Combinatorial Immunotherapy of Polyinosinic–Polycytidylic Acid and Blockade of Programmed Death-Ligand 1 Induce Effective CD8 T-cell Responses against Established Tumors

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Abstract

Purpose: Epitope-based cancer vaccines capable of inducing CD8 T-cell responses to tumor-associated antigens (TAA) expressed by tumor cells have been considered as attractive alternatives for the treatment of some types of cancer. However, reliable TAAs have not been identified for most malignant diseases, limiting the development of epitope-based vaccines. Herein, we report that the combinatorial therapy of polyinosinic–polycytidylic acid (poly-IC) and antiprogrammed death-ligand 1 (PD-L1) monoclonal antibody (mAb) can be implemented with good results for tumors where no known TAAs have been identified.

Experimental Design: Three cancer mouse models (melanoma, lung, and colon) were used to evaluate therapeutic efficacy and examine the immunologic mechanisms of the poly-IC/anti–PD-L1 mAb therapy.

Results: The combined administration of poly-IC and anti–PD-L1 mAb into tumor-bearing mice generated potent immune responses resulting in the complete eradication or remarkable reduction of tumor growth. In some instances, the poly-IC/anti–PD-L1 mAb therapy induced long-lasting protection against tumor rechallenges. The results indicate that CD8 T cells but not CD4 T cells or NK cells mediated the therapeutic efficacy of this combinatorial therapy. Experiments using genetically deficient mice indicate that the therapeutic efficacy of this combinatorial therapy depended in part by the participation of type-I IFN, whereas IFN-γ did not seem to play a major role.

Conclusions: The overall results suggest that immunotherapy consisting of the combination of poly-IC/anti–PD-L1 mAb could be a promising new approach for treating patients with cancer, especially those instances where no reliable TAAs are available as a therapeutic vaccine. Clin Cancer Res; 1–12. ©2014 AACR.

Introduction

Conventional treatments for cancer such as surgery, radiotherapy, and chemotherapy are commonly associated with suboptimal therapeutic efficacy and detrimental side effects. Therefore, different treatment modes such as immunotherapy using therapeutic vaccines or monoclonal antibodies (mAb) that enhance ongoing antitumor immune responses are being explored as alternatives or adjunct treatments (1, 2). Especially, the use of cancer vaccines that induce tumor-reactive CD8 T cells is being considered as a strategy to treat established tumors and prevent recurrences (3–5). Nevertheless, to develop such therapeutic vaccines it is necessary to identify tumor-associated antigens (TAA) containing peptide epitopes for tumor-reactive CD8 T cells (6, 7). Several investigators including us have considered using synthetic peptides representing defined CD8 T-cell epitopes derived from TAAs such as melanosomal differentiation proteins, as vaccines for treating melanoma (8–11). Being cognizant that significant challenges exist regarding the use of peptide vaccines such as those related to MHC restriction, which limit the use of a peptide to a subset of patients expressing a particular MHC class I (MHC-I) allele. In addition, for many tumor types such as lung cancer, no reliable TAAs capable of eliciting effective antitumor T-cell responses have been identified. In view of this, alternative approaches to generate antitumor CD8 T-cell responses should be explored. One strategy would be to generate or enhance existing tumor-specific CD8 T-cell responses via the combinatorial use of strong immune adjuvants such as Toll-like receptor (TLR) ligands and mAbs that block immune regulatory pathways that suppress CD8 T cells. While studying the therapeutic effects of peptide vaccines administered in combination of polyinosinic–polycytidylic acid (poly-IC) and antiprogrammed death-ligand 1 (PD-L1) mAb in a mouse model of melanoma, we observed significant therapeutic effects in mice that received an irrelevant control peptide (11). These results suggested that the combined administration of these
Translational Relevance
For many malignant diseases, few if any reliable tumor-associated antigens (TAA) capable of eliciting effective antitumor CD8 T-cell responses have been identified, limiting the development of epitope-based vaccines. Thus, there is a clear need to explore alternative and novel immunotherapeutic approaches to induce and amplify tumor-reactive CD8 T cells without depending on the use of defined TAAs. Here we describe a non–antigen-specific immunotherapy consisting of repeated co-administration of polyinosinic–polycytidylic acid (poly-IC) and antiprogrammed death-ligand 1 (PD-L1) antibodies that results in dramatic antitumor effects in several cancer mouse models, which were mediated by CD8 T cells. Because both poly-IC and anti–PD-L1 monoclonal antibody are available for clinical use, we believe that our preclinical studies can readily translate into the treatment for patients with cancer, especially in those instances where reliable TAAs have not been identified.

immune modulating agents could provide a therapeutic benefit against established tumors. Here, we report that non–antigen-specific immunotherapy consisting of repeated co-administration of poly-IC and anti–PD-L1 mAb resulted in dramatic antitumor responses in several cancer mouse models, which were mediated by CD8 T cells.

