Response to Programmed Cell Death-1 Blockade in a Murine Melanoma Syngeneic Model Requires Costimulation, CD4, and CD8 T Cells

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Abstract

The programmed cell death protein 1 (PD-1) limits effector T-cell functions in peripheral tissues, and its inhibition leads to clinical benefit in different cancers. To better understand how PD-1 blockade therapy modulates the tumor–host interactions, we evaluated three syngeneic murine tumor models, the BRAFV600E–driven YUMM1.1 and YUMM2.1 melanomas, and the carcinogen-induced murine colon adenocarcinoma MC38. The YUMM cell lines were established from mice with melanocyte-specific BRAFV600E mutation and PTEN loss (BRAFV600E/PTEN−/−). Anti–PD-1 or anti–PD-L1 therapy engendered strong antitumor activity against MC38 and YUMM2.1, but not YUMM1.1. PD-L1 expression did not differ between the three models at baseline or upon interferon stimulation. Whereas mutational load was high in MC38, it was lower in both YUMM models. In YUMM2.1, the antitumor activity of PD-1 blockade had a critical requirement for both CD4 and CD8 T cells, as well as CD28 and CD80/86 costimulation, with an increase in CD11c+ CD11b+ MHC-IIhigh dendritic cells and tumor-associated macrophages in the tumors after PD-1 blockade. Compared with YUMM1.1, YUMM2.1 exhibited a more inflammatory profile by RNA sequencing analysis, with an increase in expression of chemokine-trafficking genes that are related to immune cell recruitment and T-cell priming. In conclusion, response to PD-1 blockade therapy in tumor models requires CD4 and CD8 T cells and costimulation that is mediated by dendritic cells and macrophages. Cancer Immunol Res; 4(10); 845–57. ©2016 AACR.

Introduction

The development of inhibitors of the programmed cell death protein 1 (PD-1) or its ligand (PD-L1) represents a paradigm shift in the treatment of advanced cancers, with significant clinical benefits demonstrated in patients with several different histologies (1–4). Tumor responses are associated with a higher number of pretreatment PD-L1–expressing tumor and myeloid cells (5, 6), a high mutational load leading to increase in antigen-specific T-cell recognition (7, 8), the ability of PD-1/PD-L1 blockade to increase antigen presentation (9, 10) and modulate the tumor microenvironment (10, 11), and pre-existing CD8 T-cell infiltration (5, 12). A higher tumor mutational load induced by carcinogens such as ultraviolet light for melanoma (13) or cigarette smoking for lung carcinomas (14) would allow T cells to better differentiate between cancer and normal cells, thereby leading to immune recognition that could be unleashed by PD-1 blockade therapy.

Despite these advances, a better understanding is needed of the tumor–host interactions and how anti–PD-1 agents modulate cellular and molecular characteristics of each individual microenvironment. It is widely accepted that PD-1 blockade agents regulate T-cell activity in peripheral tissues in the context of infection or in tumors where PD-1/L1 checkpoint is the dominant inhibitory pathway. However, anti–PD-1 interacts earlier with T cells positively regulated by B7-CD28 costimulation (15), and this interaction is less well characterized (16–18).

In this study, we analyzed different tumor–host characteristics that might influence the effects of PD-1 blockade in murine models with a fully functional immune system. We conclude that T-cell priming and costimulation are required for anti–PD-1 therapy response to be effective in the melanoma tumor models in vivo.

