PD-1/PD-L1 Blockade Enhances T-cell Activity and Antitumor Efficacy of Imatinib in Gastrointestinal Stromal Tumors

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Abstract

Purpose: Tyrosine kinase inhibitors are effective in gastrointestinal stromal tumors (GISTs) but often are of transient benefit as resistance commonly develops. Immunotherapy, particularly blockage of the inhibitory receptor programmed death 1 (PD-1) or the ligand programmed death ligand 1 (PD-L1), has shown effectiveness in a variety of cancers. The functional effects of PD-1/PD-L1 blockade are unknown in GISTs.

Experimental Design: We analyzed tumor and matched blood samples from 85 patients with GISTs and determined the expression of immune checkpoint molecules using flow cytometry. We investigated the combination of imatinib with PD-1/PD-L1 blockade in Kit(V558D × Kit(V558D)/+ mice that develop GISTs.

Results: The inhibitory receptors PD-1, lymphocyte activation gene 3, and T-cell immunoglobulin mucin-3 were upregulated on tumor-infiltrating T cells compared with T cells from matched blood. PD-1 expression on T cells was highest in imatinib-treated human GISTs. Meanwhile, intratumoral PD-L1 expression was variable. In human GIST cell lines, treatment with imatinib abrogated the IFNγ-induced upregulation of PD-L1 via STAT1 inhibition. In Kit(V558D × Kit(V558D)/+ mice, imatinib downregulated IFNγ-related genes and reduced PD-L1 expression on tumor cells. PD-1 and PD-L1 blockade in vivo each had no efficacy alone but enhanced the antitumor effects of imatinib by increasing T-cell effector function in the presence of Kit and IDO inhibition.

Conclusions: PD-1/PD-L1 blockade is a promising strategy to improve the effects of targeted therapy in GISTs. Collectively, our results provide the rationale to combine these agents in human GISTs. Clin Cancer Res; 23(2); 454–65. ©2016 AACR.

Introduction

The advent of targeted molecular therapy has revolutionized the treatment of many cancers, including gastrointestinal stromal tumors (GISTs). The majority of GISTs contain an activating mutation in either the KIT or PDGFRA oncogene (1, 2). Imatinib mesylate (Gleevec) is a tyrosine kinase inhibitor that specifically targets KIT and PDGFRA (3). The tumor response to imatinib in GISTs is impressive, but most often transient. Resistance commonly develops within 2 years, often due to a secondary KIT mutation (4, 5). Imatinib acts primarily via direct effects on tumor cells. However, we previously showed that imatinib also inhibits tumor cell production of the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO; ref. 6).

Many tumors express ligands that engage inhibitory receptors on T cells and decrease T-cell activation and function within the tumor microenvironment. There is growing evidence that tumor cells commonly exploit the programmed death 1 (PD-1, PDCD1) and programmed death ligand 1 (PD-L1, PDCD1LG1) axis to evade the immune system (7). PD-1 is an inhibitory receptor that is upregulated after T-cell activation and remains elevated with antigen persistence and therefore is often increased on tumor-infiltrating T cells (8–10). The ligands for PD-1 are PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273, PDCD1LG2). PD-L1 is expressed widely on immune cells and can be upregulated by proinflammatory stimuli, such as interferons, but is also expressed by tumor cells in a variety of cancers (11). PD-L1 expression on tumors can lead to impaired T-cell proliferation and effector function, leading to apoptosis of tumor-specific T cells (12). PD-L2 expression is restricted mostly to hematopoietic cells. PD-1/PD-L1 blockade has demonstrated encouraging antitumor effects in several solid tumors, including kidney, bladder, and lung cancer, as well as melanoma (13–19).

Despite the efficacy of tyrosine kinase inhibition, nearly all patients with a metastatic GIST develop tumor progression and eventually succumb to their disease. There has not been any improvement in the first-line therapy for GISTs since imatinib was approved in 2002. In this study, we analyzed freshly isolated T cells from the tumor and peripheral blood of patients with GISTs for the expression of inhibitory receptors. We determined the effects of imatinib on IFNγ-related genes and the PD-1/PD-L1 axis.
Translational Relevance

Although GISTs are often initially sensitive to imatinib or other tyrosine kinase inhibitors, resistance generally develops, necessitating additional therapeutic strategies. There has not been any improvement in the first-line therapy for GISTs since imatinib was approved in 2002. Immunotherapy is being tested in a variety of cancers. Our findings provide new insights into the combination of tyrosine kinase inhibition and immunotherapy and provide a strong incentive to clinically combine imatinib and anti-PD-1/PD-L1 blockade.