Materials and Methods

Mice and cell lines
Six- to 8-week-old female C57BL/6 (B6) mice were from the National Cancer Institute/Charles River Program (Wilmington, MA). IFN-γ deficient (IFN-γ−/−) mice (B6 background) were from Jackson Laboratories. IFN-αβ receptor-deficient (IFN-αβR−/−) mice (B6 background) were obtained from Dr. Philippa Marrack (National Jewish Medical and Research Center, Denver, CO). All animal care and experiments were conducted according to our institutional guidelines. Lewis lung carcinoma (LLC)-A9F1 cells are a subclone of LLC (12) provided by Lea Eisenbach (Weizmann Institute of Science, Rehovot, Israel). Mouse colorectal adenocarcinoma MC38 cells were provided by Dmitry Gabrilovich (Moffitt Cancer Center, Tampa, FL). Mouse melanoma B16F10 cells were provided by Alan Houghton (Memorial Sloan Kettering Cancer Center, New York, NY). Mouse thymoma EL4 cells were from the American Type Culture Collection. All of the cell lines were cultured as recommended by the providers and were not authenticated by the authors.

Reagents and antibodies
Poly-IC was provided by Andres Salazar [poly-lysine and carboxymethylcellulose (poly-ICLC)/Hiltonol; Oncovir, Inc.] or purchased from InvivoGen (Poly-IC HMW). Cpg-1826 was prepared by the Mayo Clinic Molecular Core Facility. Anti-mouse PD-L1 (10F.9G2), anti-NK 1.1 (PK136), anti-CD4 (GK1.5), anti-CD8 (2.43), and anti-mouse interleukin-2 (IL-2; JES6-5H4) mAbs were from BioXCell. Anti-mouse–programmed death-1 (PD-1; RMPI-14) mAb was provided by Hideo Yagita (Juntendo University School of Medicine, Tokyo, Japan). Recombinant mouse IL-2 and IFN-γ were from PeproTech. Fluorescence labeled Abs were from eBioscience and BD Biosciences.

Flow cytometric analysis
The expression of MHC molecules and PD-L1 on LLC-A9F1, MC38, and B16F10 cells pretreated or not for 24, 40, or 48 hours with 100 ng/mL IFN-γ was evaluated by flow cytometry using FITC-conjugated anti–H-2Dβ, PE-conjugated anti–H-2-Kβ, APC-conjugated anti-MHC class II (MHC-II), and PE-conjugated anti–PD-L1 Abs. Cell lines also were stained by 7-AAD to exclude nonviable cells. Fluorescence was measured using a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software.

Therapeutic protocols and evaluation of antitumor effects
Mice were injected subcutaneously with 5 × 10⁵ LLC-A9F1, 5 × 10⁵ MC38, or 4 × 10⁵ B16F10 cells in a shaved rear flank. Seven (B16F10 tumor model), 8 (MC38 tumor model), or 9 days (LLC-A9F1 tumor model) later, poly-IC or Cpg-1826 was administered intravenously at 50 μg/dose. The administration of poly-IC or Cpg-1826 was repeated 3 times, 5 days apart. Anti–PD-L1 or PD-1 mAb was administered intraperitoneally on days 1 and 3 after each poly-IC or Cpg-1826 administration at a 200 μg/dose. IL-2/anti–IL-2 mAb complexes (IL-2/anti–IL-2 mAb complexes) were prepared by incubating 2 μg recombinant mouse IL-2 with 10 μg anti-mouse IL-2 mAb per dose for 18 hours at 4°C. IL-2/anti–IL-2 mAb complexes also were stained by 7-AAD to exclude nonviable cells. For in vivo cell depletions (CD8 T cells, CD4 T cells, or NK cells), mice received the following mAbs via intraperitoneal injections: anti–CD8, 500 μg/injection; anti–CD4, 200 μg/injection; or anti–NK1.1, 300 μg/injection on days −3, −1, and +4 of the first poly-IC administration. Depletions were confirmed by flow cytometry analysis of blood samples (data not shown). Tumor growth was monitored every 2 to 3 days in individually tagged mice by measuring 2 opposing diameters with a set of calipers. Mice were euthanatized when the tumor area reached 400 mm². Results are presented as the mean tumor size (area in mm²) ± SD for every treatment group at various time points until the termination of the experiment.

