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PD-L1/B7H-1 Inhibits the Effector Phase of Tumor Rejection by T Cell Receptor (TCR) Transgenic CD8$^+$ T Cells

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ABSTRACT

Although increased circulating tumor antigen-specific CD8$^+$ T cells can be achieved by vaccination or adoptive transfer, tumor progression nonetheless often occurs through resistance to effector function. To develop a model for identifying mechanisms of resistance to antigen-specific CTLs, poorly immunogenic B16-F10 melanoma was transduced to express the K^b-binding peptide SIYRYYGL as a green fluorescent protein fusion protein that should be recognized by high-affinity 2C TCR transgenic T cells. Although B16.SIY cells expressed high levels of antigen and were induced to express K^b in response to IFN-γ, they were poorly recognized by primed 2C/RAG2$^{-/-}$ T cells. A screen for candidate inhibitory ligands revealed elevated PD-L1/B7H-1 on IFN-γ-treated B16-F10 cells and also on eight additional mouse tumors and seven human melanoma cell lines. Primed 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ T cells showed augmented cytokine production, proliferation, and cytolytic activity against tumor cells compared with wild-type 2C cells. This effect was reproduced with anti-PD-L1 antibody present during the effector phase but not during the priming culture. Adoptive transfer of 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ T cells *in vivo* caused tumor rejection under conditions in which wild-type 2C cells or CTLA-4-deficient 2C cells did not reject. Our results support interfering with PD-L1/PD-1 interactions to augment the effector function of tumor antigen-specific CD8$^+$ T cells in the tumor microenvironment.

INTRODUCTION

The molecular characterization of antigens preferentially expressed by tumor cells has generated tremendous interest in the development of tumor antigen-based therapeutic vaccines. Multiple immunization strategies have been pursued, both in preclinical models and in clinical trials with advanced cancer patients. In some immunization studies of patients with melanoma, relatively high frequencies of tumor antigen-specific CD8$^+$ T cells have often been observed as detected in the peripheral blood using tetramer staining and by direct *ex vivo* functional assays such as IFN-γ ELISpot (1–3). We recently observed frequencies of CD8$^+$ T cells as high as 1% specific for the Melan-A/MART-1$_{27–35}$ epitope in patients (4). Some of these patients nonetheless had progressively growing melanoma, arguing that a relatively high frequency of specific effector T cells is not always sufficient for rejection of established metastatic cancer. These observations have motivated investigations into mechanisms of tumor resistance to antitumor T cell responses (5–7).

Although there are numerous potential mechanisms that could contribute to the resistance of solid tumors to immune effector mechanisms, a major consideration is the engagement of negative regulatory receptors on activated T cells by ligands expressed in the tumor microenvironment. CD8$^+$ effector cells can express several receptors that are thought to down-regulate T-cell responses, including CTLA-4 (8), natural killer inhibitory receptors (9, 10), and PD-1 (11). Recent work has suggested that blockade of CTLA-4 can augment antitumor T-cell responses, in both preclinical (12, 13) and clinical (14) experiments. However, the ligands for CTLA-4, B7-1 and B7-2, are predominantly expressed by antigen-presenting cells and not on tumor cells. Therefore, inhibition of CTLA-4 signaling might not restore optimal T-cell effector function during the process of tumor cell recognition within the tumor microenvironment.

PD-1 is expressed on activated T cells and appears to negatively regulate T-cell activation (15). The cytoplasmic tail contains an ITIM motif and can interact with the phosphatase SHP2 (16). Moreover, PD-1-deficient mice develop autoimmune syndromes that are potentiated on certain genetic backgrounds (17, 18). Although the identified ligands for PD-1, PD-L1 (B7H-1) and PD-L2, can be expressed by antigen-presenting cells (19), PD-L1 mRNA shows a broad tissue distribution (15, 20), and PD-L1 protein expression has been observed on tumor cells (21). Recent evidence suggests that blockade of PD-1/PD-L1 interactions might augment antitumor T-cell responses (21, 22), although the phase of T-cell activation regulated by PD-1 is not clear, and a comparison with other inhibitory receptors, such as CTLA-4, has not been explored.

B16-F10 melanoma is a poorly immunogenic tumor that lacks *de novo* class I MHC expression and serves as an ideal substrate for investigating mechanisms of tumor resistance to T-cell effector function. Surprisingly, after transfection to express the model antigen SIYRYYGL as a green fluorescent protein (GFP) fusion protein, we observed suboptimal cytokine production and cytolysis by high-affinity 2C T cell receptor (TCR) transgenic T cells, even with pretreatment of the tumor cells with IFN-γ to restore class I MHC expression. We detected high expression of PD-L1 after IFN-γ treatment on B16-F10 and all mouse and human tumor cell lines tested, and we found that the effector function of 2C T cells could be restored by eliminating PD-1 engagement. *In vivo*, PD-1-deficient 2C cells were superior to wild-type or CTLA-4-deficient 2C cells at tumor rejection, supporting the notion that strategies to interfere with PD-1/PD-L1 interactions in human cancer patients should be developed for clinical translation.