Materials and Methods

Patient samples

Tumor specimens and matched peripheral blood were obtained from 85 patients with GISTs who underwent surgery at our institution and were consented to a protocol approved by the Institutional Review Board. Blood was drawn before surgical incision, and peripheral blood mononuclear cells were isolated by density centrifugation over Ficoll–Plaque PLUS (GE Healthcare). Tumor tissue was subjected to mechanical dissociation to obtain single-cell suspensions, as described previously (6). After procurement, all specimens were processed, and cells were immediately analyzed with flow cytometry. Tumor (KIT+ ) and stromal cells (KIT−) were isolated using human CD117 microbeads (Miltenyi Biotec). The purity of isolated cells was greater than 90% by flow cytometry.

Cell lines and treatments

The human GIST cell lines GIST-T1 (KIT exon 11 mutant; ref. 20), HG129 (also KIT exon 11 mutant; ref. 21), and GIST882 (KIT exon 13 mutant; provided by Jonathan Fletcher [Department of Pathology, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA]) were maintained at 37°C in RPMI1640 medium supplemented with 10% FBS, 2 mmol/L L-glutamine, 50 U/mL penicillin–streptomycin, 0.1% 2-mercaptoethanol, and 10 mmol/L Hepes. Cells were incubated with recombinant human IFNγ (100 ng/mL; R&D Systems), imatinib (100 nmol/L; Novartis), or the pan-JAK inhibitor tetracyclic pyridine (RMP1-14; BioX cell) or anti-PD-L1 (10F.9G2; BioX cell) blockade.

Mice and treatments

Kit+/Sema4A−/− mice (23) that were 6 to 12 weeks old were maintained in a specific pathogen-free animal facility, age- and sex-matched for experiments, and used in accordance with an institution-approved protocol. Tumors were minced, incubated in 5 mg/mL collagenase IV (Sigma-Aldrich) plus 0.5 mg/mL DNase I (Roche Diagnostics) in HBSS for 30 minutes at 37°C, then quenched with FBS, and washed through 100- and 40-μm nylon cell strainers (Falcon, BD Biosciences) in PBS with 1% FBS. Tumor-draining lymph nodes and spleens were mechanically dissociated as described previously (6). Cells were immediately analyzed with flow cytometry. Imatinib was administered at 90 mg/kg per day in the drinking water. 1-Methyl-D-tryptophan (1-MT; Sigma-Aldrich) was prepared and administered twice daily by oral gavage at 400 mg/kg per day as before (6). Anti-PD-1 (RMP1-14; Bio X cell) or anti-PD-L1 (10F.9G2; Bio X cell) blocking antibodies or isotypes (rat IgG2a or rat IgG2b) were administered intraperitoneally at a dose of 250 and 200 μg per mouse, respectively, at the indicated time points. Murine T cells were purified from mesenteric lymph nodes from C57BL/6 mice (B6, The Jackson Laboratory) and tumors from Kit+/Sema4A−/− mice using CD3-biotin and anti-biotin MicroBeads (Miltenyi Biotec). Positive selection was performed using two sequential MACS columns. CD3+ T cells were then cultured in anti-CD3–coated 96-well plates (BD Biosciences) with 10 μg/mL of either anti-PD-1 antibody or isotype, and supernatant was harvested at 48 hours.

