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+R-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+R 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 cytolysis 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-127–35 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-L1 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 (B7H1) 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-2b) 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): CD8a-phycocerythrin (PE); CD8a-PerCP; Kb-biotin; interleukin (IL)-2 uncoupled; IL-2-biotin; IFN-γ uncoupled; and IFN-γ-biotin. Antimurine PD-
Flow Cytometry. Flow cytometric analysis was performed as described previously (25) using FACSscan (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 cell lines were purchased from American Type Culture Collection. The M357 methylcholanthrene-induced fibrosarcoma cell line, Ag104 spontaneous fibrosarcoma, 412pro UV-light induced sarcoma-like tumor, and C3 mouse embryonal cells trans vectored 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 plasmacytoma 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, 244, 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 CaCl2 transfection of PHOENIX cells with the above-mentioned plasmid vectors. Forty-eight h later, supernatants were collected, and B16-F10 tumor cells were trans vectored using a Polybrene (8 μg/ml)-containing infection mixture. Transvectored 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-γ.

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-primeid 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 pulse labeling with [3H]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 × 10⁷ cells/ml, and titrated in duplicate in V-bottomed microtiter plates to give the indicated E:T ratios along with 2000 ⁵¹Cr-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.

B16.SIY Cells Poorly Stimulate 2C/RAG2−/− TCR Transgenic T Cells. To render B16-F10 melanoma cells (H-2b) recognizable by 2C TCR transgenic T cells, we used retroviral transduction to introduce a cDNA encoding the Kβ-binding peptide SIYRYYG (SIY),
fused in frame with enhanced GFP (28). The SIY/Kb 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 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/H9253 (30). Indeed, IFN-γ pretreatment substantially up-regulated Kb expression (Fig. 1B) and preserved SIY-GFP expression (Fig. 1A). 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. 1C). In contrast, PD-L2 was not detectably expressed (Fig. 1D) compared with a positive control transfectant (Fig. 1E). 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. 4A). By comparison, 2C/RAG2−/−/PD-1−/− T cells showed no detectable expression. Both naive and primed 2C T cells also expressed PD-L1 (Fig. 4B). 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. 4C, when anti-PD-L1 was included throughout the priming cultures of 2C/RAG2−/− T cells, no improvement in cytolytic function was detected with T cells alone. Similar results were observed in at least three experiments.
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/RAG2−/−/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).

Fig. 4. A, PD-1 expression on 2C T cells during priming. Purified 2C/RAG2−/− (top row) and 2C/RAG2−/−/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-1 expression by 2C T cells. Purified 2C/RAG2−/− (top row) and 2C/RAG2−/−/PD-1−/− (bottom row) T cells were primed with mitomycin C-treated P815.B71 and stained for PE anti-PD-1 on day 0 (left panels) and day 2 (right panels). C, augmentation of T-cell activation with anti-PD-1 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-1 mAb or control immunoglobulin. At day 8, cells were collected, purified by Ficoll-Hypaque centrifugation, and stained with B16.SIY-IFN (left panel). Control primed 2C cells were also stimulated in the presence of anti-PD-1 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.

A

B16.SIY + 2C/RAG2−/−

B16.SIY-IFN + 2C/RAG2−/−

B

B16.SIY + 2C/RAG2−/−/PD-1−/−

B16.SIY-IFN + 2C/RAG2−/−/PD-1−/−

C

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 upregulated expression of the inhibitory ligand PD-L1 on 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. 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-
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 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-

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−/− (C) and 2C/RAG2−/−/PD-1−/− (D) 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. [3H]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 P14RAG2−/− mice were challenged s.c. with 10⁶ HTR.C cells after i.v. adoptive transfer of 10⁶ 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.
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 antimurine T-cell effector function in vivo. Translating these concepts to human cancer patients should be a high priority in future studies.

ACKNOWLEDGMENTS

We thank Barbara Spies and Candace Cham for technical assistance, Janet Washington for assistance with mouse breeding, Mike Nishimura and Hans Schreiber for tumor cell lines, and Marisa Alegre for careful reading of the manuscript.

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Cancer Res 2004;64:1140-1145.

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