MATERIALS AND METHODS

Mice. 2C/RAG2$^{-/-}$, 2C/RAG2$^{-/-}$/CTLA4$^{-/-}$, 2C/RAG2$^{-/-}$/PD-1$^{-/-}$, and P14/RAG2$^{-/-}$ mice (H-2^b) were maintained in a specific pathogen-free barrier facility at the University of Chicago. The 2C/RAG2$^{-/-}$, 2C/RAG2$^{-/-}$/CTLA4$^{-/-}$, and 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice have been described previously (8, 22, 23), and P14/RAG2$^{-/-}$ mice were purchased from Taconic (Germantown NY). Animals were maintained and used in agreement with our Institutional Animal Care and Use Committee according to the NIH guidelines for animal use.

Antibodies. Antibodies against the following molecules coupled to the indicated fluorochromes were purchased from BD PharMingen (San Diego, CA): CD8α-phycoerythrin (PE); CD8α-PerCP; Kb-biotin; interleukin (IL)-2 uncoupled; IL-2-biotin; IFN-γ uncoupled; and IFN-γ-biotin. Antimurine PD-

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1-FITC, anti-PD-L1-PE, anti-PD-L1 uncoupled, anti-PD-L2-PE, and antihuman PD-L1-biotin were obtained from ebioscience (San Diego, CA). Streptavidin-conjugated PerCP and PE were obtained from PharMingen. The 2C TCR was stained using monoclonal antibody (mAb) 1B2 (24) that was either FITC- or biotin-coupled in our laboratory.

Flow Cytometry. Flow cytometric analysis was performed as described previously (25) using FACScan (Becton Dickinson) flow cytometers and FlowJo software (TreeStar, San Carlos, CA).

T-Cell Purification. Spleens were harvested from 2C/RAG2$^{-/-}$, 2C/RAG2$^{-/-}$/CTLA4$^{-/-}$, or 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice and prepared into single cell suspensions. CD8^{+} T cells were purified by negative selection separation system SpinSep according to the manufacturer's instructions (StemCell Technologies, Vancouver, Canada). An aliquot of purified cells was routinely stained with 1B2-FITC and CD8α-PE for analysis by flow cytometry. T-cell purity was generally >95%.

Tumor Cells. Tumor cell lines were cultured in complete DMEM and 10% FCS. The P815.B71 mastocytoma cell line was generated previously and maintained as described in the presence of Geneticin (1 mg/ml; Ref. 26). The M-MSV/BALB/3T3 Moloney murine sarcoma virus-transformed embryonal fibroblast nonproducer cell line, B16-F10 spontaneous melanoma cell line, and SKMel23 and SkMel28 human melanoma cells lines were purchased from American Type Culture Collection. The MC57 methylcholanthrene-induced fibrosarcoma cell line, Ag104 spontaneous fibrosarcoma, 4120pro UV-light induced sarcoma-like tumor, and C3 mouse embryonal cells transfected with E6/E7 and RAS (27) were provided by Dr. Hans Schreiber (University of Chicago, Chicago, IL). The EL4 chemically induced T lymphoma was stocked in our own laboratory. The PD-L2-transfected murine plasmocytoma cell line J558-B7DC and control-transfected line J558-neo were provided by Dr. Yang Liu (Ohio State University, Columbus, OH). The human melanoma cell lines 1088, 624, 537, 586, and 888-A2 were provided by Dr. Mike Nishimura (University of Chicago, Chicago, IL).

SIY Transduction. B16.SIY and B16.C tumor cell lines were obtained by retroviral transduction of B16-F-10 murine melanoma cell line with pLEGFP-SIY or empty pLEGFP vectors, provided by Dr. Hans Schreiber, as described previously (28). Retrovirus was obtained by $CaCl_2$ transfection of PHOENIX cells with the above-mentioned plasmid vectors. Forty-eight h later, supernatants were collected, and B16-F10 tumor cells were transfected using a Polybrene (8 μg/ml)-containing infection mixture. Transfected cells were selected in the presence of Geneticin (5 mg/ml).

IFN-γ Treatment. Tumor cell lines were cultured for 48 h with 20 ng/ml murine IFN-γ (R&D Systems, Minneapolis, MN) and washed three times. No IFN-γ was detected in supernatants of tumors alone after treatment. IFN-γ-treated tumor lines are denoted by the suffix "-IFN."

Cytokine and Proliferation Assays. Primed 2C T cells were obtained by incubation of purified T cells with mitomycin C-treated P815.B71 for 4 days and repetition of that treatment for an additional 4 days. Eight day-primed T cells were cocultured with mitomycin C-treated or irradiated (1000 rad) B16.SIY-IFN or mitomycin C-treated HTR.C (each at 50,000 cells/well). Supernatants were collected at 18 h, and the concentration of IL-2 or IFN-γ was detected by ELISA using the above-mentioned antibody pairs as instructed by the manufacturer (BD PharMingen). Parallel plates were cultured for 48 h and analyzed for proliferation by pulsing with [^{3}H]thymidine (1 μCi/well) for the last 6 h of the culture. Cells were harvested, and radioactivity was counted as described previously (8) using a TopCount-NXT instrument (Packard).

