Combinatorial Immunotherapy of Polyinosinic-Polycytidylic Acid and Blockade of Programmed Death-1 Induces Effective CD8 T Cell Responses against Established Tumors

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Running title: Combinatorial Immunotherapy of Poly-IC and Anti-PD-L1 mAb

Keywords: B16 melanoma, lung cancer, colon cancer, immunotherapy, poly-IC, PD-1 blockade

Conflict of Interest: The authors declare no conflict of interest.
Translational Relevance

For many malignant diseases, few if any reliable tumor-associated antigens (TAAs) capable of eliciting effective anti-tumor 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 poly-IC and anti-PD-L1 antibodies that results in dramatic anti-tumor effects in several cancer mouse models, which were mediated by CD8 T cells. Because both poly-IC and anti-PD-L1 mAb are available for clinical use, we believe that our preclinical studies can readily translate into the treatment for cancer patients, especially in those instances where reliable TAAs have not been identified.
Abstract

Purpose: Epitope-based cancer vaccines capable of inducing CD8 T cell responses to tumor-associated antigens (TAAs) 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 anti-programmed 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 immunological 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 interferon, whereas interferon-γ did not appear 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 cancer patients, especially those instances where no reliable TAAs are available as a therapeutic vaccine.
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 (mAbs) that enhance ongoing anti-tumor 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 (TAAs) 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 anti-tumor T cell responses have been identified. In view of this, alternative approaches to generate anti-tumor 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 anti-programmed 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 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 anti-tumor 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). Interferon-gamma (IFNγ)-deficient (IFNγ−/−) mice (B6 background) were from Jackson Laboratories (Bar Harbor, ME). IFNαβ receptor-deficient (IFNαβR−/−) 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 animal care and use committee (IACUC) 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 (Manassas, VA). 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-ICLC/Hiltonol™, Oncovir, Inc., Washington, DC) or purchased from InvivoGen (Poly-IC HMW; San Diego, CA).

CpG-1826 was prepared by the Mayo Clinic Molecular Core Facility (Rochester, MN).

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 (West Lebanon, NH). Anti-mouse programmed death-1 (PD-1; RMP1-14) mAb was provided by Hideo Yagita (Juntendo University School of Medicine, Tokyo, Japan). Recombinant mouse IL-2 and IFNγ were from PeproTech (Rocky Hill, NJ). Fluorescence labeled Abs were from eBioscience (San Diego, CA) and BD Biosciences (San Jose, CA).

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 h with 100 ng/mL IFNγ was evaluated by flow cytometry using FITC-conjugated anti-H-2Db, PE-conjugated anti-H-2Kb,
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 (Ashland, OR).

**Therapeutic protocols and evaluation of anti-tumor effects**

Mice were injected s.c. with $5 \times 10^5$ LLC-A9F1, $5 \times 10^5$ MC38, or $4 \times 10^5$ 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 i.v. 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 i.p. 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-2Cx) were prepared by incubating 2 μg recombinant mouse IL-2 with 10 μg anti-mouse IL-2 mAb per dose for 18 h at 4°C. IL-2Cx were administered i.p. on 2 and 1 days before the first poly-IC administration. Survivor mice were rechallenged s.c. with the same number of tumor cells (in their opposite flanks).
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 2-3 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 IFNγ EliSpot assays were performed as described (13), using purified spleen CD8 T cells (Miltenyi Biotec; Auburn, CA). CD8 T cells were incubated at 1 × 10⁵ together with 1 × 10⁵ stimulator cells (EL4, LLC-A9F1, and MC38 cells pretreated or not for 24 or 48 h with 100 ng/mL IFNγ). Cultures were incubated at 37°C for 20 h and spots (IFNγ producing cells) were developed as described by the EliSpot kit manufacturer (Mabtech, Inc., Mariemont,
Spot counting was done with an AID EliSpot Reader System (Autoimmun Diagnostika GmbH, Strassberg, Germany).

Statistical analyses

Statistical significance to assess the numbers of tumor-specific CD8 T cells (EliSpot) was determined by unpaired Student’s 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 analysis of variance (ANOVA). All analyses and graphics were done using GraphPad Prism 6.02 (GraphPad Software, San Diego, CA). All experiments were repeated at least twice with similar results.