Materials and Methods

Mice, cell lines, and reagents

C57Bl/6 mice, B6.Cg-BrafL1MmcmPtentm1HwuTg(Tyr-cre/ERT2)13Bos/BosJ, B6.129S2-Cd28tm1Mak/J, and B6.129S4-Cd8βtm1Flv/J were purchased from The Jackson Laboratory (Bar Harbor, ME). C57Bl/6 mice, B6.Cg-BrafL1MmcmPtentm1HwuTg(Tyr-cre/ERT2)13Bos/BosJ, B6.129S2-Cd28tm1Mak/J, and B6.129S4-Cd8βtm1Flv/J were purchased from The Jackson Laboratory (Bar Harbor, ME).
Cd80tm1Shr Cd86tm2Shr/J mice (Jackson Laboratories) were bred and kept under defined-flora pathogen-free conditions at the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved animal facility of the Division of Experimental Radiation Oncology, UCLA, and used under the UCLA Animal Research Committee protocol #2004-159-23. Cell lines were cultured in DMEM media (Invitrogen) supplemented with 10% FBS (Omega Scientific) and 2 nmol/L l-glutamine (Invitrogen). YUMM1.1 and YUMM1.7 cell lines were obtained from induced tumors in conditional mouse models of melanoma based on melanocyte-specific BRAFV600E-mutating activation and PTEN loss (BRAFV600E/PTEN-/-). YUMM2.1 was obtained from BRAFV600E/PTEN-/- mice crossed with mice bearing a Cmb11 allele (19), which targets exon 3, resulting in removal of the G53Kb kinase sites in β-catenin that are needed for ubiquitin-mediated destruction. However, analysis of the YUMM2.1 cell line showed that it had not recombined the β-catenin site (see below). YUMM cell lines were tested and authenticated by PCR and exome sequencing. Recombinant murine interferon gamma (IFNγ) was obtained from Peprotech. Tumors were followed by caliper measurement three times per week, and tumor volume was calculated using the formula: tumor volume = ((width)^2 x length)/2. Mean and SD of the tumor volumes per group were calculated.

Antitumor studies in mouse models

To establish subcutaneous (s.c.) tumors, 3 × 10^3 MC38, 1 × 10^6 YUMM2.1, or 1 × 10^6 YUMM1.1 cells per mouse were injected into the flanks of C57BL/6 mice. When tumor diameter reached 4 to 5 mm, four doses of 300 μg of anti–PD-1 (Cat. No. BE0146, clone RMP1-14), anti–PD-L1 (Cat. No. BE0101, clone 10F.9G2), or isotype control antibody (Cat. No. BE0090, clone LIT-2), all from BioXCell, were injected intraperitoneally (i.p.) every 3 days. For T-cell subset depletion studies, 250 μg of anti-CD8 (Cat. No. BE0117, clone YTS 169.4), 250 μg of anti-CD4 (Cat. No. BE0003-2, clone OX-4), both from BioXCell, or the combination were administered every 2 days starting the day before anti–PD-1 was initiated and through the duration of the experiment. For CD103 depletion, 200 μg of CD103 (Cat. No. BE0026, clone M290) from BioXCell was administered starting the day before anti–PD-1 treatment was initiated and administered i.p. every 2 days until the end of the experiment.

Whole-exome sequencing: Mutation calling and copy-number analysis

Sequencing of the MC38, YUMM2.1, and YUMM1.1 tumors and spleens were harvested from mice at predesigned time points. Tumors were digested with collagenase D (Roche) and stained with antibodies to CD3 BV605, Ly6C FITC, PD-L1/CD274 PE, CD8α BV421, CD45RA/B220, CD11b BV85, CD11c PE-Cy7, CD103 PerCP Cyanine 5.5, MHC Class II (I-A/I-E) FITC (Biolegend), Ly6G (Gr-1) PerCP Cyanine 5.5, F4/80 Pacific blue/eFluor450, CD25 APC, CD4 FITC (eBioscience). Intracellular staining of Foxp3 PE (eBioscience) was done according to the manufacturer's recommendations. Cells were analyzed with an LSR-II or FACScalibur flow cytometer (BD Biosciences), followed by Flow-Jo software (Tree-Star) analysis (28).

Western blotting and immunofluorescence staining

Western blotting was performed using standard methods on lysates from cultured murine melanoma cell lines using primary antibodies to β-catenin, GAPDH and histone H3, and secondary anti-rabbit IgG horseradish peroxidase–linked antibody, all from Cell Signaling Technology, and Pdcd1-l1 (H-130) and gr100 (H-300) from Santa Cruz Biotechnology. Nuclear and cytoplasmic extraction reagents were obtained from Thermo Scientific. Proteins were visualized using ImageQuant 4000 scanner. Immunofluorescence staining was performed on tumor sections of frozen sections using Anti-FITC (BD Biosciences) and anti-rabbit IgG (H+L) antibody from Jackson ImmunoResearch Laboratories (ref. 29).
Topflash analysis