Measurement of immune responses
For detection of CD8 T cells, secreting IFN-γ EliSpot assays were performed as described (13), using purified spleen CD8 T cells (Miltenyi Biotec). CD8 T cells were incubated at 1 × 10⁵ together with 1 × 10⁴ stimulator cells.
Therapeutic effects of the combinatorial immunotherapy with poly-IC and anti–PD-L1 mAb against established B16 melanoma

In a recent study, we observed a significant antitumor effect in a control group of mice that received an irrelevant peptide vaccine combined with poly-IC [TLR3 ligand and melanoma differentiation-associated protein 5 (MDA5) agonist] and anti–PD-L1 mAb against established subcutaneous B16 tumors (11). In view of this interesting observation, we first explored the therapeutic efficacy and examined the immunologic mechanisms involved of the combined administration of poly-IC and anti–PD-L1 mAb in the B16 mouse melanoma model. Mice were inoculated subcutaneously on day 0 with $4 \times 10^5$ B16F10 cells and later treated on days 7, 12, and 17 with poly-IC at 50 μg/dose given intravenously. Anti–PD-L1 mAb (200 μg/dose) was administered intraperitoneally 1 and 3 days after each poly-IC treatment. Various subsets of immune cells (CD8 T cells, CD4 T cells, or NK cells) were depleted using mAb 3 and 1 days before and 4 days after receiving the first poly-IC treatment. Nontreated mice were included as controls. As noted, some mice received poly-IC alone or anti–PD-L1 mAb alone. Points, mean for each group of mice; bars, SD. P values were compared with no treatment group and calculated using 2-way ANOVA tests ($^* P<0.05$, $^*^* P<0.001$, $^*^*^* P<0.00001$).

Next, we determined whether the therapeutic efficacy of poly-IC/anti–PD-L1 mAb combinatorial therapy would extend to other tumor types. For these studies, we selected the transplantable LLC and the MC38 colon carcinoma. Because the tumor recognition of T cells and the efficacy of anti–PD-L1 mAb may depend on the expression levels of MHC-I (H-2Db and H-2Kb), which were somewhat increased by IFN-γ, B16F10 melanoma cells were also included in these evaluations. Both LLC-A9F1 and MC38 cells that were pretreated or not with IFN-γ increased its expression only with B16F10 cells and 7 days later they received poly-IC alone, anti–PD-L1 mAb alone, or poly-IC plus anti–PD-L1 mAb. As shown in Fig. 1, tumors grew at a somewhat lower rate in mice that received poly-IC or anti–PD-L1 mAb as compared with the untreated group. In contrast, the combined administration of poly-IC/anti–PD-L1 mAb resulted in a remarkable synergistic therapeutic effect. Notably, depletion of CD4 T cells or NK cells did not reduce the effectiveness of the combination therapy. However, depletion of CD8 T cells abrogated the antitumor effect. Although B16 tumor growth was slowed down by the poly-IC/anti–PD-L1 mAb combination therapy, none of the mice rejected their tumors.

Therapeutic effects of poly-IC/anti–PD-L1 mAb combinatorial immunotherapy against established lung and colon tumors

Figure 1. Therapeutic effects induced by the combinatorial therapy of poly-IC/anti–PD-L1 mAb against established B16F10 tumors. B6 mice (5 per group) were inoculated subcutaneously on day 0 with $4 \times 10^5$ B16F10 cells and later treated on days 7, 12, and 17 with poly-IC at 50 μg/dose given intravenously. Anti–PD-L1 mAb (200 μg/dose) was administered intraperitoneally 1 and 3 days after each poly-IC treatment. Various subsets of immune cells (CD8 T cells, CD4 T cells, or NK cells) were depleted using mAb 3 and 1 days before and 4 days after receiving the first poly-IC treatment. Nontreated mice were included as controls. As noted, some mice received poly-IC alone or anti–PD-L1 mAb alone. Points, mean for each group of mice; bars, SD. P values were compared with no treatment group and calculated using 2-way ANOVA tests ($^* P<0.05$, $^*^* P<0.001$, $^*^*^* P<0.00001$).

Statistical analyses

Statistical significance to assess the numbers of tumorspecific CD8 T cells (EliSpot) was determined by unpaired Student t tests. As required by our IACUC guidelines, the numbers of mice included in each treatment group were selected based on the expected outcomes and variability between mice in each group (observed in previous experiments), which were taken into account to assess statistical significance of the therapy. Tumor sizes between 2 populations throughout time were analyzed for significance using 2-way ANOVA. All analyses and graphics were done using GraphPad Prism 6.02 (GraphPad Software). All experiments were repeated at least 2 times with similar results.
The effectiveness of poly-IC/anti–PD-L1 mAb therapy was evaluated against 9-day established subcutaneous LLC-A9F1 tumors. Tumor growth was effectively controlled in mice receiving the combination of poly-IC/anti–PD-L1 mAb (Fig. 3A). Interestingly, administration of poly-IC alone resulted in an equally significant antitumor effect. However, anti–PD-L1 mAb alone had a substantially lower, but statistically significant antitumor effect. The use of a different TLR ligand (CpG, a TLR9 agonist) with anti–PD-L1 mAb did not increase the effectiveness of the therapy as compared with the use of anti–PD-L1 mAb alone. Administration of IL-2/anti–IL-2 mAb complexes (IL-2C/C2) has been shown to potentiate in vivo CD8 T-cell expansion (14, 15), and increase the antitumor effects of the T cells (10, 16). The addition of IL-2C/C2 did not further improve the effectiveness of the combination therapy. Most remarkably, the administration of poly-IC alone and the combination of poly-IC/anti–PD-L1 mAb (with or without IL-2C/C2) resulted in complete tumor eradications in 80% (4/5) of mice. To assess the generation of long-term systemic immunity, the mice that rejected their tumors in the 3 groups mentioned above were rechallenged on day 39 with the LLC-A9F1 tumor. One half of mice that received the combinatorial therapy plus IL-2C/C2 showed no significant response to the LLC-A9F1 tumor (Fig. 3D, right). A comparison of the levels of antigen-reactive CD8 T cells observed during tumor rejection (day 12, Fig. 3C) and after rejection (day 32, Fig. 3D) indicates that during the course of tumor rejection a marked reduction (approximately 10-fold) in tumor-reactive T cells occurred in this model system, which would explain the lack in the ability of some mice to resist a tumor rechallenge (Fig. 3B). These results also suggest that the administration of IL-2C/C2 before the injection of the combinatorial therapy may be detrimental for the acquisition of long-term immunity in this tumor model.