Results
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 anti-tumor effect in a control group of mice that received an irrelevant peptide vaccine combined with poly-IC (TLR3 ligand and 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 immunological mechanisms involved of the combined administration of poly-IC and anti-PD-L1 mAb in the B16 mouse melanoma model. Mice were inoculated s.c. 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 to 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. On the other hand, depletion of CD8 T cells abrogated the anti-tumor 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**

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 Lewis lung carcinoma (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 and PD-L1 molecules, we evaluated the expression of MHC-I, MHC-II, and PD-L1 on LLC, clone A9F1 (LLC-A9F1) and MC38 cells that were pretreated or not with IFNγ. B16F10 melanoma cells were also included in these evaluations. Both LLC-A9F1 and MC38 expressed high levels of MHC-I (H-2D^b and H-2K^b), which were somewhat increased by IFNγ treatment (Fig. 2). The B16F10 cells expressed low levels of MHC-I, but these were dramatically increased by IFNγ. All 3 tumors did not express MHC-II and IFNγ treatment was able to upregulate its expression only on B16F10. Expression of PD-L1
was found expressed on all 3 tumors and treatment with IFNγ enhanced its expression by ~10-fold.

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 anti-tumor effect. On the other hand, anti-PD-L1 mAb alone had a substantially lower, but statistically significant anti-tumor 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 to the use of anti-PD-L1 mAb alone. Administration of IL-2/anti-IL-2 mAb complexes (IL-2Cx) has been shown to potentiate in vivo CD8 T cell expansion (14, 15), and increase the anti-tumor effects of the T cells (10, 16). The addition of IL-2Cx 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-2Cx) resulted in complete tumor eradication 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 combination of poly-IC/anti-PD-L1 mAb or poly-IC alone were able to reject the second tumor challenge (Fig. 3B). Surprisingly, in the case of mice treated with the combinatorial therapy plus IL-2Cx, no rejections were observed after tumor rechallenge.

To assess whether tumor-reactive CD8 T cells were induced in this tumor model, CD8 T cells were isolated from spleens of LLC-A9F1-bearing mice that were treated with poly-IC/anti-PD-L1 mAb on day 12, when the average tumor size had decreased by ~50%. The CD8 T cells were effective in recognizing LLC-A9F1 cells and this recognition was increased by IFNγ pretreatment of the tumor cells (Fig. 3C).

Interestingly, while the CD8 T cells did not produce IFNγ spots when cultured alone, they did recognize an irrelevant tumor (EL4 thymoma). These results suggest that the CD8 T cells induced by this therapy in this tumor model may recognize shared antigens expressed by LLC-A9F1 and EL4 tumor cells. The presence of anti-tumor CD8 T cells was also evaluated in spleens of mice treated with poly-IC/anti-PD-L1 mAb with or without IL-2Cx on day 32, after their tumors had been completely rejected. CD8 T cells
from mice treated with poly-IC/anti-PD-L1 mAb significantly recognized the LLC-A9F1 cells and the response was increased by IFNγ pretreatment of the tumor cells (Fig. 3D, left). However, CD8 T cells from the mice that received the therapy plus IL-2Cx did not show a 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 (~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-2Cx 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-2Cx) was inhibited significantly as compared with the non-treated 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-2Cx completely rejected their tumors. Mice that failed to reject their tumors had relatively small tumors (<100 mm²) at the end of the experiment. On the other hand, in mice treated with poly-IC alone, although tumors significantly grew at a slow rate as compared with no treatment, only 1/5 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 anti-tumor 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 IFNγ (Fig. 4B, middle panel). 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 panel), as compared 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-2Cx to the combinatorial therapy (Fig. 4B, right panel), suggesting that the use of IL-2Cx before administarting 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 since almost no reactivity was observed towards EL4 and LLC-A9F1 cells (Fig. 4B).

The anti-tumor effect of anti-PD-L1 mAb is most likely due to 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. Since 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.

**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 anti-tumor 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-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 non-depleted animals rejected the secondary tumor challenge (Fig. 5C), suggesting that long-term anti-tumor 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 rechallenges and were analyzed for their ability to react with tumor cells, it was evident that the responses of CD8 T cells from the CD4-depleted mice were ~40% lower as compared to the responses of the untreated mice (Fig. 5D).