Topflash vectors were obtained from Addgene (M51 Super 8x TOPFlash/TOPFlash mutant, Cat. No. 12457; M50 Super 8x TOPFlash, Cat. No. 12456). YUMM1.7 and YUMM2.1 cells (±10 μmol/L tamoxifen) were plated to achieve 70% confluency in 6-well plates. Cells were cotransfected with pTK-RLuc (green Renilla luciferase) along with either Topflash or Fopflash vectors. After 48 hours, cells were harvested and luciferase activity was measured using Dual-Luciferase Reporter Assay System (Cat. No. E1910) from Promega, where firefly luciferase signal was normalized to its corresponding Renilla luciferase signal. Topflash/Fopflash signal was determined from each treatment and graphed using Graphpad/Prism.

β-Catenin downregulation

β-catenin shRNA lentiviral vector (Cat. No. 29210-V) and the negative control shRNA lentiviral vector (Cat. No. 108080) were purchased from Santa Cruz Biotechnology. YUMM2.1 and YUMM1.1 cells were transduced at a multiplicity of infection of 1 to 10 in media containing 5 μg/mL polybrene and then selected in complete DMEM with 2.5 μg/mL of puromycin for 3 weeks.

Statistical analysis

Data were analyzed with GraphPad Prism (version 5) software (GraphPad Software). Descriptive statistics such as number of observations, mean values, and SD were reported and presented graphically for quantitative measurements. Normality assumption was checked for outcomes before statistical testing. For measurements such as tumor volume or percentage of tumor-infiltrating lymphocytes (TIL), pairwise comparisons between treatment groups were performed by unpaired t tests. All hypothesis testing was two-sided, and a significance threshold of 0.05 for P value was used.

Results

In vivo syngeneic animal models with differential responses to PD-1 pathway blockade

In order to have animal models that consistently respond to anti–PD-1 therapy, we tested four melanoma models, three derived from Brachyury(+/−)/PTEN(−/−) genetically engineered mice (Supplementary Fig. S1A) and B16, and compared them with MC38, a cell line that has been previously shown to respond well to PD-1 blockade therapy (30, 31). In three replicate studies, we observed antitumor activity of anti–PD-1 or anti–PD-L1 antibody therapy against MC38 (Fig. 1A) and YUMM2.1 (Fig. 1B), but no antitumor activity against YUMM1.1 (Fig. 1C), YUMM1.7, or B16 (Supplementary Fig. S1B). Of note, these responses to anti–PD-1 antibody are incomplete, and both MC38 and YUMM2.1 tumors start regrowing around days 35 to 40 after tumor injection. We decided to focus our further mechanistic studies in MC38 for a tumor that is known to respond to anti–PD-1, and studied the differential responses in YUMM1.1 and YUMM2.1.

Similar PD-L1 expression induced in MC38, YUMM2.1, and YUMM1.1 by IFNγ

In order to investigate the mechanism of response to anti–PD-1 therapy, we first focused on induced PD-L1 expression in these three cell lines. Total cellular PD-L1 increased upon exposure to IFNγ in the three cell lines, with a higher magnitude of increase in MC38 cells than in YUMM2.1 and YUMM1.1 cells (Fig. 2A).

Figure 1.
Enhanced in vivo antitumor activity with anti–PD-1 or anti–PD-L1 in MC38 and YUMM2.1 tumor models compared with YUMM1.1. Tumor growth curves of MC38 (A), YUMM2.1 (B), and YUMM1.1 (C), with 4 mice in each group (mean ± SD) after treatment with anti–PD-1, anti–PD-L1, or isotype control. The arrow indicates the day when treatment with anti–PD-1, anti–PD-L1 or isotype control was started. *P < 0.001 by unpaired t test on day 20, anti–PD-1 versus isotype control, anti–PD-L1 versus isotype control in MC38, anti–PD-L1 versus isotype control in YUMM1.1 cells (Fig. 2B).

