increased risk of cardiovascular complications, metabolic abnormalities, and psychiatric manifestations, all of which may lead to devastating morbidity and higher mortality (49, 50). The lack of viable treatment options poses a great need for novel therapies, and our study suggests checkpoint blockade may be a promising strategy. Further preclinical study can be aimed at investigating other checkpoint blockade modalities such as anti–PD-1, anti–CTLA-4, or the combination.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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