PD-L1 Inhibition. Naive T cells were primed for 8 days with P815.B71 in the presence or absence of 10 μg/ml sterile anti-PD-L1 mAb or control immunoglobulin (ebioscience, San Carlos, CA). Primed T cells were then stimulated with irradiated B16.SIY-IFN tumor cells. T cells primed for 8 days with P815.B71 in the absence of mAbs were stimulated with irradiated B16.SIY-IFN tumor cells in the presence or absence of 10 μg/ml sterile anti-PD-L1 mAb or control immunoglobulin. Supernatants of all groups were collected at 18 h and analyzed by ELISA.

Cytolytic Assay. Primed T cells were collected, purified by Ficoll-Hypaque centrifugation, adjusted to 2×10^6 cells/ml, and titrated in duplicate in V-bottomed microtiter plates to give the indicated E:T ratios along with 2000 ^{51}Cr-labeled target cells (either B16.SIY-IFN or B16.C-IFN) in a volume of 200 μl. Supernatants (50 μl) were collected after 4 h, and radioactivity was measured using a 96-well plate gamma counter (TopCount; Packard). The percentage of specific lysis was calculated using the instrument software.

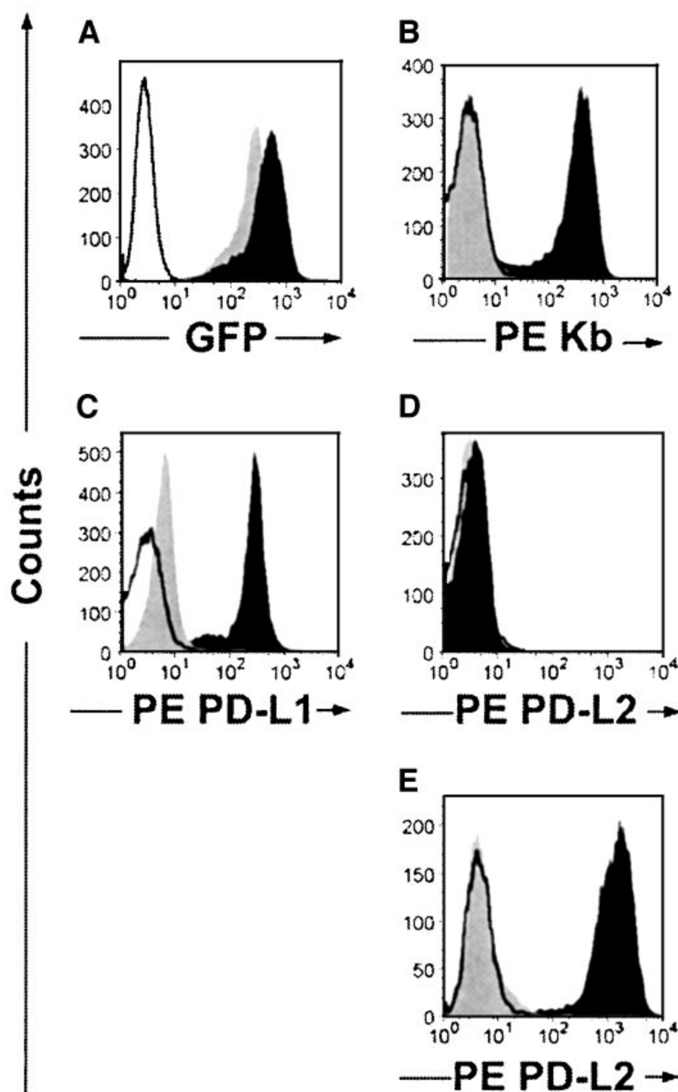

Fig. 1. Flow cytometric analysis of B16.SIY cells. *A–D,* B16.SIY tumor cells were cultured in the presence (*solid black*) or absence (*solid gray*) of 20 ng/ml murine IFN-γ for 48 h and stained as indicated. *A,* analysis for green fluorescent protein expression of B16.SIY or B16.SIY-IFN and B16-F10 as negative control (*black line*). *B,* analysis of K^b expression. Control immunoglobulin staining is represented by the *black line*. *C,* analysis of PD-L1 expression. Control immunoglobulin staining is represented by the *black line*. *D,* analysis of PD-L2 expression. Control immunoglobulin staining is represented by the *black line*. *E,* positive control of PD-L2 expression on J558-B7DC transfectants (*solid black*), J558-neo (*solid gray*), and control immunoglobulin (*black line*).

***In Vivo* Tumor Experiments.** Purified naive 2C/RAG2$^{-/-}$, 2C/RAG2$^{-/-}$/CTLA4$^{-/-}$, or 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ T cells were transferred i.v. into P14/RAG2$^{-/-}$ mice (H-2^b) by retro-orbital injection. The following day, HTR.C cells were washed with PBS, and 10^6 living cells were injected in 100 μl of PBS via a 27-gauge needle s.c. on the left flank. Tumor size was assessed twice per week using calipers, the longest and the shortest diameters were measured, and a mean was calculated. Data of groups of five mice (positive control groups of tumor + PBS-injected mice consisted of only two mice) were analyzed at each time point, and a mean and SD were determined using Microsoft EXCEL software. Measurements were continued for at least 3 weeks.