Increased mutational load in MC38 compared with YUMM1.1 and YUMM2.1

Next, we determined whether mutational load is a contributor to the observed differential response to anti–PD-1 therapy. MC38, which was established from a mouse exposed to the carcinogen dimethylhydralazine (32), has a higher mutational load (2,778 mutations), compared with the much lower mutational rates in YUMM1.1 and YUMM2.1 (128 and 68 nonsynonymous variants, respectively; Supplementary Fig. S1C). Despite independent derivation, 26 variants are shared by YUMM1.1 and YUMM2.1, which likely represent SNPs not found in the sequenced strain-matched control or in the National Center for Biotechnology Information database of genetic variation. Copy-number
Figure 2. IFNγ modulates PD-L1 expression in MC38, YUMM2.1, and YUMM1.1. 
A, Western blot analysis of PD-L1. MC38, YUMM2.1, and YUMM1.1 cells were cultured with or without IFNγ for 24 hours. 
B, expression of PD-L1 by flow cytometry on MC38, YUMM2.1, and YUMM1.1 cells at baseline and after 24 hours of stimulation with IFNγ. 
C, chromosomal copy-number variation in MC38, YUMM2.1, and YUMM1.1 cell lines. Y-axis represents Log2 depth ratio vs. matched normal.
CD8 and CD4 T cells in response to PD-1 blockade in MC38 and YUMM2.1

To elucidate the role of CD8 and CD4 T cells in anti–PD-1 activity, both cell subtypes were depleted in C57Bl/6 mice bearing MC38 or YUMM2.1 tumors. Antibody-mediated depletion was confirmed in YUMM2.1 tumors and spleens (Supplementary Fig. S2A and S2B). In the absence of CD8 cells, CD4 cells, or both, antitumor response diminished in both MC38 and YUMM2.1 models (Fig. 3A and B). Of note, CD8 cell depletion (anti–PD-1+CD8) in the YUMM2.1 tumor model only partially abrogated the response to anti–PD-1 therapy, whereas CD4 cell depletion, or CD4 plus CD8 depletion, completely abrogated this response (Fig. 3B).

Increased TILs in MC38, but decreased in YUMM2.1, upon PD-1 blockade

Three and ten days after starting treatment with anti–PD-1 or isotype control, tumors and spleens were harvested and stained for CD3, CD4, and CD8 (Supplementary Fig. S2C and S2D). CD8 T-cell infiltration increased in MC38 tumors (calculated as percentage of all cells in the tumor) on day 3 and day 10 of treatment with anti–PD-1 when compared with isotype control (Fig. 3C), whereas CD8 T cells in the corresponding spleens of MC38 tumor–bearing mice remained unchanged (Supplementary Fig. S2E). The percentage of intratumoral CD11c+CD8 T cells on day 10 and no change in spleen (Fig. 3G). Immunochemistry of tumors and spleens of mice in the YUMM2.1 group collected after anti–PD-1 treatment was analogous to nondepleted mice, with or without the addition of anti–PD-1 (Fig. 5D). Growth of tumors in mice that were CD103-depleted was analogous to nondepleted mice, with or without the addition of anti–PD-1 (Fig. 3E). Of note, anti–PD-1-treated YUMM2.1 tumors exhibited a significant increase in CD11c+CD11b–MHC-II+DCs compared with isotype control–treated tumors (Fig. 5F). This finding was not present in MC38 tumors.

Increased tumor-associated macrophages in YUMM2.1 tumors treated with anti–PD-1

Another immune cell subtype potentially implicated in T-cell priming are tumor-associated macrophages (TAM). CD11b+ F4/80+ TAMs were gated after the exclusion of dead cells

**Requirement of costimulation with PD-1 blockade in YUMM2.1**

The evidence that both CD4 and CD8 cells are required for response to PD-1 blockade in the MC38 and YUMM2.1 models suggests that T-cell priming and CD4 helper function may be needed to induce the cytotoxic response to the tumors, which was further studied. The antitumor activity of PD-1 blockade against YUMM2.1 was completely abolished in CD28 knockout (KO; Fig. 4B and C). Silencing β-catenin did not change the antitumor response in the YUMM2.1 model (Fig. 4D), nor did it change in the nonresponsive YUMM1.1 model (Fig. 4E).