Flow cytometry and cytokine detection

Human-specific antibodies were purchased from BD Biosciences (CD45, 2D1; CD3, SK7; CD4, RPA-T4; PD-1, MIH4), eBioscience (CD8, RPA-T8), BioLegend (PD-L1, 29E.2A3), R&D Systems (TIM-3, 344823), and Enzo Life Sciences (LAG-3, 17B4). Mouse-specific antibodies were purchased from BD Biosciences (CD3, 125-2C11; CD4, GK1.5; CD44, IM7; CD62L, ME-14; PD-L1, MH5; CD69, H1.2F3; TNF-γ, XMG1.2; TNF, MP6-XT22), eBioscience (CD8, 53-6.7; PD-1, J43; PD-L2, 122; Foxp3, FJK-16s; Ki67, SolA15; Granzyme B, NGZB), and BioLegend (CD45, 30-F11; PD-L1, 10F.9G2; CD25, PC61). Appropriate isotype controls were used where applicable. A viability dye was typically used to exclude dead cells. Intracellular staining was performed using the eBioscience Fixation and Permeabilization Buffer Kit. For intracellular cytokine staining, cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 50 ng/mL) and ionomycin (750 ng/mL) for 4 hours at 37°C, 5% CO2 in the presence of 1 μg/mL brefeldin A (BD Biosciences). Surface staining was performed and all cells were fixed and permeabilized with the BD Cytofix/Cytoperm Kit and stained for IFNγ and TNFα. Supernatant cytokines were measured by cytometric bead array according to the manufacturer’s instructions (Mouse Inflammation Kit; BD Biosciences). Data were acquired using a BD FACSAria or Fortessa LSR flow cytometer and analyzed using FlowJo software (Tree Star).

Immunofluorescence and IHC

Formalin-fixed and paraffin-embedded specimens were sectioned at 5-μm thickness and mounted on glass slides. The PD-L1 (clone 5H1) antibody was obtained from Lieping Chen (Department of Immunobiology, Yale University School of Medicine, New Haven, CT) (12). In brief, antigen retrieval was achieved with citrate buffer. Anti-PD-L1/B7-H1 murine IgG (1:1,000) was applied on tissue sections overnight at 4°C. After washing with TBS (0.05 mol/L Tris base, 0.9% NaCl, pH 8.4), tissue sections were incubated with biotinylated anti-mouse IgG (1:100; BA-2001, Vector Laboratories), followed by incubation with Elite ABC Kit for 30 minutes at room temperature. Antibody binding was detected with the TSA Biotin System (NEL7000001KT, PerkinElmer) and DAB (DAKO, K3468).
Murine PD-L1 staining was performed using the polyclonal antibody against mouse B7-H1/PD-L1 antibody (1:100, R&D Systems). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and KIT immunostaining (1:200, D13A2; Cell Signaling Technology) were performed as described previously (24). Slides were analyzed and imaged on the Axio Imager 2 wide-field microscope (Zeiss).

qRT-PCR
Total RNA was extracted from each human GIST specimen, cell line, or murine GIST specimen, reverse transcribed, and amplified with PCR TaqMan probes for human PDCD1LG1 (i.e., PD-L1, Hs01125351_m1), IFNG1R1 (Hs00988303_m1), IFI1 (Hs00971960_m1), GAPDH (Hs02758991_g1, murine Pdh1 (Mm00452054_m1), Id1 (Mm00492590_m1), and Gapdh (Mm99999913_g1, all Applied Biosystems). Quantitative PCR was performed using a ViaATM real-time PCR system (Applied Biosystems). Relative expression was calculated by the 2−ΔΔCT method according to the manufacturer’s protocol and expressed as the fold increase over the indicated control.

STAT1 knockdown
For transient STAT1 knockdown, GIST-T1 cells were transfected with 30 nmol/L of ON-TARGET plus SMARTpool siRNA specific for human STAT1 (L-003543-00) or a nontarget control siRNA (D-01810-10-05; Thermo Scientific) using Lipofectamine RNAiMAX (Invitrogen) for 48 hours. GIST-T1 cells were treated with PBS or IFNγ (100 ng/mL) for 6 hours.

Western blot and cytokine array
Western blot analysis of whole-protein lysates from frozen tumor tissues or cells was performed as described previously (6). Antibodies for PD-L1 (E1L3N), phosphorylated SRC (Tyr416), phosphorylated STAT1 (Tyr701), phosphorylated STAT3 (Ser727 or Tyr705), total and phosphorylated KIT (Tyr719), phosphorylated ERK1/2 (Thr202/Tyr204), phosphorylated SMAD2 (Ser465/467), TGFβ, and GAPDH were purchased from Cell Signaling Technology. Anti-IDO antibody (clone 10.1) was purchased from EMD Millipore. Whole protein from frozen tumor tissues was lysed, and total protein was tested for cytokine/chemokine expression using a Proteome Profiler Array (R&D Systems). Densitometry was conducted on blots using ImageJ (NIH, Bethesda, MD).

Statistical analysis
Data are expressed as mean ± SEM or median. Unpaired two-tailed Student t test or one-way ANOVA comparisons were performed as applicable using GraphPad Prism 6.0 (GraphPad Software). P < 0.05 was considered significant.