RESULTS

B16.SIY Cells Poorly Stimulate 2C/RAG2$^{-/-}$ TCR Transgenic T Cells. To render B16-F10 melanoma cells (H-2^b) recognizable by 2C TCR transgenic T cells, we used retroviral transduction to introduce a cDNA encoding the K^b-binding peptide SIYRYYGL (SIY),

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fused in frame with enhanced GFP (28). The SIY/K^b complex (29) and the SIY-GFP fusion (28) have previously been described to be recognized by the 2C TCR. Flow cytometric analysis revealed high expression of the fusion protein as assessed by GFP fluorescence (Fig. 1). However, B16.SIY cells were nonetheless poorly lysed by primed 2C T cells (data not shown) and produced only low levels of IL-2 and IFN-γ (Fig. 2, *B* and *C*). We initially presumed this result to be due to defective class I MHC surface expression, which has been reported to be overcome by treatment of B16-F10 melanoma with IFN-γ (30). Indeed, IFN-γ pretreatment substantially up-regulated K^b expression (Fig. 1*B*) and preserved SIY-GFP expression (Fig. 1*A*). However, IFN-γ-treated B16.SIY cells were still poorly recognized by primed 2C T cells (Fig. 2). These results suggested that IFN-γ-treated B16.SIY cells were either deficient in expression of another positive regulator or expressed high levels of a negative regulator of T-cell activation.

IFN-γ-Treated B16.SIY Cells Up-Regulate PD-L1 but not PD-L2. We screened IFN-γ-treated B16.SIY cells for expression of candidate ligands that could engage inhibitory receptors on primed 2C T cells. Neither B7-1 nor B7-2, which could engage CTLA-4, were detected (data not shown). However, the PD-1 ligand PD-L1 was expressed at high levels (Fig. 1*C*). In contrast, PD-L2 was not detectably expressed (Fig. 1*D*) compared with a positive control transfectant (Fig. 1*E*). Thus, PD-L1 was a strong candidate for inhibiting the function of primed 2C cells.

PD-1-Deficient 2C/RAG2$^{-/-}$ T Cells Show Augmented Cytokine Production and Cytolytic Activity. The high up-regulation of PD-L1 on IFN-γ-treated B16.SIY cells led us to investigate whether engagement of PD-1 by PD-L1 could be preventing optimal T cell activation. This was addressed first by using 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice. As shown in Fig. 2, primed T cells from 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ mice showed augmented lysis and robust cytokine production against IFN-γ-treated B16.SIY cells. Although the maximal percentage of specific lysis was only around 17% in a 4-h chromium-release assay, the cytolytic effect of PD-1-deficient 2C cells was even more striking when visualized after a 3-day culture (Fig. 3), at which time tumor cells were no longer visible, and T cells had expanded. These results demonstrate that the poor responsiveness of 2C T cells to IFN-γ-treated B16.SIY tumor cells could be overcome by elimination of PD-1.

Anti-PD-L1 mAb Augments 2C T-Cell Responses in the Effector Phase but not the Priming Phase. The use of PD-1-deficient T cells eliminated a potential negative effect of PD-1 during both the *in vitro* priming of the 2C T cells and the effector assays. Examination of PD-1 expression on 2C cells by flow cytometry revealed maximal induction 24–48 h after initial activation, but more sustained expression during the second 4-day priming culture (Fig. 4*A*). By comparison, 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ T cells showed no detectable expression. Both naive and primed 2C T cells also expressed PD-L1 (Fig. 4*B*). Moreover, the P815.B71 cells used to activate the 2C T cells also were found to express PD-L1 (data not shown). Thus, there was ample opportunity for PD-L1/PD-1 interactions to occur during the priming culture.

To distinguish between a negative regulatory role for PD-1 during the priming phase *versus* the effector phase, neutralizing anti-PD-L1 mAb was used with wild-type 2C cells. As shown in Fig. 4*C*, when anti-PD-L1 was included throughout the priming cultures of 2C/RAG2$^{-/-}$ T cells, no improvement in cytokine production was observed on restimulation with IFN-γ-treated B16.SIY. However, the addition of anti-PD-L1 only to the restimulation culture with IFN-γ-treated B16.SIY significantly augmented IL-2 production (Fig. 4*C*) and IFN-γ production (data not shown). These results suggest that the major inhibitory effect of PD-L1/PD-1 interactions occurs at the effector phase of the CD8$^+$ T-cell response.

Multiple Tumor Cell Lines Express PD-L1 but not PD-L2 Constitutively and/or on Stimulation with IFN-γ. To determine whether PD-L1 expression by tumor cells was commonly observed, a