Next, the effectiveness of the poly-IC/anti–PD-L1 mAb combination therapy was examined in the MC38 colon cancer model. Here, tumor growth in mice that were treated with poly-IC/anti–PD-L1 mAb (with or without IL-2C/C2) was inhibited significantly as compared with the nontreated or poly-IC alone-treated mice (Fig. 4A). In this tumor model, 60% (3/5) of mice that received the poly-IC/anti–PD-L1 mAb treatment and 80% (4/5) of those receiving the combinatorial therapy plus IL-2C/C2 completely rejected their tumors. Mice that failed to reject their tumors had relatively small tumors (<100 mm²) at the end of the
Combinatorial Immunotherapy of Poly-IC and Anti-PD-L1 mAb

Figure 3. Therapeutic effects induced by the combinatorial therapy of poly-IC/anti–PD-L1 mAb against established lung carcinoma tumors. A, B6 mice (5 per group) were inoculated subcutaneously on day 0 with $5 \times 10^5$ LLC-A9F1 cells and injected intravenously on days 9, 14, and 19 with poly-IC or CpG at 50 μg/dose. Anti–PD-L1 mAb was administered intraperitoneally 1 and 3 days after each poly-IC or CpG administration at 200 μg/dose. IL-2C was administered intraperitoneally 2 and 1 days before the first poly-IC administration. Points, mean for each group of mice; bars, SD. P values were compared with no treatment group and calculated using 2-way ANOVA test (**, P < 0.01; ***, P < 0.001; ****, P < 0.0001). Mice with complete tumor rejections: poly-IC = 4/5; poly-IC/αPD-L1 = 4/5; Poly-IC/αPD-L1+IL-2C = 4/5. B, at the termination of the experiment presented in A, on day 39, mice (4 per group) that were originally treated with poly-IC alone or poly-IC/αPD-L1 mAb with or without IL-2C and had successfully rejected the tumors were rechallenged subcutaneously with $5 \times 10^5$ LLC-A9F1 cells on their flanks contralateral to the initial tumor challenge. Each line corresponds to the tumor size of each individual mouse. C, CD8 T cells were purified from pooled splenocytes of mice treated with the combination of poly-IC/anti–PD-L1 mAb on day 12, and tumor cell recognition was evaluated using an IFN-γ Elispot assay. Stimulator cells: LLC-A9F1 cells previously treated or not with IFN-γ (100 ng/mL, 24 hours) and EL4 cells. Photos in left panel represent examples of wells obtained using $1 \times 10^5$ CD8 T cells and $1 \times 10^5$ tumor cells per well. D, on day 32, IFN-γ Elispot assay using purified CD8 T cells from mice treated with the combination of poly-IC/anti–PD-L1 mAb with or without IL-2C were performed using EL4 cells and IFN-γ-treated (100 ng/mL, 24 or 48 hours) or nontreated LLC-A9F1 cells as stimulator cells. Results of C (right) and D represent the average number of spots from triplicate wells with SD (error bars) of the mean. P values of D were compared with CD8 alone and calculated using unpaired Student t test (*, P < 0.05; **, P < 0.01).

Experiment. However, in mice treated with poly-IC alone, although tumors significantly grew at a slow rate as compared with no treatment, only 1 of 5 mice rejected its tumor (generating large error bars in Fig. 4A). Notably, in this model none of survivor mice developed tumors after a tumor rechallenge on day 39, regardless of their initial treatments (data not shown).