It is known that CD8 T cells can exert their anti-tumor function through the secretion of cytostatic lymphokines such as IFN\(\gamma\) (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\(\gamma\) (IFN\(\gamma^{-/-}\)) or type-I IFN receptors (IFN\(\alpha\beta\)R\(^{-/-}\)). Surprisingly, IFN\(\gamma^{-/-}\) mice treated with the combination of poly-IC/anti-PD-L1 mAb with IL-2Cx completely rejected their tumors (Fig. 6A). On day 35, the IFN\(\gamma^{-/-}\) 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\(\gamma\) is not required for tumor eradication and
long-term protection in the LLC-A9F1 tumor model. A different outcome was observed in IFNαβR−/− mice, where only 33% (2/6) animals treated with poly-IC/anti-PD-L1 mAb with IL-2Cx 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 anti-tumor 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 DCs, enhancing tumor antigen cross-presentation to CD8 T cells. One could easily envision that tumor-infiltrating DCs 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 anti-tumor 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 anti-tumor 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 due to 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 DCs (which express PD-L1 and PD-L2) rescues the T cells to expand and become more potent effector cells (28, 29). Since the tumor cells themselves express PD-L1 (30), which is enhanced by IFNγ (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 IFNγ 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 anti-tumor effects of poly-IC.

Our results indicate that the anti-tumor 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-B). In addition, the EliSpot assays clearly showed that tumor-reactive CD8 T cells were induced by this combinatorial therapy (Figs. 3C and 4B). While 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 appeared to recognize antigen(s) not present in other tumor cells (EL4 and LLC-A9F1). At present, we do not know the nature of TAAs 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 p15E_{604-611} (KSPWFTTL) peptide, an immunodominant H-2K^{b} 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 p15E_{604-611} were barely recognized by tumor-reactive CD8 T cells (comprising only ~3% of the response observed with MC38 tumor cells, data not shown). Future and complex studies will be required to identify the TAAs recognized by the CD8 T cells.

The overall effectiveness of tumor immunotherapy will not only depend in achieving an initial anti-tumor 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. On the other hand, 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 IFNγ−/− mice that rejected their LLC-A9F1 primary tumors were able to resist a subsequent tumor rechallenge (data not shown), indicating that IFNγ 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 TAAs 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 ~40% as compared to the non-depleted mice (Fig.
5D). On the other hand, 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
DCs via CD40 ligand/CD40 interactions. Nevertheless, our results indicate that
administration of IL-2 (as IL-2Cx) 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-2Cx 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 TAAAs, 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.

IFNγ has been considered to be a essential cytokine for the anti-tumor effects of CD8 T cells (19). Specifically, IFNγ increases the expression of MHC-I molecules on tumor cells, which in many instances enhances their recognition by CD8 T cells. In addition, IFNγ has direct anti-tumor activity, limiting cell proliferation (40, 41). In fact, as shown here, B16F10, LLC-A9F1, and MC38 cells treated with IFNγ increased their levels of MHC-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). While IFNγ clearly has a positive anti-tumor effect, this cytokine can also exhibit immunosuppressive activities (20). Specifically, many tumors including the ones used in the present study when exposed to IFNγ increase their expression of PD-L1 (Fig. 2), which inhibits the function of T cells (42). Furthermore, although IFNγ 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 non-cognate peptide/MHC-I complexes that limit antigen-specific T cell recognition (9). In addition,
IFNγ 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 IFNγ 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 (WT) B6 mice receiving this therapy rejected their tumors (Fig. 3A), 100% of the IFNγ−/− 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 anti-tumor effects in IFNγ−/− mice against B16 melanoma and a human papilloma virus mouse tumor model (8, 9, 25). In these instances, the anti-tumor effect of the CD8 T cells was mediated by perforin-mediated cytolysis, but not IFNγ.

Poly-IC is recognized by TLR3 and cytoplasmic RIG-I-like receptors, such as the melanoma differentiation-associated protein 5 (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 TNFα, IL-6, and IL-12 (21, 22). Since 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 IFNαβR−/− mice. Indeed, the anti-tumor effects of the poly-IC/anti-PD-L1 mAb combination therapy in the LLC-A9F1 model were reduced in IFNαβR−/− mice (Fig. 6B) as compared to the WT B6 mice (Fig. 3A). Nevertheless, this therapy still elicited significant anti-tumor effects in 4 of 6 mice, where 2 animals rejected their tumors and 2 had a substantial decrease in tumor growth rate as compared to the untreated controls. These results suggest that type-I IFN signals are important but not absolutely required for inducing the anti-tumor effects. The anti-tumor effects of the combinatorial therapy in the absence of type-I IFN signals may be due to 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™, which was used in the present 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 anti-tumor 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). Since 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 cancer patients, especially in those instances where no reliable TAAs have been identified.