**Increased antigen-presenting dendritic cells in anti–PD-1–treated YUMM2.1 tumors**

The next step was to identify the cells involved in antigen presentation and costimulation. We phenotyped the different subtypes of dendritic cells (DC) by staining for CD11c+ B220− (conventional) and CD11c+ B220+ (plasmacytoid) subsets. Conventional DCs can be further subdivided into CD11c+ B220− CD8− DCs, which are CD103+ in peripheral tissues and have been reported to mediate antigen cross-presentation to CD8 T cells (35), and CD11c−CD11b− MHC-II+DCs, which are considered to be dedicated antigen-presenting cells (APCs) that present peptides on MHC-II molecules to CD4 T cells (ref. 36; gating strategy in Supplementary Fig. S4A and S4B). The percentage of CD11c+ B220− CD8− DCs in MC38, YUMM2.1, or YUMM1.1 was not significantly different across time points or with PD-1 blockade therapy. A very small percentage of CD11c+ B220− CD8− cells in YUMM2.1 tumors were present (Fig. 5D). Growth of tumors in mice that were CD103-depleted was analogous to nondepleted mice, with or without the addition of anti–PD-1 (Fig. 3E). Of note, anti–PD-1–treated YUMM2.1 tumors exhibited a significant increase in CD11c+CD11b–MHC-II+DCs compared with isotype control–treated tumors (Fig. 5F). This finding was not present in MC38 tumors.

**Basis of Response to Programmed Cell Death-1 Blockade**

Published OnlineFirst September 2, 2016; DOI: 10.1158/2326-6066.CIR-16-0060
Figure 3.
Both CD8 and CD4 cells mediate response to PD-1 blockade in MC38 and YUMM2.1. Tumor growth curves of MC38 (A) and YUMM2.1 (B) after anti-PD-1 and either anti-CD8 (anti-PD-1aCD8), anti-CD4 (anti-PD-1aCD4), anti-CD8 + anti-CD4 (anti-PD-1aCD8/4) or isotype control; 4 mice in each group, mean ± SD.

(∗, P < 0.001 isotype control, anti-PD-1aCD8, anti-PD-1aCD4, anti-PD-1aCD8/4 versus anti-PD-1 in MC38, P < 0.001 isotype control, anti-PD-1aCD8, anti-PD-1aCD4, anti-PD-1aCD8/4 versus anti-PD-1 in YUMM2.1, unpaired t test, n = 4). ∗, P = 0.003 anti-PD-1aCD8 versus anti-PD-1, unpaired t test, n = 4. The arrow indicates the day treatment with anti-PD-1 or isotype control was started. This experiment was performed in triplicate. (Continued on the following page)
No change in MDSCs or regulatory T cells with PD-1 blockade therapy

To evaluate the effect of anti–PD-1 on other cellular components of the tumor microenvironment, we harvested tumors 10 days after anti–PD-1 treatment was started and analyzed the two main subsets of myeloid-derived suppressor cells (MDSC): monocytederived MDSCs (MO-MDSC, CD11b<sup>+</sup>Ly6Chi<sub>High</sub>Ly6Glo<sub>Low</sub>) and polymorphonuclear MDSCs (PMN-MDSC, CD11b<sup>+</sup>Ly6Chi<sub>Low</sub>Ly6Go<sub>High</sub>; Supplementary Fig. S4D). Anti–PD-1 did not change the percentage of MO-MDSCs or PMN-MDSC in any tumors compared with isotype control (Fig. 6C). Another immune-suppressive cell population, regulatory T cells (T<sub>reg</sub>; Supplementary Fig. S4E; T<sub>reg</sub> CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup>), showed a nonstatistically

(Continued) On days 3 (d3) and 10 (d10) after treatment with anti–PD-1 or isotype control was started, MC38 and YUMM1.1 tumors were isolated and stained with fluorescent-labeled antibodies, analyzed by FACS. C and D, percentage of CD3<sup>+</sup>CD8<sup>+</sup> (CD8 T cells) and CD3<sup>+</sup>CD4<sup>+</sup> (CD4 T cells) in MC38 (C) and YUMM1.1. D, tumors are shown (mean ± SD). *, P = 0.03 anti–PD-1 d10 versus control d10 in MC38. P = 0.03 anti–PD-1 d10 versus control d10 in YUMM2.1 (unpaired t test, n = 4). Results were consistent in 6 replicate experiments. E and F, statistical analysis of the 2C total number of CD8 T cells per gram of tumor in MC38 (E) and (F) YUMM2.1 tumors. *, P = 0.05 anti–PD-1 d10 versus control d10 in MC38, P = 0.02 anti–PD-1 d10 versus control d10 in YUMM2.1, unpaired t test, n = 8). G, representative immunofluorescence of CD8 T cells stained in YUMM2.1 tumors and spleens d10 after treatment with anti–PD-1 or isotype control was started.