To assess the antitumor CD8 T-cell responses, Elispot was performed using spleen CD8 T cells from survivor mice on day 55, after the rechallenge tumors were rejected. The CD8 T cells from the poly-IC/anti–PD-L1 mAb treated mice exhibited high responses against MC38 cells regardless of whether the tumor cells were treated or not with IFN-γ (Fig. 4B, middle). In contrast, the CD8 T-cell responses from the single mouse that was treated with poly-IC alone, that was able to reject the initial tumor and the tumor rechallenge, showed approximately 5-fold lower reactivity (Fig. 4B, left), as compared with mice that received the poly-IC/anti–PD-L1 mAb combinatorial therapy. Interestingly, the tumor recognition of CD8 T cells was somewhat decreased by the addition of IL-2C to the combinatorial therapy (Fig. 4B, right), suggesting that the use of IL-2C before administrating poly-IC and anti–PD-L1 mAb may be unfavorable for the generation
or persistence of the tumor-reactive CD8 T cells. It should be noted that in the colon cancer model, the CD8 T cells specifically recognized the MC38 cells because almost no reactivity was observed toward EL4 and LLC-A9F1 cells (Fig. 4B).

The antitumor effect of anti–PD-L1 mAb is most likely because of its blocking effect on the PD-1 inhibitory pathway. However, it is possible that the anti–PD-L1 mAb could have a direct cytolytic effect on the tumor cells, for example via ADCC. Because PD-1 blockade can also be achieved using antibodies specific for the PD-1 receptor (expressed on T cells), we compared the efficacy of anti–PD-1 and anti–PD-L1 mAbs in combination with poly-IC against established MC38 tumors. As shown in Fig. 4C, both anti–PD-1 and anti–PD-L1 mAbs were equally effective in controlling tumor growth when administered in combination with poly-IC.

**Figure 4.** Therapeutic effects induced by the combinatorial therapy of poly-IC/anti–PD-L1 mAb against established colon carcinoma tumors. A, B6 mice (5 per group) were inoculated subcutaneously on day 0 with $5 \times 10^5$ MC38 cells and injected intravenously on days 8, 13, and 18 with poly-IC at 50 μg/dose. Anti–PD-L1 mAb and IL-2 were administered as described in Fig. 3. Points, mean for each group of mice; bars, SD. P values were compared with no treatment group and calculated using 2-way ANOVA test ($^* P < 0.05$, $^{***} P < 0.0001$). Mice with complete tumor rejections: poly-IC = 1/5; poly-IC/αPD-L1 = 3/5; poly-IC/αPD-L1 + IL-2C = 4/5. B, CD8 T cells were purified from pooled splenocytes of each group on day 55 (after a successfully rejecting as tumor rechallenge), and tumor cell recognition was evaluated using IFN-γ Elispot assays. Stimulator cells were as follows: MC38 and LLC-A9F1 cells previously treated or not with IFN-γ (100 ng/mL, 24 or 48 hours) and EL4 cells. Results represent the average number of spots from triplicate wells with SD (error bars) of the mean. Photos represent examples of wells obtained using 1 × 10^5 CD8+ T cells per well. C, B6 mice (4–5 per group) were inoculated with MC38 cells and injected with poly-IC as described above in A. Anti–PD-L1 or PD-1 mAbs were administered intraperitoneally 1 and 3 days after each poly-IC administration at 200 μg/dose. Points, mean for each group of mice; bars, SD. Mice with complete tumor rejections: poly-IC/αPD-L1 = 3/4; poly-IC/αPD-1 = 4/5.

**Effector mechanism of poly-IC/anti–PD-L1 mAb therapy against lung and colon tumors**

To assess the contribution of various lymphocyte subsets in the rejection of LLC-A9F1 and MC38 tumors, the antitumor efficacy of the poly-IC/anti–PD-L1 mAb combinatorial therapy was evaluated in mice depleted of CD8 T cells, CD4 T cells, or NK cells. In both the LLC-A9F1 and MC38 tumor models, the therapeutic effects of the combination therapy disappeared when CD8 T cells were depleted (Fig. 5A and B). Conversely, the elimination of CD4 T cells and NK cells had no significant deleterious effect. It has been reported that CD8 T cells require help of CD4 T cells to become functional long-term memory cells (17, 18). Thus, the depletion of CD4 T cells may impair the acquisition of long-term immunity in mice treated with the combination of poly-IC/anti–PD-L1 mAb. To examine whether CD4 T-cell depletion during the treatment for the primary MC38
tumor challenge affected the generation of long-term immunity, the surviving mice from the experiment shown in Fig. 5B were rechallenged on day 39 with fresh MC38 cells. Notably, 80% (4/5) of the CD4 T-cell depleted mice and 100% (5/5) of nondepleted animals rejected the secondary tumor challenge (Fig. 5C), suggesting that long-term antitumor immunity in this tumor model could be generated in the absence of CD4 T cells. Nevertheless, when CD8 T cells were isolated from the spleens of the 2 groups of mice (CD4 depleted and untreated) that rejected the MC38 tumors, and tumor cell recognition was evaluated using IFN-γ EliSpot assays. Stimulator cells were as follows: MC38 cells previously treated or not with IFN-γ (100 ng/mL, 24 hours) and EL4 cells. Results represent the average number of spots from triplicate wells with SD (error bars) of the mean. Photos represent examples of wells obtained using 1 x 10^5 CD8 T cells and 1 x 10^5 tumor cells per well.