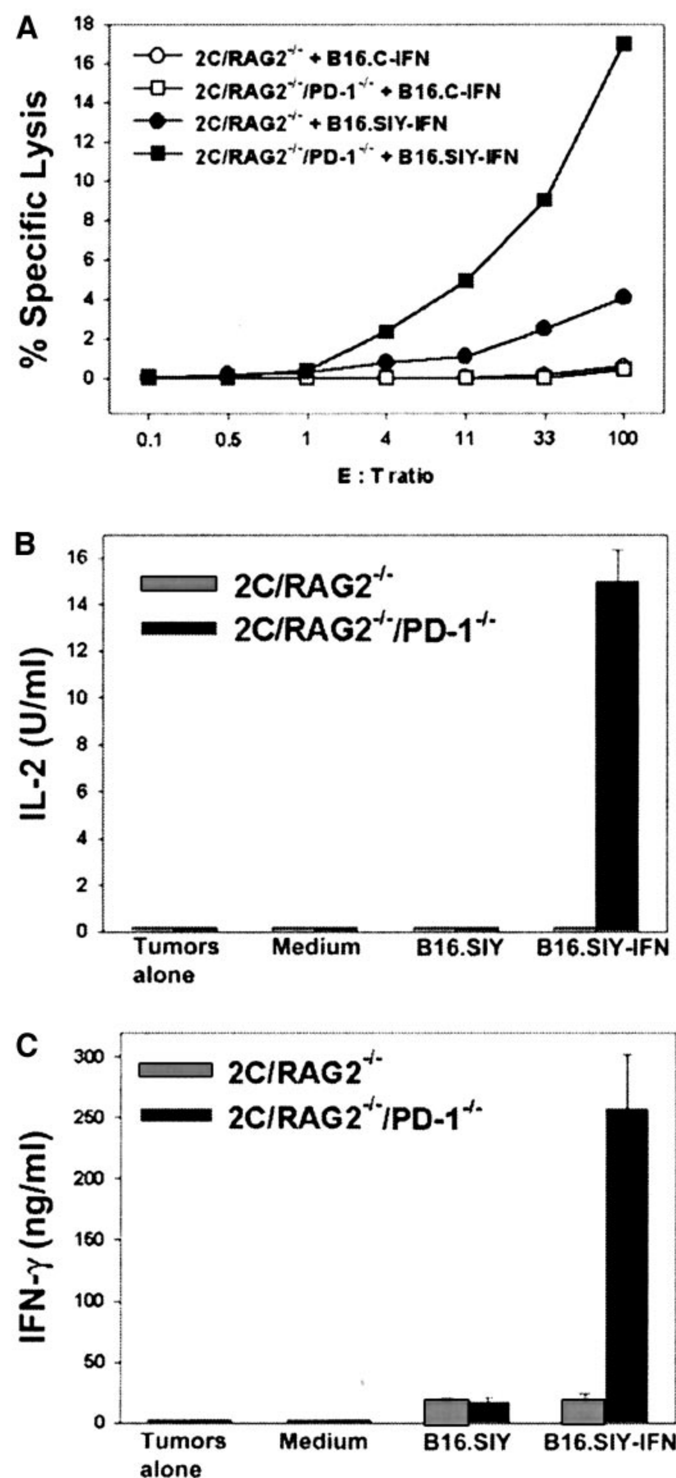

Fig. 2. Markedly improved 2C effector function in the absence of PD-1. *A*, cytolytic activity. Primed 2C/RAG2$^{-/-}$ or 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ T cells were cocultured with B16.C-IFN or B16-SIY-IFN (48 h IFN-γ pretreatment) for 4 h at the indicated E:T cell ratios, and lytic function was determined by ^{51}Cr release. The difference between wild-type and PD-1$^{-/-}$ 2C cells was significant using an unpaired *t* test ($P < 0.001$). *B* and *C*, cytokine production. Primed 2C/RAG2$^{-/-}$ or 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ T cells were stimulated with B16.SIY or B16.SIY-IFN cells, and supernatants were collected after 18 h. Levels of IL-2 (*B*) and IFN-γ (*C*) were determined by ELISA. *Tumors alone* indicates cytokine levels detected without T cells. *Medium* indicates cytokine levels detected with T cells alone. Similar results were observed in at least three experiments.

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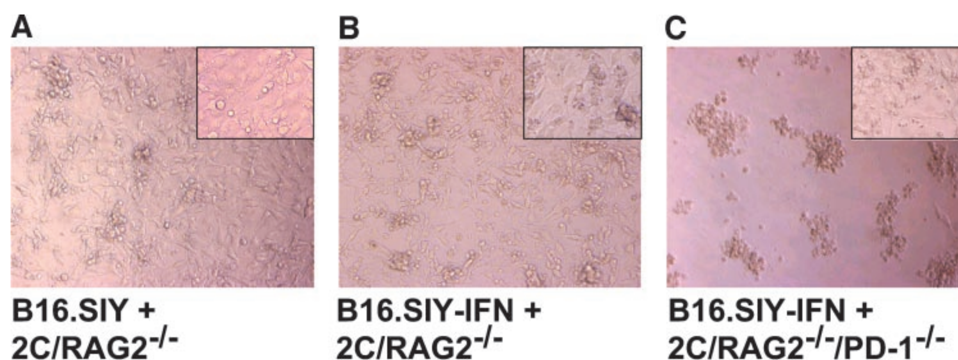

Fig. 3. Improved tumor cell elimination *in vitro* by PD-1-deficient 2C cells. Equal numbers of primed 2C/RAG2$^{-/-}$ T cells (*A* and *B*) or 2C/RAG$^{-/-}$/PD-1$^{-/-}$ cells (*C*) were cocultured with B16.SIY (*A*) or IFN-γ-pretreated B16-SIY (*B* and *C*) in 24-well plates. Areas representative of the cell density were photographed at day 3. Similar results were found on days 4 and 5 and in at least three experiments (data not shown).

panel of tumor cell lines of a variety of histologies was examined, with or without exposure to IFN-γ. As shown in Fig. 5*A*, all eight tumors examined expressed PD-L1 on IFN-γ treatment; both HTR.C and J558 showed high constitutive expression. None of the tumor lines expressed detectable PD-L2 (data not shown).