**Disclosure of Potential Conflicts of Interest**

The authors do not have any potential conflict of interest.

**Authors’ Contributions**

**Conception and design:** E. Celis, Y. Harabuchi
Development of methodology: E. Celis, T. Nagato, Y. Harabuchi, YR. Lee

Acquisition of data (performed experiments): T. Nagato, YR. Lee

Data analysis and statistical tests: T. Nagato, Y. Harabuchi

Manuscript preparation: E. Celis, T. Nagato

Acknowledgements

We thank Dr. Andres Salazar (Oncovir, Inc.) for kindly providing Poly-ICLC (Hiltonol™).

Grant Support

This work was supported by NIH grants R01CA136828 and R01CA157303.
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**Figure legends**

**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 s.c. on day 0 with $4 \times 10^5$ B16F10 cells and later treated on days 7, 12, and 17 with poly-IC at 50 $\mu$g/dose given i.v. Anti-PD-L1 mAb (200 $\mu$g/dose) was administered i.p. one 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. Non-treated 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*<0.0001).

**Figure 2.** Expression levels of MHC-I (H-2Db and H-2Kb), MHC-II, and PD-L1 on LLC-A9F1, MC38, and B16F10 cells. LLC-A9F1, MC38, and B16F10 cells were incubated or not with 100 ng/mL of IFN$\gamma$ for 24, 40, or 48 h, and stained with specific
Abs as indicated, followed by flow cytometric analysis.

**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 s.c. on day 0 with $5 \times 10^5$ LLC-A9F1 cells and injected i.v. on days 9, 14, and 19 with poly-IC or CpG at 50 $\mu$g/dose. Anti-PD-L1 mAb was administered i.p. 1 and 3 days after each poly-IC or CpG administration at 200 $\mu$g/dose. IL-2Cx was administered i.p. 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-2Cx = 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/anti-PD-L1 mAb with or without IL-2Cx and had successfully rejected the tumors were rechallenged s.c. 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 IFNγ EliSpot assay. Stimulator cells: LLC-A9F1 cells previously treated or not with IFNγ (100 ng/mL, 24 h) and EL4 cells. Photos in left panel represent examples of wells obtained using 1 × 10⁵ CD8 T cells and 1 × 10⁵ tumor cells per well.

D, on day 32, IFNγ EliSpot assay using purified CD8 T cells from mice treated with the combination of poly-IC/anti-PD-L1 mAb with or without IL-2Cx were performed using EL4 cells and IFNγ-treated (100 ng/mL, 24 or 48 h) or non-treated LLC-A9F1 cells as stimulator cells. Results of C (right panel) 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’s t test (*P<0.05, **P<0.01).

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 s.c. on day 0 with 5 × 10⁵ MC38 cells and injected i.v. on days 8, 13, and 18 with poly-IC at 50 μg/dose. Anti-PD-L1 mAb and IL-2Cx were
administered as described in Fig. 3 legend. 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-2Cx = 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 IFNγ EliSpot assays. Stimulator cells were as follows: MC38 and LLC-A9F1 cells previously treated or not with IFNγ (100 ng/mL, 24 or 48 h) 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 and 1 × 10^5 tumor 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 i.p. 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.
**Figure 5.** Role of lymphocyte subsets in the anti-tumor effects of poly-IC/anti-PD-L1 mAb therapy. A and B, B6 mice (5 per group) were inoculated s.c. on day 0 with \(5 \times 10^5\) LLC-A9F1 cells (A) or MC38 cells (B). Poly-IC and anti-PD-L1 mAb were administered as described in Fig. 3 (for LLC-A9F1) and 4 (for MC38). Various subsets of immune cells (CD8 T cells, CD4 T cells, or NK cells) were depleted using mAb as described in Fig. 1. Points, mean for each group of mice; bars, SD. C, at the termination of the experiment presented in B, on day 39, mice (5 per group) that had successfully rejected the tumors were rechallenged s.c. with \(5 \times 10^5\) MC38 cells on their flanks contralateral to the initial challenge. Points, mean for each group of mice; bars, SD. Right portion of C, tumor growth curves are shown for individual mice from the CD4-depleted group. D, at the termination of the experiment presented in C, on day 17 after tumor rechallenge (day 56 after initial tumor challenge), CD8 T cells were purified from pooled splenocytes of mice that completely rejected their rechallenged tumors, and tumor cell recognition was evaluated using IFN\(\gamma\) EliSpot assays. Stimulator cells were as follows: MC38 cells previously treated or not with IFN\(\gamma\) (100 ng/mL, 24 h) and EL4 cells. Results represent the average number of spots from triplicate wells with SD.
bars) of the mean. Photos represent examples of wells obtained using $1 \times 10^5$ CD8 T cells and $1 \times 10^5$ tumor cells per well.