Results
T cells infiltrating human GISTs express high levels of inhibitory receptors compared with blood
The inhibitory receptors PD-1, LAG-3, and TIM-3 (HAVCR2) are typically expressed on antigen-specific and dysfunctional T cells within tumors and during chronic viral infection (10, 25). To identify the inhibitory receptors that are relevant in GISTS, we performed flow cytometry on T cells from 106 tumor specimens and matched blood, freshly obtained from 85 patients with GISTS undergoing surgery (Supplementary Table S1). The percentages of CD4+ and CD8+ T cells among leukocytes were lower in tumors than in matched blood. CD8+ T cells were less frequent than CD4+ T cells in both compartments (Fig. 1A). Intratumoral T cells displayed greater cell-surface expression of PD-1, LAG-3, and TIM-3 compared with T cells from matched blood (Fig. 1B–D). PD-1 was expressed at the highest levels on both intratumoral CD4+ and CD8+ T cells (48% and 39%, respectively). Moreover, the percentage of PD-1 on CD4+ and CD8+ T cells correlated within the same tumor specimen (Fig. 1E).

Patterns of PD-1 expression in T cells in human GISTS
The expression of inhibitory receptors on tumor-infiltrating T cells suggested that T-cell immune evasion occurs in human GISTS. PD-1+ T cells tended to also express LAG-3 and less frequently TIM-3 (Fig. 2A). In contrast, PD-1+ T cells had little expression of LAG-3 or TIM-3. The majority of T cells from matched blood did not express any inhibitory receptors (Fig. 2B). Previously, we showed that tumors sensitive to tyrosine kinase inhibition (based on serial radiologic assessment prior to surgery) contained more CD3+ and CD8+ T cells, but a lower percentage of CD4+ T cells and T regulatory cells (Treg) compared with untreated and resistant tumors (6). Here, we found that compared with untreated tumors, there was higher PD-1 expression on CD4+ T cells in sensitive tumors and CD8+ T cells in tumors with acquired resistance to imatinib (i.e., increasing tumor size or vascularity after a period of stable or shrinking disease by serial radiologic assessment; Fig. 2C). There was no association between PD-1 expression and type of oncogene mutation (KIT, PDGFRA, or wild-type), tumor location, size, or mitotic rate (data not shown). In 10 patients who had multiple tumors removed during surgery, we found a similar expression pattern of inhibitory receptors within individuals, despite differences in tumor location, size, and mitotic rate (Fig. 2D).

PD-L1 is expressed in a subset of human GISTS
To assess the expression of PD-L1 in human GISTS, we evaluated human GIST specimens by IHC. PD-L1 expression was low in most cases and tended to be focal (Fig. 3A). PD-L1 was present on tumor cells, but also on some intratumoral leukocytes. To ascertain the relative location of PD-L1 within the tumor, we measured PD-L1 mRNA levels in freshly isolated tumor (CD45+ KIT+) stroma (CD45+ KIT−) cells from two resistant human GISTS. PD-L1 mRNA was equally distributed between these two compartments (Fig. 3B). Furthermore, we performed real-time PCR on 41 bulk human GIST samples. The PD-L1 mRNA level was variable but did not appear to correlate with treatment response to tyrosine kinase inhibition (Fig. 3C), mutation (Fig. 3D), or tumor size (Fig. 3E). There was a weak inverse relationship between mitotic rate and PD-L1 expression (Fig. 3F). Overall, intratumoral PD-L1 expression was variable and heterogeneous.