We also examined a panel of human melanoma cell lines for expression of PD-L1. A representative flow cytometry histogram is shown in Fig. 5*B*. All seven cell lines showed augmented expression of PD-L1 after exposure to human IFN-γ (Fig. 5*C*). Thus, the potential inhibitory effect of PD-L1 on T-cell effector function could extend to the human system as well.

Absence of PD-1 Allows Tumor Rejection by 2C T Cells Under Conditions in Which WT 2C Cells and CTLA-4-Deficient 2C T Cells Fail to Reject. We wished to examine the effect of PD-1 deficiency on tumor rejection *in vivo* but sought out a tumor cell line that did not require IFN-γ pretreatment for MHC/peptide recognition. As with IFN-γ-treated B16.SIY cells, 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ T cells showed augmented proliferation in response to HTR.C tumor cells, which express L^d and are thus recognized by the 2C TCR, to the same extent as that seen with B16.SIY cells (Fig. 6). Cytokine production also was augmented, and similar results were observed with J558 tumor cells as stimulators (data not shown). Of note, both wild-type and PD-1-deficient 2C cells responded comparably to anti-CD3/anti-CD28 mAb stimulation, consistent with the notion that the augmented function of PD-1$^{-/-}$ T cells depends on ligand expression by the stimulator cell.

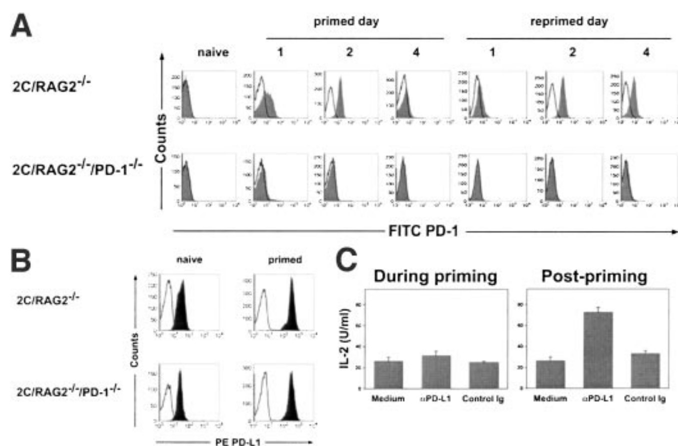

Fig. 4. *A,* PD-1 expression on 2C T cells during priming. Purified 2C/RAG2$^{-/-}$ (*top row*) and 2C/RAG$^{-/-}$/PD-1$^{-/-}$ (*bottom row*) T cells were primed with mitomycin C-treated P815.B71, stained with FITC anti-PD-1, and analyzed by flow cytometry at the indicated time points. At day 4, the cells were collected, purified by Ficoll-Hypaque centrifugation, reprimed for another 4 days, and analyzed similarly. *B,* PD-L1 expression by 2C T cells. Purified 2C/RAG2$^{-/-}$ (*top row*) and 2C/RAG$^{-/-}$/PD-1$^{-/-}$ (*bottom row*) T cells were primed with mitomycin C-treated P815.B71 and stained for PE PD-L1 on day 0 (*left panels*) and day 2 (*right panels*). *C,* augmentation of T-cell activation with anti-PD-L1 antibody during the effector phase but not the priming phase. Purified 2C/RAG2$^{-/-}$ cells were primed for 4 days and reprimed for 4 days with mitomycin C-treated P815.B71 in the absence or presence of anti-PD-L1 mAb or control immunoglobulin. At day 8, cells were collected, purified by Focoll-Hypaque centrifugation, and stimulated with B16.SIY-IFN (*left panel*). Control primed 2C cells were also stimulated in the presence of anti-PD-L1 mAb or control immunoglobulin (*right panel*). After 18 h, supernatants were collected, and levels of interleukin 2 were determined by ELISA. Similar results were seen for IFN-γ (data not shown) and in two independent experiments.

HTR.C tumor cells were thus chosen for *in vivo* experiments. To this end, purified 2C/RAG2$^{-/-}$ or 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ T cells were adoptively transferred into P14/RAG2$^{-/-}$ recipient mice. These mice were chosen as recipients because homeostatic proliferation of transferred 2C T cells is blocked by the irrelevant P14 TCR transgenic population (data not shown) and also because they lack any other T cells that could contribute to tumor rejection. 2C/RAG2$^{-/-}$/CTLA-4$^{-/-}$ T cells were also administered as a comparison. One day later, mice were challenged with HTR.C cells s.c., and tumor measurement was recorded over time. As shown in Fig. 7, only PD-1-deficient T cells were capable of rejecting HTR.C tumors, whereas tumor growth in mice receiving wild-type 2C cells was comparable with that in mice receiving PBS alone. In fact, even CTLA-4-deficient 2C cells failed at tumor rejection in this experimental setting, arguing that the inhibitory effect of PD-1 in this model is dominant. When compiled over two independent experiments using tumor measurements at day 24 as an indicator, these differences were statistically significant (wild type *versus* PD-1$^{-/-}$, $P < 0.001$; PD-1$^{-/-}$ *versus* CTLA-4$^{-/-}$, $P < 0.001$; wild type *versus* CTLA-4$^{-/-}$, $P = 0.697$). Thus, absence of PD-1 can lead to potent tumor rejection *in vivo*.