It is known that CD8 T cells can exert their antitumor function through the secretion of cytostatic lymphokines such as IFN-γ (19, 20). Furthermore, stimulation of TLR3 and RIG-I–like receptors by poly-IC induces the activation of antigen-presenting cells (APCs) and the generation of high amounts of type-I IFN (21, 22), which is implicated in the potentiation of CD8 T-cell responses (23, 24). Thus, the efficacy of poly-IC/anti–PD-L1 mAb therapy against LLC-A9F1 was evaluated in mice deficient for IFN-γ (IFN-γ−/−) or type-I IFN receptors (IFN-αβR−/−). Surprisingly, IFN-γ−/− mice treated with the combination of poly-IC/anti–PD-L1 mAb with IL-2C× completely rejected their tumors (Fig. 6A). On day 35, the IFN-γ−/− mice were rechallenged with live LLC-A9F1 cells and although the tumors started growing, they were all rejected (data not shown). These results indicate that IFN-γ is not required for tumor eradication and long-term protection in the LLC-A9F1 tumor model. A different outcome was observed in IFN-αβR−/− mice, where only 33% (2/6) animals treated with poly-IC/anti–PD-L1 mAb with IL-2C× rejected their tumors (Fig. 6B). These results indicate that type-I IFN plays an
important role in generating immunity necessary to achieve effective therapeutic responses against established LLC-A9F1 tumors.

Discussion

Numerous groups including ours are involved in developing T-cell epitope-based vaccination strategies for malignant diseases such as melanoma, cervical cancer, and breast carcinoma. These malignancies were selected because of the existence of defined TAAs that can be used to stimulate antigen-specific, tumor-reactive CD8 T-cell responses (8, 13, 25). However, for many other tumor types including lung and colon carcinomas, which are the leading worldwide causes of cancer death (26), few if any reliable TAAs for triggering tumor-specific CD8 T-cell responses have been identified, limiting the development of epitope-based vaccines. Thus, we explored an alternative and novel immunotherapeutic approach to induce and efficiently amplify tumor-reactive CD8 T cells without depending on the use of defined TAAs. To achieve this goal, we took advantage of a recent unexpected observation where immunization with a control-irrelevant peptide in combination with poly-IC and PD-1 blockade substantially decreased the rate of tumor growth in the B16 mouse melanoma model (11). We hypothesize that the CD8 T-cell responses that in many instances are naturally generated against TAAs throughout the course of the disease are in general ineffective and that the administration of poly-IC and anti–PD-L1 mAb somehow improves these responses, or alternatively generates new antitumor T-cell responses that result in therapeutic effectiveness. On one hand, poly-IC, a TLR3 and RIG-I–like receptor agonist is known to stimulate various immune cells including professional APCs such as dendritic cells, enhancing tumor antigen cross-presentation to CD8 T cells. One could easily envision that tumor-infiltrating dendritic cells that capture TAAs (either in the form of shed antigens or dead tumor cells) after exposure to poly-IC would become potent APCs capable of priming a new CD8 T-cell response (or alternatively of expanding and reactivating an existing suboptimal response) capable of delaying tumor growth and even in some instances eradicating disease. In the case of the LLC-A9F1 tumor, poly-IC by itself was effective in eliciting outstanding antitumor effects that were not further enhanced by PD-1 blockade (Fig. 3A). On the other hand, co-administration of poly-IC and anti–PD-L1 mAb was required to obtain similar remarkable antitumor effects in the B16 melanoma and MC38 colon carcinoma models (Figs. 1 and 4A). The additive effect of anti–PD-L1 mAb in the combination therapy could be because of various reasons depending on the specific tumor model and stage of disease. For example, it is possible that TAA-reactive CD8 T cells naturally generated before therapy express the inhibitory PD-1 receptor, which is a marker of exhausted T cells (27) and that PD-1 blockade during their interactions with dendritic cells (which express PD-L1 and PD-L2) rescues the T cells to expand and become more potent effector cells (28, 29). Because the tumor cells themselves...
express PD-L1 (30), which is enhanced by IFN-γ (Fig. 2), it is also likely that PD-1 blockade enhances the effector phase of the CD8 T-cell response increasing tumor killing and perhaps promoting T-cell survival and proliferation at the tumor site (31). Previous studies in mouse models of immunotherapy have shown remarkable therapeutic effects of PD-1 blockade (9–11, 32, 33). Poly-IC is well known to induce high levels of type-I IFN, which has been shown to induce the expression of PD-1 on T cells limiting their function (34, 35). In addition, poly-IC has been reported to stimulate the production of IFN-γ by NK cells (36), which will contribute to enhance the expression of PD-L1 on the tumor cells. Thus, it should be of no surprise that in most instances PD-1 blockade would synergize with the antitumor effects of poly-IC.