Imatinib abrogates IFNγ-induced upregulation of PD-L1 on human GIST cell lines
Next, we found that PD-L1 induction was low on three imatinib-sensitive human GIST cell lines (Fig. 4A). Because IFNγ is known to induce PD-L1 expression (12), we tested its effect on GIST cell lines. IFNγ upregulated PD-L1 mRNA in GIST-T1 cells over time as measured by real-time PCR (Fig. 4B). Similar results were observed in GIST882 and HG129 cells (data not shown). IFNγ-induced upregulation of PD-L1 transcripts was abolished with either the JAK inhibitor pyridine 6 or the KIT inhibitor
Imatinib. IFN-γ treatment also induced PD-L1 protein expression, but imatinib abrogated the effect as measured by flow cytometry (data not shown) and Western blot analysis (Fig. 4C). The mechanism appeared to be partly through the inhibition of STAT-1 activation, as JAK inhibition by pyridine 6 also reduced PD-L1 upregulation and, like imatinib, was associated with decreased phosphorylated STAT1 (Fig. 4C) and STAT1 mRNA levels (data not shown). Furthermore, STAT1 knockdown by siRNA in GIST-T1 cells blocked IFN-γ-induced upregulation of PD-L1 protein and mRNA (Fig. 4D and E). STAT1 knockdown was confirmed by Western blot analysis (Fig. 4D). We next measured the mRNA levels of IFNγ receptor (IFNGR1) and its major downstream signaling component IFN response factor 1 (IRF1). Notably, imatinib reduced the IFNγ-mediated increase in IFNGR1 and IRF1 mRNA (Fig. 4F). Collectively, IFNγ played a significant role in the regulation of PD-L1 expression in human GIST cells, but its effect was inhibited by imatinib.

Imatinib modulates IFNγ-related genes and PD-L1 expression in GISTs

To determine whether imatinib also alters tumor IFNγ signaling in KITV558/+ mice, we assessed IFNγ-related genes in our previously published mRNA microarray data (6). After 1 week of imatinib therapy, the expression of multiple IFNγ-related genes was reduced in bulk tumor (Fig. 5A). Pdil1 was not present on the array. Notably, PD-L1 expression was markedly reduced after 4 weeks of imatinib by IHC (Fig. 5B). To further investigate the extent to which imatinib treatment modulates PD-1 and PD-L1 expression in GISTs, we performed flow cytometry on intratumoral T cells and tumor cells (Fig. 5C). PD-L1 was present on tumor cells and decreased with 1 week of imatinib treatment. PD-L2 expression was minimal (data not shown). T-cell subsets within the tumor expressed both PD-1 and PD-L1 at baseline, but imatinib had little to no effect on expression levels. CD8+ T cells from the tumor-draining lymph node and spleen generally had very low PD-1 expression (Fig. 5D). Compared with intratumoral PD-1+ CD8+ T cells, PD-1+ CD8+ T cells from the tumor produced only minimal amounts of IFNγ after stimulation with PMA and ionomycin, suggesting that these cells are dysfunctional (Fig. 5D). However, in vitro treatment of bulk intratumoral T cells from untreated KITV558/+ mice with anti-PD-1 antibody increased their production of both TNF and IFNγ (Fig. 5E). In contrast, T cells from the mesenteric lymph node of wild-type mice had little cytokine production at baseline or after PD-1 blockade. Thus, PD-1 blockade preferentially affected tumor-infiltrating T cells compared with T cells from the mesenteric lymph node, which essentially lacked PD-1 expression.

PD-1/PD-L1 blockade enhances the antitumor effects of imatinib in murine GISTs

Given the presence of PD-1 and PD-L1 in both human and murine GISTs, we hypothesized that blockade of the PD-1/PD-L1
axis would have an antitumor effect. We treated KitV558/D mice with imatinib or vehicle combined with PD-1 or PD-L1 antibodies or isotype control for 4 weeks. The combination of imatinib and PD-1/PD-L1 blockade was not associated with noticeable toxicity in our model, and mice did not show signs of autoimmunity. Notably, anti-PD-1 and anti-PD-L1 altered tumor weight.

**Figure 2.** Patterns of PD-1 expression in T cells in human GISTs. **A,** Expression of individual or the combination of inhibitory receptors (PD-1, LAG-3, and TIM-3) on CD4+ and CD8+ T cells in 63 human GIST specimens by Boolean gate analysis. Data, mean ± SEM. *, P < 0.05. **B,** Total number of inhibitory receptors expressed by CD4+ (top) and CD8+ T cells (bottom) from the blood (left) and tumor (right). **C,** Percentage of PD-1+ cells among CD4+ (top) and CD8+ T cells (bottom) in blood and tumor from untreated (n = 36), imatinib-sensitive (n = 38), and imatinib-resistant (n = 32) GIST specimens. Each dot represents a separate specimen. Horizontal red line, median values. *, P < 0.05. **D,** Expression of inhibitory receptors on tumor-infiltrating T cells from patients with GISTs with multiple metastases (n = 10). Each dot represents a separate tumor specimen.
only when combined with imatinib (Fig. 6A). The combination treatment decreased phosphorylated KIT, phosphorylated STAT1, IDO, and TGFβ and its upstream mediator phosphorylated SMAD2 (Fig. 6B). Furthermore, Pdl1 and Ido1 mRNA levels were downregulated after 4 weeks of treatment (Fig. 6B). Importantly, the enhanced antitumor effect of imatinib and anti-PD-1 persisted at 3 months of treatment (Fig. 6D) and was detectable as early as 1 week (Fig. 6E). After 1 week of combined treatment, we noticed decreased KIT staining, indicating a reduction of tumor cells, especially in tumors from mice treated with imatinib plus anti-PD-L1, and increased TUNEL staining, indicating increased tumor cell apoptosis (Fig. 6F). There was no change in the frequency of