Continued
Figure 5.
Increased antigen-presenting DCs in anti–PD-1-treated YUMM2.1 tumors. A, tumor growth curves of CD28KO or C57BL/6 mice bearing YUMM2.1 treated with anti–PD-1 or isotype control. B, tumor growth curves of CD80/86KO or C57BL/6 mice bearing YUMM2.1 treated with anti–PD-1 or isotype control. Four mice in each group (mean ± SD). The arrow indicates the day treatment with anti–PD-1 or isotype control was initiated. C, on day 10 after starting treatment, MC38, YUMM2.1, and YUMM1.1 tumors were isolated and stained with fluorescent-labeled antibodies and analyzed by FACS, with 3 mice in each group (mean ± SD), B220– and B220+ cells presented as percentage of CD11c+ cells. *, P = 0.04 anti–PD-1 versus isotype control, CD11c+ B220– cells in MC38 tumors, unpaired t test, n = 3. D, B220−CD8+ and B220+CD103+ presented as percentage of CD11c+ cells. E, in vivo YUMM2.1 growth curve after anti–PD-1 ± anti–CD103 or isotype control ± anti–CD103, 4 mice in each group (mean ± SD). The arrow indicates the day anti–PD-1 or isotype control treatment was started. F, CD11b+ and CD11b+MHC-IIhigh DCs presented as percentage of CD11c+ cells. *, P = 0.04 anti–PD-1 versus control, P = 0.01 anti–PD-1 versus control in YUMM2.1 tumors, unpaired t test, n = 5.
Figure 6.
Modulation of the tumor microenvironment by anti–PD-1 in MC38, YUMM2.1, and YUMM1.1. On day 10 after anti–PD-1 or isotype control, MC38, YUMM2.1, and YUMM1.1 tumors were isolated and stained with fluorescent-labeled antibodies and analyzed by FACS, with 3 mice in each group (mean ± SD). 


B, TAMs MHC-IIhigh (M1 TAMs, CD11b+ F4/80+ MHC-IIhigh) and TAMs MHC-IIlow (M2 TAMs, CD11b+ F4/80+ MHC-IIlow). *P = 0.04 anti–PD-1 d10 versus control d10 TAMs; F4/80+ versus control d10 TAMs in YUMM2.1 tumors, unpaired t test, n = 3.

C, MO-MDSC (CD11b+ Ly6C+ Ly6G+) and PMN-MDSC (CD11b+ Ly6C– Ly6G+) presented as percentage of CD11b+ cells.

D, analysis of Tregs (CD4+ CD25+ FoxP3+).

E, representative FACS plots in tumors.
significant trends toward a decrease in MC38 and YUMM2.1 tumors with anti–PD-1 and an increase in YUMM1.1 (Fig. 6D). Representative flow charts of TAMs, MDSCs, and Tregs are shown in Fig. 6E.

A more inflammatory gene signature profile in YUMM2.1 compared with YUMM1.1

RNA was extracted from cultured YUMM1.1 and YUMM2.1 and subjected to RNA sequencing. GSEA and pathway analyses indicated that immune response, cytokine production, and inflammatory-related genes were strongly represented in YUMM2.1 compared with YUMM = 1.1 cells (Fig. 7A). Corresponding normalized enrichment scores (NES), P values, and FDR of the GSEA plots are included (Fig. 7B). Analysis of genes that code for secreted proteins with a log_2-fold higher than 1 in YUMM2.1 compared with YUMM1.1 cells revealed an increase in inflammatory and chemotaxis-related genes (Supplementary Fig. S4F).

Discussion

Immunological checkpoint blockade with anti–PD-1 or anti–PD-L1 antibodies reverses cancer immunosuppression and promotes antitumor immune responses in several cancer types. Long-term responses with minimal side effects have been reported in patients with melanoma, lung, liver, kidney, bladder, mismatch repair–deficient colon cancers, and hematologic malignancies, among others (1–4, 31). Why these agents exhibit antitumor responses in certain histologies and only in a percentage of patients with the same type of tumor remains unknown. Here, we studied tumor models that respond differently to anti–PD-1 treatment and tested the reasons for anti–PD-1 activity in MC38 and YUMM2.1 tumors.