**Figure 6.** Role of IFNγ and type-I IFN in the therapeutic anti-tumor effects of poly-IC/anti-PD-L1 mAb. IFN$\gamma^{/-}$ mice (A) and IFN$\alpha\beta R^{/-}$ mice (B) were inoculated s.c. on day 0 with $5 \times 10^5$ LLC-A9F1 cells and treated with the combination of poly-IC/anti-PD-L1 mAb with IL-2Cx in the same manner as described in Fig. 3. Non-treated mice were included as controls. Tumor sizes were determined in individual mice by measuring 2 opposing diameters and are presented as tumor areas in square millimeters. For A, *points*, mean for each group of mice; *bars*, SD. For B, *lines*, tumor size in area of each individual mouse.
Figure 2

**H-2D<sup>b</sup>**

- **LLC-A9F1**
- **MC38**
- **B16F10**

**H-2K<sup>b</sup>**

**MHC class II**

**PD-L1**

- Isotype
- No IFN<sub>γ</sub>
- IFN<sub>γ</sub> 24h
- IFN<sub>γ</sub> 48h
- IFN<sub>γ</sub> 40h
Figure 3
Figure 4

A

- No treatment
- Poly-IC
- Poly-IC/αPD-L1
- Poly-IC/αPD-L1+IL-2Cx

Tumor Size (mm²)

Days Post Tumor Injection

B

Poly-IC

CD8 alone
MC38
MC38 + IFNγ 24 h
MC38 + IFNγ 48 h
LLC-A9F1
LLC-A9F1 + IFNγ 48 h
EL4

Poly-IC/αPD-L1

Poly-IC/αPD-L1 + IL-2Cx

C

- No treatment
- Poly-IC/αPD-L1
- Poly-IC/αPD-1

Tumor Size (mm²)

Days Post Tumor Injection

IFNγ Spots / 1 x 10⁶ CD8+ T cells

Days Post Tumor Injection

Author Manuscript Published OnlineFirst on January 3, 2014; DOI: 10.1158/1078-0432.CCR-13-2781

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Figure 5

A) Tumor Size (mm²) vs. Days Post Tumor Injection for different treatments:
- Poly-IC/αPD-L1
- αCD8+Poly-IC/αPD-L1
- αCD4+Poly-IC/αPD-L1
- αNK1.1+Poly-IC/αPD-L1

B) Tumor Size (mm²) vs. Days Post Tumor Injection for different treatments:
- No treatment
- Poly-IC/αPD-L1
- αCD8+Poly-IC/αPD-L1
- αCD4+Poly-IC/αPD-L1

C) Tumor Size (mm²) vs. Days Post Tumor Rechallenge for different treatments:
- Poly-IC/αPD-L1
- αCD4+Poly-IC/αPD-L1

D) IFNγ Spots / 1x10⁵ CD8⁺ T cells for different conditions:
- CD8 alone
- MC38
- MC38 + IFNγ 24h
- EL4

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Figure 6

A

![Graph showing tumor size over days post tumor injection for different treatments: No treatment and Poly-IC/α PD-L1+IL-2Cx.]

B

![Graphs comparing tumor size over days post tumor injection for No treatment and Poly-IC/α PD-L1+IL-2Cx treatments.]

Combinatorial Immunotherapy of Polyinosinic-Polyctidylic Acid and Blockade of Programmed Death-1 Induces Effective CD8 T Cell Responses against Established Tumors

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Clin Cancer Res  Published OnlineFirst January 3, 2014.

Updated version

Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-13-2781

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