Figure 3.
PD-L1 is expressed in a subset of human GISTs. A, Representative IHC with anti-PD-L1 (clone 5H1) shows membranous and cytoplasmic expression in human GISTs (black arrow; scale bar, 50 μm). B, Freshly isolated KIT⁻ (stroma) and KIT⁺ (tumor) cells from two human GISTs were analyzed for PD-L1 mRNA by real-time PCR. Bars, mean ± SEM. C, RNA was isolated from fresh-frozen untreated (n = 14), imatinib-sensitive (n = 9), and imatinib-resistant (n = 18) human GISTs and analyzed for PD-L1 mRNA using real-time PCR. Bars, means. D, PD-L1 mRNA relative expression in 25 GIST samples with confirmed mutation. Horizontal red line, median values. E, Scatter plots to analyze the correlation of PD-L1 mRNA with tumor size and (F) mitotic count. HPF, high-power field. Values are log transformed.
CD4+ or CD8+ T cells or Tregs after 1 or 4 weeks of combination treatment (data not shown). Remarkably, intratumoral CD8+ T cells showed increased proliferation and inflammatory cytokine production upon combination treatment for 1 week compared with imatinib alone (Fig. 6G). In accordance with our observations from the mRNA microarray, IFNγ protein and two of the chemokines it induces, CXCL9 and CXCL10, were downregulated after 1 week of treatment (Fig. 6H). Furthermore, PD-1 blockade...
Imatinib modulates IFNγ-related genes and PD-L1 expression in GISTs. A, Bar graph showing mRNA levels of IFNγ-related genes determined by microarray analysis of tumors from KitV558+/− mice treated with imatinib for 1 week. Data are shown as log2 fold change compared with vehicle-treated KitV558+/− mice (3/group). Bars, mean. B, Representative IHC with anti-PD-L1 of tumors from KitV558+/− mice treated with vehicle or imatinib for 4 weeks (scale bar, 100 μm). C, Histograms of PD-1 and PD-L1 expression on tumor cells (CD45+/−, tumor-associated macrophages (TAMs, CD45+/F4/80+/CD11b−), and T-cell subsets (CD4+ Tconv cells: CD45+/CD4+/Foxp3−, Tregs: CD45+/CD3+/CD4+/CD25+/Foxp3+, and CD8+ T cells: CD45+/CD3+/CD8+)) in GIST-bearing KitV558+/− mice treated with vehicle or imatinib for 1 week (3–5/group). Representative plots are depicted with isotype control (gray), vehicle (blue), and imatinib (red). D, Expression of IFNγ in stimulated CD8+ T cells from spleens, tumor-draining lymph nodes (TdLN), and tumors from untreated KitV558+/− mice. CD8+ T cells from the indicated tissues were isolated, stimulated in vitro with PMA and ionomycin, and then analyzed by flow cytometry. E, Effect of PD-1 blockade on cytokine production in T cells. T cells were harvested from the mesenteric lymph node (mLN) from B6 mice [wild-type (WT)] or the tumor from untreated KitV558+/− mice and cultured in vitro in the presence of anti-PD-1 (10 μg/mL) or isotype control. After 48 hours, culture supernatant was collected, and cytokines were measured by cytometric bead array. Data, mean ± SEM. *, P < 0.05.
combined with the IDO inhibitor 1-MT was more effective after 1 week of treatment in our GIST mouse model than IDO inhibition alone (Fig. 6I). Thus, IDO inhibition (which also occurs after imatinib therapy alone) is sufficient to enable the antitumor effect of anti-PD-1. Taken together, we showed that KIT inhibition combined with PD-1/PD-L1 blockade reduced tumor weight in KitV558/+ mice, which was associated with increased tumor cell apoptosis and enhanced frequency of cytokine-producing CD8+ T cells.