Upregulation of PD-L1 and its ligation to PD-1 on activated T cells is a well-described mechanism by which cancer tissues limit the host immune response, termed adaptive immune resistance (37). High baseline PD-L1–expressing tumor cells have been positively correlated with response to PD-1 blockade in patient samples (5, 6). However, PD-L1 was markedly increased upon IFNγ exposure in the three murine cell lines studied, which does not provide an explanation for the different responses to anti–PD-1.

Mutational load has been associated with a higher clinical benefit to immunotherapy (38–40). A greatly increased number of somatic mutations were observed in MC38 compared with YUMM2.1 and YUMM1.1, accompanied by high copy-number variation, consistent with its origin as a carcinogen-induced cell
line. The high mutational load could be at least partially responsible for the effectiveness of anti–PD-1 therapy in MC38 tumors. However, both YUMM2.1 and YUMM1.1 displayed a very low number of new somatic mutations, consistent with tumors arising from genetically engineered mice driven by a strong driver oncogene and avoidance of senescence.

T-cell response has been widely accepted to be crucial for effective anti–PD-1/PD-L1 antitumor activity (41). We confirmed the essential roles of both the CD8 and CD4 T cells in anti–PD-1 effect in both MC38 and YUMM2.1 tumor models. Depletion of CD8 cells completely abrogated the antitumor effect of PD-1 blockade in the MC38 model but only had a partial effect in the YUMM2.1 model, whereas CD4 depletion completely reversed the antitumor effect in both models. Considering that anti–PD-1 also controls key T-cell inhibitory interactions between PD-L1 on APCs and PD-1 on T cells (17, 42) and that PD-1 limits CD4 T-cell clonal expansion in response to an immunogenic stimulus (43), it is not surprising that CD4 T cells are required for anti–PD-1/PD-L1 tumor response. However, another group has reported opposite observations, with increased antitumor effect seen with CD4 cell depletion combined with PD-1/PD-L1 blockade (44). Of note, none of the tumor models evaluated by this group was responsive to anti–PD-1/PD-L1 itself. The authors suggested that CD4 cell depletion effect was partially attributed to a removal of CD4-positive immunosuppressive Tregs. However, in another report (31), Tregs increased after very early analysis (48 and 72 hours) following treatment with anti–PD-1 in MC38, whereas in our tumor models, Tregs did not change with anti–PD-1 when analyzed up to 14 days after starting therapy.

Next, we characterized anti–PD-1 modulation of the cellular components in the tumor microenvironment. CD8 T cells were expected to increase in both anti–PD-1-responsive tumors. This was true for MC38, but in YUMM2.1, CD8 T cells decreased over time with anti–PD-1 therapy, implying that CD8 T cells may have an early role in this antitumor response. Therefore, the early activation of CD8 T cells could take place during antigen presentation to naive T cells, where PD-1/PD-L1 costimulation has been shown to lead to T-cell receptor (TCR) downmodulation (16, 17, 42). DCs have been reported to hyperactivate CD8 T cells in the absence of PD-1/PD-L1 costimulation, which was accompanied by a higher TCR surface level and an increase in IFNγ (17). Depending on where PD-1/PD-L1 blockade takes place, T-cell activity may vary. It is unknown if the location of PD-1/PD-L1 interaction and its consecutive blockade is tumor-dependent in a short-term implanted tumor model. Functional studies to determine T-cell activity shortly after anti–PD-1 are administered, and further characterization of the specific CD8 T-cell phenotype could provide some explanation on how CD8 T cells exhibit their effect in this tumor model. The role of natural killer (NK) cells in this setting is unknown and technically challenging because of their low frequency in the tumor microenvironment, but certainly interesting to explore. Differences in PD-1 expression on the CD8 T cells could also be informative to address PD-1 responsiveness in the YUMM2.1 tumor model, as shown by others (31).