Our results indicate that the antitumor effects of the poly-IC/anti–PD-L1 mAb combination therapy were mediated principally by CD8 T cells and that CD4 T cells and NK cells played a minimal role, if any (Figs. 1 and 5A and B). In addition, the EliSpot assays clearly showed that tumor-reactive CD8 T cells were induced by this combinatorial therapy (Figs. 3C and 4B). Although in the LLC-A9F1 lung cancer model, the majority of the CD8 T cells induced by the combination therapy seemed to recognize a shared antigen present in another completely different tumor (EL4 thymoma; Fig. 3C), the CD8 T cells generated by the combination therapy in the MC38 colon carcinoma seemed to recognize antigen(s) not present in other tumor cells (EL4 and LLC-A9F1). At present, we do not know the nature of TAA recognized by the tumor-reactive CD8 T cells induced by poly-IC/anti–PD-L1 mAb therapy. In MC38 tumor model, we examined whether spleen CD8 T cells from mice treated with the combination therapy could recognize EL4 cells pulsed with the p15E04–611 (KSPWFTITL) peptide, an immunodominant H-2Kb restricted CD8 T-cell epitope derived from an endogenous murine leukemia virus expressed by numerous tumors including MC38 (37, 38). Nevertheless, in EliSpot assays EL4 cells pulsed with p15E04–611 were barely recognized by tumor-reactive CD8 T cells (comprising only approximately 3% of the response observed with MC38 tumor cells, data not shown). Future and complex studies will be required to identify the TAA recognized by the CD8 T cells.

The overall effectiveness of tumor immunotherapy will not only depend on achieving an initial antitumor response, hopefully capable of reducing tumor masses to an undetectable level, but one would also hope that the immune response would persist for long-time periods to prevent tumor recurrences and metastatic spread. Using tumor rechallenges in mice that rejected their initial tumors as a way to evaluate long-term immunity allowed us to evaluate the establishment of CD8 T-cell memory by the poly-IC/anti–PD-L1 mAb combination therapy. However, the 2 tumor models where complete rejections were achieved by this therapy gave somewhat divergent results. In the MC38 colon carcinoma model, all the mice that rejected their original tumors resisted the tumor rechallenges, indicating the establishment of effective CD8 memory T cells. However, in the LLC-A9F1 lung cancer model, only one half of the mice that rejected their primary tumors resisted a tumor rechallenge. However, 100% of IFN-γ−/− mice that rejected their LLC-A9F1 primary tumors were able to resist a subsequent tumor rechallenge (data not shown), indicating that IFN-γ decreases the generation of long-lived (memory) CD8 T cells as previously noted in a microbial infection model (39). Numerous additional factors could determine the generation of long-term CD8 T-cell memory in this mode of immunotherapy, such as the nature of the TAA recognized by the T cells and the immune suppressive effect of the tumor microenvironment that may facilitate the establishment of exhausted CD8 T cells incapable of reacting to a subsequent tumor encounter. It is well known that helper CD4 T cells play a role in the establishment of memory CD8 T cells (17, 18). Our results in the MC38 tumor model showed that the majority (80%) of the mice that were depleted of CD4 T cells while receiving poly-IC/anti–PD-L1 mAb therapy and rejected the original tumor were able to resist a tumor rechallenge (Fig. 5C). However, the level of CD8 T-cell responses was reduced by approximately 40% as compared with the nondepleted mice (Fig. 5D). However, in the LLC-A9F1 tumor model, removal of CD4 T cells reduced the level of protection against tumor rechallenge from 50% (Fig. 3B) to 0% (data not shown). Thus, our results suggest that indeed, CD4 T cells may play a role in promoting long-term survival of tumor reactive CD8 T cells, which in some instances such as with the LLC-A9F1 tumor, determines the ability to resist a tumor rechallenge. The mechanism(s) by which CD4 T cells may facilitate the generation of long-lived CD8 T cells could be numerous, including the production of IL-2 and enhancing the function of dendritic cells via CD40 ligand/CD40 interactions. Nevertheless, our results indicate that administration of IL-2 (as IL-2×) decreased the ability of mice to resist a tumor rechallenge (Fig. 3B) and decreased the levels of tumor-reactive CD8 T cells (Figs. 3D and 4B). It should be noted that the type of IL-2× we used has been reported to enhance proliferation and survival of memory CD8 T cells and NK cells but does not result in stimulation of CD4 T regulatory cells (14, 15). An important issue that remains to be determined is whether the poly-IC/anti–PD-L1 mAb therapy generates CD4 T cells reactive with TAA, which could be somehow involved in CD8 long-term immunity, or whether the role of the CD4 T cells in this process is independent of their antigen specificity.