DISCUSSION

In the current study, we were surprised to observe that B16-F10 melanoma cells expressing the SIY antigen as a GFP fusion protein (28) were poorly recognized by 2C T cells. Even after induction of class I MHC expression using IFN-γ, poor lysis and cytokine production were observed. We found that IFN-γ also up-regulated expression of the inhibitory ligand PD-L1/B7H1 and that interference with PD-1 engagement could restore effector function of primed 2C T cells *in vitro* and *in vivo*. These observations support the pursuit of similar strategies to overcome tumor resistance to T-cell function in the clinic.

There is some controversy regarding whether PD-L1 and PD-L2 exert positive or negative regulatory effects on T cells (15, 20, 31, 32). It seems unlikely that a positive costimulatory ligand would be found to be so widely expressed on tumor cells as we have observed for PD-L1. In addition, PD-L1 was expressed on the surface of naive T cells, which are unlikely to mediate spontaneous self-costimulation through homotypic interactions. However, it is possible that positive *versus* negative regulatory effects could vary with the level of PD-L1 expression or posttranslational modification of the molecule. For PD-L2, there is clear evidence that it can induce a positive costimu-

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latory signal through an as yet unidentified alternative receptor other than PD-1 (32, 33). We did not observe PD-L2 expression on any tumor cell lines, even with IFN-γ treatment. A recent study has indicated that induction of PD-L2 on macrophages occurs with IL-4 treatment or interaction with Th2 cells (34), so it is possible that other cytokines could up-regulate PD-L2 expression on tumor cells.

Although previous work has shown that tumors transfected to express high levels of PD-L1 grew more aggressively *in vivo* (35), our current study revealed that all tumor cells tested to date up-regulated PD-L1 expression in response to IFN-γ. Because a goal of many immunotherapy protocols is to induce a type 1 T-cell phenotype (28, 4), this result suggests that PD-L1 may be a frequent mechanism for

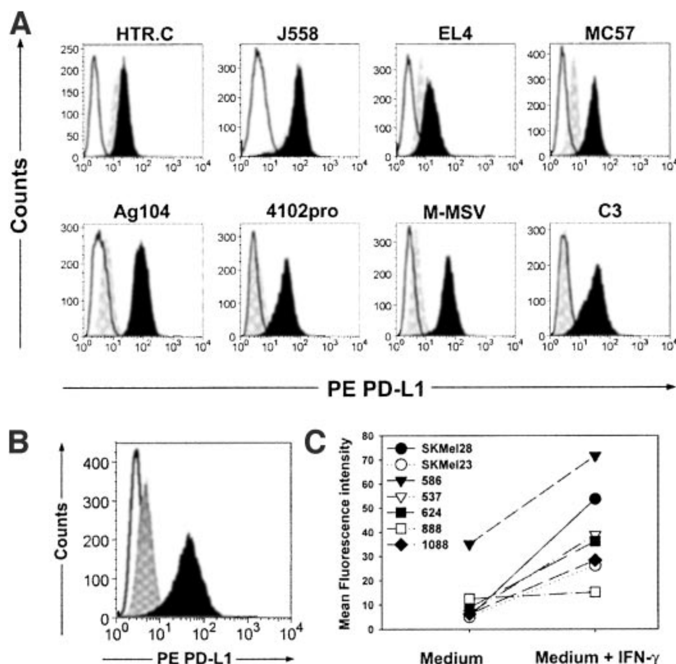

Fig. 5. *A,* PD-L1 expression on murine tumor cell lines. The indicated tumor cell lines were cultured in complete medium with 10% FCS in the absence (*solid gray*) or presence (*solid black*) of 20 ng/ml murine IFN-γ for 48 h, stained with PE-anti-PD-L1, and analyzed by flow cytometry. Isotype control antibody staining was performed in parallel (*black line*). No expression of PD-L2 was observed on any tumor cell line (data not shown). Similar results were observed in at least two experiments. *B* and *C,* PD-L1 expression on human melanoma cell lines. The indicated tumor cell lines were cultured in complete medium with 10% FCS in the absence or presence of 200 ng/ml human IFN-γ for 48 h, stained with PE-antihuman PD-L1, and analyzed by flow cytometry. Isotype control antibody staining was performed in parallel. Representative flow cytometry histogram of SKMel28, showing control immunoglobulin staining (*black line*) and PE PD-L1 without (*solid gray*) or with (*solid black*) pretreatment with IFN-γ, is shown in *B*. Changes of mean fluorescence intensity of all tumor lines analyzed are shown in *C*. Similar results were observed in at least two experiments.