Discussion

In this study, we found that the PD-1/PD-L1 axis contributes to tumor immune evasion in GISTS. Among the inhibitory receptors analyzed on intratumoral T cells in human GISTS, PD-1 was expressed at the highest frequency. Notably, the PD-1 expression on CD4+ and CD8+ T cells within sensitive and resistant human GISTS had a bimodal distribution, with a distinct population having very high expression, suggesting that a subset of patients might particularly benefit from PD-1/PD-L1 blockade. The level of PD-1 expression on intratumoral CD4+ T cells correlated with that on CD8+ T cells within a patient. Furthermore, in patients with multiple tumors, the different tumors had similar amounts of inhibitory receptors on intratumoral T cells, despite differences in tumor location, size, and mitotic rate. This observation does not resolve whether the antitumor immune response is either tumor driven, despite the known heterogeneity among tumor subclones, or patient driven, as systemic host factors may be responsible for a consistent T-cell response. PD-1 expression has been demarcated as a marker recognizing tumor-specific proteins (26). PD-1+ CD8+ T cells accumulated in murine GISTS but produced less IFNγ upon in vitro stimulation compared with PD-1− CD8+ T cells, suggesting that PD-1 marks functionally impaired intratumoral T cells in our model. Nevertheless, bulk intratumoral T cells made inflammatory cytokines after treatment with anti-PD-1 in vitro, as did intratumoral CD8+ T cells from KitV558/+ mice that had been treated with anti-PD-1 in vivo.

PD-L1 expression in human GISTS was variable and did not correlate with treatment status, tumor mutation, and tumor size. Tumors with a lower mitotic rate had a very modest correlation with higher PD-L1 expression, which has been suggested by prior data (27). We showed that PD-L1 expression in human GISTS was similar between tumor and stromal cells. It is unclear whether PD-L1 expression by either tumor or immune cells is required for response to anti-PD-1/PD-L1 therapy. In a previous report, a subset of melanoma patients with PD-L1− tumors responded to PD-1 blockade, suggesting that PD-L1 expression may not be necessary for response (28).

Investigation of this question is hampered by the suboptimal antibodies for PD-L1 IHC. In fact, in several human GISTS, we found only focal immunostaining despite relatively high PD-L1 mRNA levels.

Multiple mechanisms have been shown to drive PD-L1 expression. First, PD-L1 can be induced by IFNγ (12, 29). Although human GIST cell lines had low PD-L1 expression, IFNγ treatment markedly increased it, likely through STAT1. IFNγ generally enhances the inflammatory response but may also promote immunosuppression by reducing tumor recognition and T cell–mediated lysis (30). PD-L1 expression has also been linked to EGFR mutations, PI3K/AKT signaling, and, in BRAF-resistant melanoma cell lines, reactivation of the MAPK pathway (31–33). In GISTS, PD-L1 expression may conceivably be induced by other cytokines, signaling pathways, and immune cells within the tumor microenvironment, as well as by IFNγ. Oncogene inhibition downregulates PD-L1 expression in melanoma cell lines (33, 34). Similarly, in our mouse model, imatinib decreased PD-L1 mRNA, protein, and cell-surface expression. In addition, imatinib treatment of GIST cell lines abrogated the effect of IFNγ on PD-L1 expression. The effects of imatinib were likely mediated by suppression of JAK/STAT and PI3K/AKT, which are both downstream of KIT, and by repression of IFNγ and IFNγ-related genes. The reduction in IFNγ is consistent with the previous finding that imatinib reduced MHC class I expression in human GISTS (35) and our demonstration that imatinib lowered class II in murine and human tumor-associated macrophages in GISTS (36).