IFN-γ has been considered to be a essential cytokine for the antitumor effects of CD8 T cells (19). Specifically, IFN-γ increases the expression of MIC-I molecules on tumor cells, which in many instances enhances their recognition by CD8 T cells. In addition, IFN-γ has direct antitumor activity, limiting cell proliferation (40, 41). In fact, as shown here, B16F10, LLC-A9F1, and MC38 cells treated with IFN-γ increased their levels of MIC-I molecules (Fig. 2) and significantly increased recognition by CD8 T cells from...
mice treated with the combinatorial therapy in the case of LLC-A9F1 (Fig. 3C). Although IFN-γ clearly has a positive antitumor effect, this cytokine can also exhibit immunosuppressive activities (20). Specifically, many tumors including the ones used in this study when exposed to IFN-γ increase their expression of PD-L1 (Fig. 2), which inhibits the function of T cells (42). Furthermore, although IFN-γ increases MHC-I expression, in some instances it may decrease CD8 T-cell recognition by either, decreasing the generation of some peptide epitopes, through the induction of immunoproteasomes (43), or through the production of excessive noncognate peptide/MHC-I complexes that limit antigen-specific T-cell recognition (9). In addition, IFN-γ may exhibit direct inhibitory/toxic effects on T cells, limiting clonal expansion (39). Irrespective of all these issues, our results with LLC-A9F1 indicate that IFN-γ did not play an essential role in limiting the tumor growth produced by the poly-IC/anti–PD-L1 mAb combination therapy (Fig. 6A). In fact, whereas 80% of wild-type B6 mice receiving this therapy rejected their tumors (Fig. 3A), 100% of the IFN-γ−/− mice eliminated the tumors (Fig. 6A). Similar to the findings presented here, we have recently described that peptide vaccination with poly-IC (with and without anti-CD40 mAb) generated remarkable antitumor effects in IFN-γ−/− mice against B16 melanoma and a human papilloma virus mouse tumor model (8, 9, 25). In these instances, the antitumor effect of the CD8 T cells was mediated by perforin-mediated cytolyis, but not IFN-γ.

Poly-IC is recognized by TLR3 and cytoplasmic RIG-I-like receptors, such as the MDA5, resulting in the activation of APCs and the generation of high levels of type-I IFN as well as other proinflammatory cytokines such as TNF-α, IL-6, and IL-12 (21, 22). Because type-I IFN has important roles for activating and expanding CD8 T cells (23, 24), we predicted that the poly-IC/anti–PD-L1 mAb therapy would be ineffective in IFN-αR−/− mice. Indeed, the antitumor effects of the poly-IC/anti–PD-L1 mAb combination therapy in the LLC-A9F1 model were reduced in IFN-αR−/− mice (Fig. 6B) as compared with the wild-type B6 mice (Fig. 3A). Nevertheless, this therapy still elicited significant antitumor effects in 4 of 6 mice, where 2 animals rejected their tumors and 2 had a substantial decrease in tumor growth rate as compared with the untreated controls. These results suggest that type-I IFN signals are important but not absolutely required for inducing the antitumor effects. The antitumor effects of the combinatorial therapy in the absence of type-I IFN signals may be because of the participation of other T-cell stimulatory cytokines such as IL-12 generated by poly-IC–stimulated APCs or could be the result of type-I IFN signals directly on the tumor cells.

Finally, it should be mentioned that the combinatorial poly-IC/anti–PD-L1 mAb cancer immunotherapy described here could be expediently taken into the clinic. Currently, there is a formulation of poly-IC being developed as a therapeutic. Hiltonol that was used in this study is a high molecular weight poly-IC formulation stabilized with poly-lysine and carboxymethylcellulose (poly-ICLC) that has already used in humans as a monotherapy or as an immune adjuvant for cancer vaccines (44–49). Furthermore, several humanized mAbs for the purpose of implementing PD-1 blockade (anti–PD-L1 or anti–PD-1) are being developed and are currently undergoing clinical testing (50, 51). Our results in the MC38 tumor model suggest that effective antitumor effects using immunotherapy with poly-IC and PD-1 blockade can be achieved with either mAb specific for the PD-1 receptor or its ligand PD-L1 (Fig. 4C). Because both poly-IC and various Abs to induce PD-1 blockade are being developed for clinical use, we believe that our preclinical studies could readily be translated into the treatment for patients with cancer, especially in those instances where no reliable TAA have been identified.

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

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Conception and design: T. Nagato, Y.-R. Lee, Y. Harabuchi, E. Celis
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Nagato, Y.-R. Lee
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Nagato, Y.-R. Lee, Y. Harabuchi, E. Celis
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Combinatorial Immunotherapy of Polyinosinic–Polycytidylic Acid and Blockade of Programmed Death-Ligand 1 Induce Effective CD8 T-cell Responses against Established Tumors

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