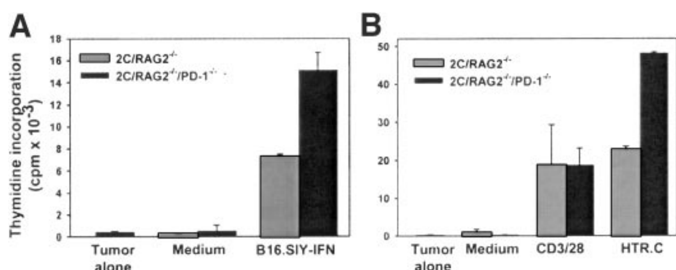

Fig. 6. Increased proliferation of primed 2C/RAG2$^{-/-}$PD-1$^{-/-}$ T cells in response to B16.SIY-IFN or HTR.C tumor cells. Purified primed 2C/RAG2$^{-/-}$ (▤) and 2C/RAG$^{-/-}$/PD-1$^{-/-}$ (■) cells were stimulated with either mitomycin C-treated B16.SIY-IFN (*A*) or HTR.C (*B*) cells or a 5:1 ratio of anti-CD3:anti-CD28-coated beads for 48 h. [^{3}H]Thymidine was added during the last 6 h, and incorporated radioactivity was measured. Similar results were observed in at least two experiments.

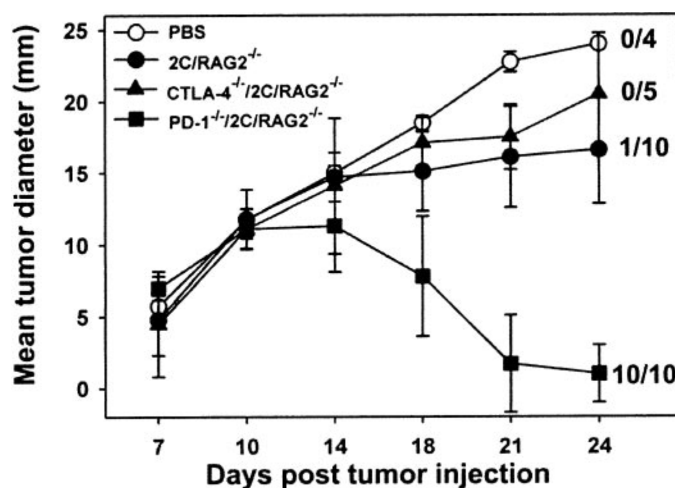

Fig. 7. Absence of PD-1 enables 2C T cells to reject HTR.C tumors *in vivo*. Groups of 4–10 of P14/RAG2$^{-/-}$ mice were challenged s.c. with 10^6 HTR.C cells after i.v. adoptive transfer of 10^6 naive 2C/RAG2$^{-/-}$ (●), 2C/RAG2$^{-/-}$/CTLA-4$^{-/-}$ (▲), or 2C/RAG2$^{-/-}$/PD-1$^{-/-}$ T cells (■) 24 h earlier. PBS-injected mice were compared as a control (○). Mean diameters were determined at the indicated time points. The numbers of animals ultimately rejecting the tumor are shown to the *right* of each curve. The data are pooled from two experiments.

resisting the effector phase of IFN-γ-producing antitumor T-cell responses. Moreover, our current results extend previous observations by supporting a role for PD1/PD-L1 blockade in adoptive T-cell therapy approaches, which have gained increased attention for clinical application (36).

In our model, PD-1-deficient T cells caused tumor rejection in a setting in which CTLA-4-deficient T cells failed. It is possible that the absence of CTLA-4 on T cells could potentiate tumor rejection in other tumor models. Of note, our experimental system focused exclusively on a monoclonal population of CD8$^+$ T cells, in the absence of CD4$^+$ cells. Because there is evidence that CD4$^+$ T cells are the dominant population that undergoes spontaneous activation in CTLA-4$^{-/-}$ mice (37), a benefit of CTLA-4 deficiency may be better observed when CD4$^+$ T cells are participating in the response. It is worth considering that the ligand for PD-1, PD-L1, was expressed directly on tumor cells, whereas for CTLA-4, the ligands would be expressed predominantly by antigen-presenting cells. Thus, PD-L1 could play a more critical role in suppressing the execution of T-cell effector function during the process of tumor cell recognition. An inhibitory effect of PD-1 at the effector phase of antiviral immunity has been reported recently (35).

Our data suggest that PD-1 engagement preferentially delivers an inhibitory signal at the effector phase of CD8$^+$ T-cell function rather than during early T-cell activation and differentiation. The mechanism for this difference is unclear, but it is similar to what we had observed for CTLA-4 in previous studies in which augmented function of 2C/RAG2$^{-/-}$/CTLA-4$^{-/-}$ T cells was only seen on secondary restimulation (8). Similarly, it has been reported that T-cell anergy is preferentially induced in primed effector cells rather than naive T cells (38). Together, these observations suggest that CD8$^+$ T cells must differentiate into a state that renders them inhibitable by these negative regulatory processes. The molecular mechanisms that govern this differential susceptibility between naive and effector cells are an attractive subject for future studies.

There are additional putative inhibitory receptors that could theoretically down-regulate T-cell effector function via ligands expressed directly on tumor cells. Natural killer inhibitory receptors can be found on CD8$^+$ effector and memory cells (9, 10) that could be engaged by the appropriate class I MHC molecules on tumor cells. In

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addition, the recently described inhibitory receptor BTLA that has functional similarities to PD-1 appears to recognize the B7 family member B7x (39, 40) that also could be expressed by tumor cells. Interfering with the interaction between these receptor/ligand pairs also might potentiate antitumor T-cell effector function *in vivo*. Translating these concepts to human cancer patients should be a high priority in future studies.

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