Tumor cells treated with targeted therapy were recently shown to induce a reactive secretome that promotes survival of sensitive cells, and the expansion and dissemination of drug-resistant clones (37). This therapeutic obstacle might be overcome by combining targeted therapy with mobilization of the immune system. Indeed, our data demonstrated that anti-PD-1 and anti-PD-L1 in KitV558/+ mice with established tumors increased the effects of imatinib by enhancing CD8+ T-cell function, resulting in substantial tumor cell apoptosis. Administered alone, anti-PD-1 and anti-PD-L1 were ineffective. It seems unlikely that pretreatment with the blocking antibodies would further improve the combination of PD-1/PD-L1 blockade and imatinib. Imatinib-induced tumor cell death could sensitize GISTS to PD-1/PD-L1 blockade through the release of endogenous tumor antigens that subsequently activate the immune system. However, the rapid tumor response by 1 week makes it more likely that a preexisting immune response was amplified. It does not seem that PD-1 inhibition in our model reduced tumor growth independent of the adaptive immune system, a mechanism recently shown in human melanoma tumor cells, which frequently express PD-1 (38).

Figure 6.

PD-1/PD-L1 blockade enhances the antitumor effects of imatinib in murine GISTS. A, Tumor weights from KitV558/+ mice (3–5/group) treated with imatinib (Im) or vehicle (Veh) for 4 weeks. Anti-PD-1 or anti-PD-L1 antibodies or their isotypes (iso) were given on days 0, 4, 8, and 12. Mice were sacrificed at day 28. B, Western blot analysis of tumor samples from KitV558/+ mice treated as in A. C, Bulk tumors were analyzed for expression of Pdcd and Idol by real-time PCR. Data are normalized to control. Data, mean of at least three samples ± SEM. *P < 0.05. D, Tumor weights after 3 months of treatment (3–5 mice/group). Antibodies or isotypes were given as in A. Mice were sacrificed at day 90. E, Tumor weights from KitV558/+ mice (3–5/group) treated with imatinib for 1 week. Anti-PD-1 or anti-PD-L1 antibodies were given on days 0, 2, 4, and 6. Mice were sacrificed at day 7. F, Tumor KIT and TUNEL staining after 1 week of treatment. Scale bars, 50 μm. G, Percentages of intratumoral CD8+ T cells that were Kit+ or IFNγ/TNF-producing intratumoral CD8+ T cells after in vitro stimulation with PMA and ionomycin. Data, means of three to four samples ± SEM. *P < 0.05. H, Cytokine array densitometry of tumors from KitV558/+ mice after 1 week of treatment. I, Tumor weights from KitV558/+ mice treated with 1-MT for 1 week (3–5/group). Anti-PD-1 antibody was given as in E. Mice were sacrificed at day 7. All tumor weights are shown normalized to vehicle control.
The other contributing factor that explains the efficacy of combining imatinib with anti-PD-1 or anti-PD-L1 is the inhibition of IDO. IDO is an enzyme that catalyzes the degradation of the essential amino acid tryptophan to kynurenine, whose metabolites suppress T cells. Previously, we showed that imatinib reduces tumor cell production of IDO in our model by decreasing the levels of the transcription factor ETV4, which regulates IDO transcription (6). Recent studies have suggested that elevated expression of metabolic enzymes (e.g., IDO) and inhibitory molecules (e.g., PD-1) is induced by IFNγ as an adaptive response by the tumor (29, 39). Our data imply that IDO inhibition by imatinib partially accounts for the antitumor efficacy of concomitant PD-1/PD-L1 blockade. This supposition is consistent with a previous report showing that IDO inhibition augmented the efficacy of T-cell immunotherapy in B16 melanoma (40). Furthermore, tumor regression after therapeutic PD-1 blockade has been demonstrated to require preexisting CD8+ T cells that are negatively regulated by PD-1/PD-L1–mediated adaptive immune resistance. Response to pembrolizumab (anti-PD-1) was associated with higher numbers of CD8+ T cells in the tumor microenvironment before treatment (41). The combination of imatinib and PD-1/PD-L1 blockade may be most effective in treatment-naïve tumors, which are the most sensitive to oncogene inhibition. Melanomas with high levels of somatic mutations have been shown to be more likely to respond to checkpoint immune blockade (42). However, our data show that murine GISTs, which have only one mutation, respond to PD-1/PD-L1 blockade in the setting of tyrosine kinase inhibition. In conclusion, PD-1 was expressed at high levels on tumor-infiltrating T cells in human GISTs, while PD-L1 levels were variable. In a mouse model of GIST, anti-PD-1 and anti-PD-L1 had no antitumor effect when used alone but did increase the efficacy of imatinib. The mechanism involved a reduction of IDO levels and an increase in CD8+ T-cell function. PD-1/PD-L1 blockade is a promising strategy to improve the effects of targeted therapy in GISTs.

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

References