The correlation between tumor-intrinsic stabilized β-catenin and both T-cell exclusion and anti–PD-1/L1 resistance in genetically engineered mice with BRAFV600E/Pten−/−/β-catenin−stabilized tumors (34) led us to investigate the effect of β-catenin downregulation in T-cell modulation and anti–PD-1 antitumor response. Although our analysis indicated that YUMM2.1 did not have recombined β-catenin allele that would render β-catenin more stable, it does have more β-catenin expression and activity compared with the other YUMM cell lines. We observed that T cells were reduced over time (but never upfront excluded) with anti–PD-1 therapy, and this phenomenon was independent from the β-catenin status. PD-1 blockade antitumor effect was not altered in the presence of a downregulated Wnt/β-catenin pathway.

Looking further into the importance of costimulatory interactions during antigen presentation to naïve T cells, we demonstrated that the absence of CD28 or CD80/86 prevented the anti–PD-1 effects in YUMM2.1 tumors. This observation does not necessarily imply that the PD-1/PD-L1 inhibitory effects only take place at the APC–T-cell synapse, but suggest that PD-L1–expressing APCs are positively enhanced upon PD-1 blockade. Indeed, the priming of CD4 and CD8 T cells is more effective in the absence of PD-1/PD-L1 signaling (45), and downmodulation of PD-L1 in DCs results in increased costimulatory molecule CD80 expression and a distinct cytokine profile (46). The same group observed strong tumor growth control when using PD-L1–silenced DCs in a mouse model of lymphoma, although with no increased cure rates, possibly due to PD-L1–expressing tumor cells that might counteract CD8 T-cell activity (47). Analysis of the different DC subsets in YUMM2.1 tumors revealed an increase in CD11c+CD11b+MHC-IIhigh DCs upon PD-1 blockade, which was not present in the other tumor models analyzed. Cross-priming of tumor antigens by BATF3-dependent DCs is crucial to the efficacy of anti–PD-1 antibodies (48). Taken together, these data imply that priming via CD4 T cells has a more important role in the antitumor efficacy of PD-1 blockade in the YUMM2.1 model.

When looking into the ability of the models to evoke an inflammatory reaction required for immune cell recruitment and DC–T-cell costimulation, YUMM2.1 exhibited an “inflammatory profile” consistent with an endogenous upregulation of immune, cytokine producing, and inflammatory response-related genes. The YUMM2.1 model could therefore intrinsically harbor inflammatory mediators necessary to couple innate recognition to T-cell–mediated immunity by DCs in vivo, which is also supported by the increase in chemotactic factors such as Cxcl10, Ccl6, or Cxcl12. This observation is consistent with other reports, where chemokine-trafficking of immune cells into tumors was observed in human melanoma cell lines (49) or in mice receiving adoptive cell therapy and anti–PD-1 blockade (50).

In conclusion, T-cell priming supports anti–PD-1 antitumor responses mediated by CD4 and CD8 T cells, critically requiring costimulation in vivo.

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

Authors’ Contributions
Conception and design: B. Homet Moreno, S. Hu-Lieskovan, A. Ribas
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Homet Moreno, L. Robert, K. Meeth, A.T. Weeraratna, S. Hu-Lieskovan, A. Ribas

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Grant Support

This study was funded in part by the NIH grants P01CA168585 (to A. Ribas and T.G. Graeber), R35 CA197633, the Ressler Family Fund, the D.S. Samuels Family Fund, the Rubby Family Fund, the Alexandra Cooper Memorial Fund, and the Garcia-Corsini Family Fund (to A. Ribas). B. Homet Moreno was supported in part by the Rio Hortega Scholarship (08/142) from the Hospital 12 de Octubre, Madrid, Spain. G. Parisi was supported in part by the Division of Medical Oncology and Immunotherapy (University Hospital of Siena). J.M. Zaretsksy is a member of the UCLA Medical Scientist Training Program supported by NIH NIGMS training grant GM08042. J. Tsioi was supported by the NIH Ruth L. Kirschstein Institutional National Research Service Award #T32 CA090120. S. Hu-Lieskovan was supported by a Young Investigator Award and a Career Development Award from the American Society of Clinical Oncology (ASCO), a Tower Cancer Research Foundation Grant an UCLA KL2 award, and a Dr. Charles Coltman Fellowship Award from the Hope Foundation.

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Received March 21, 2016; revised July 12, 2016; accepted August 4, 2016; published OnlineFirst September 2, 2016.

References

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