Cancer Research

Combined Blockade of IL6 and PD-1/PD-L1 Signaling Abrogates Mutual Regulation of Their Immunosuppressive Effects in the Tumor Microenvironment

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Abstract

Recently emerging cancer immunotherapies combine the applications of therapeutics to disrupt the immunosuppressive conditions in tumor-bearing hosts. In this study, we found that targeting the proinflammatory cytokine IL6 enhances tumor-specific Th1 responses and subsequent antitumor effects in tumor-bearing mice. IL6 blockade upregulated expression of the immune checkpoint molecule programmed death-ligand 1 (PD-L1) on melanoma cells. This PD-L1 induction was canceled in IFNγ-deficient mice or $CD4^+$ T cell–depleted mice, suggesting that $CD4^+$ T cell–derived IFNγ is important for PD-L1 induction in tumor-bearing hosts. In some patients with melanoma, however, treatment with the anti–PD-1 antibody nivolumab increased systemic levels of IL6, which was associated with poor clinical responses. This PD-L1 blockade-evoked induction of IL6 was reproducible in melanoma-bearing mice. We found that PD-1/PD-L1 blockade prompted $PD\text{-}1^+$ macrophages to produce IL6 in the tumor microenvironment. Depletion of macrophages in melanoma-bearing mice reduced the levels of IL6 during PD-L1 blockade, suggesting macrophages are responsible for the IL6-mediated defective $CD4^+$ Th1 response. Combined blockade of the mutually regulated immunosuppressive activities of IL6 and PD-1/PD-L1 signals enhanced expression of T cell–attracting chemokines and promoted infiltration of IFNγ-producing $CD4^+$ T cells in tumor tissues, exerting a synergistic antitumor effect, whereas PD-L1 blockade alone did not promote Th1 response. Collectively, these findings suggest that IL6 is a rational immunosuppressive target for overcoming the narrow therapeutic window of anti–PD-1/PD-L1 therapy.

Significance: These findings advance our understanding of IL6-PD1/PD-L1 cross-talk in the tumor microenvironment and provide clues for targeted interventional therapy that may prove more effective against cancer. *Cancer Res; 78(17); 5011–22.* ©2018 AACR.

Introduction

Melanoma is one of the leading causes of cancer mortality. Surgery, radiotherapy, and/or systemic therapies including targeted drugs offer a chance for cure in patients with early-stage melanoma, but the vast majority of patients with advanced or metastatic diseases are rarely cured (1). In such situations, there are strong correlations between the number or type of tumor-infiltrating T cells and favorable outcomes (2). However, the spontaneous antitumor immune response is relatively weak because of the detrimental effects of immunosuppressive factors or cells such as regulatory T cells (Treg), tumor-associated macrophages (TAM), and myeloid-derived suppressor cells (MDSC; ref. 3). Ligation of programmed cell death (PD)-1 on tumor-specific T cells with its ligands, PD-L1, is also involved in tumor-induced immunosuppression (4). Treatment with antibodies (Ab) that disrupts this interaction has provided dramatic objective response rates ranging from 30% to 40% in patients with advanced melanoma (4–6). However, although some clinical studies suggested that PD-L1 expression in tumor tissues was correlated with the response to this therapy (6), a substantial population of patients do not respond despite the measurable PD-L1 expression (4, 5). These observations raise the requirement of strategies to predict which patients will benefit from these agents and to overcome the insufficient therapeutic efficacy in nonresponders.

Many comprehensive studies have shown that IFNγ-producing $CD4^+$ Th1 cells exert a critical role in antitumor responses (7–10), and thus, their infiltration into tumor tissue is an indicator of better prognosis (11). In contrast, patients with cancer have profound systemic Th2 bias rather than Th1 polarization (12, 13). Notably, a beneficial effect induced by PD-1/PD-L1 blockade is not obvious in $CD4^+$ T cell–mediated antitumor Th1 responses *in vivo* (14, 15), although cytotoxic activity, proliferation, and IFNγ production in both $CD8^+$ and $CD4^+$ T cells were recovered by inhibiting the

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PD-1/PD-L1 interaction *in vitro* (16, 17). Furthermore, the effect of PD-1/PD-L1 blockade on other immune cells in tumor microenvironment remains unclear, despite the PD-1 expression in some myeloid cells such as macrophages (18). A better understanding of the effect of PD-1/PD-L1 blockade on these tumor-associated immune cells is required to design a rationale-based strategy for improving its therapeutic efficacy.

Inflammation is closely linked to the prognosis of patients with cancer. Chronically elevated levels of proinflammatory cytokine, IL6, which promotes tumor cell survival, is a poor prognostic factor in patients with many types of cancers including melanoma (9, 19, 20). Hence, a therapeutic approach for IL6 blockade using humanized IL6/IL6R Abs has been developed to abrogate its direct effect on tumor growth/survival (20). In addition, tumor cell–extrinsic effects of IL6 have been demonstrated in antitumor immune responses through myeloid-lineage cells and T cells (8, 9, 21, 22). Furthermore, the higher level of IL6, which is referred to as cytokine release syndrome ranged from mild to life-threatening symptoms, is observed in some patients undergoing immunotherapies such as adoptive T-cell transfer (23) or PD-1 blockade (24, 25). However, the antitumor immunologic relevance of inflammation in such potent immunotherapies remains unclear.

In this study, we found that anti-IL6 Ab treatment augmented Th1 responses, but in turn, induced upregulation of PD-L1 expression on melanoma cells through $CD4^+$ T cell–derived IFNγ. On the other hand, treatment with anti–PD-L1 Ab prompted TAMs to produce IL6 counteracting Th1 responses in melanoma-bearing mice. Consistent with this, vigorous increase of circulating IL6 was observed in a certain population of patients with melanoma treated with anti–PD-1 therapy, which was associated with a poor clinical response to this therapy. These findings suggest that combined blockade of IL6 signaling and PD-1/PD-L1 pathways disrupts the mutual "see-saw" interplay between these immunosuppressive events, resulting in synergistic antitumor effects.

Materials and Methods

Mice, tumor cells, and Ab treatment

Male C57BL/6NCrSlc and Balb/cCrSlc mice were purchased from Japan SLC, Inc. IL6-deficient mice were obtained from The Jackson Laboratory. All the mice including IFNγ-deficient mice (26) were housed at the Center for Animal Resources and Development, Kumamoto University (Kumamoto, Japan), and all the experimental procedures were approved by the Institutional Animal Committee of Kumamoto University and performed in accordance with the guidelines.

B16-F10 melanoma and CT26 colon carcinoma were authenticated by simple sequence length polymorphism or isozyme analysis and provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer (Tohoku University, Sendai, Japan), and RIKEN BRC Cell Bank, respectively. Ovalbumin (OVA)-expressing melanoma MO4 (27) were kindly provided by Dr. Kenneth L. Rock (Department of Pathology, University of Massachusetts Medical School, Worcester, MA). RMA lymphoma (9, 28) was kindly provided by Dr. Akira Shibuya (Department of Immunology, University of Tsukuba, Tsukuba, Japan). These cell lines were not further authenticated, and *Mycoplasma* testing on these cell lines was not performed in our laboratory. However, routine confirmation of *in vitro* growth properties, morphology, and tumor formation in syngeneic mouse strain provide evidence of correct cell identity. Mice were inoculated subcutaneously with 3×10^5 B16-F10, CT26, MO4, or RMA. Tumor size was expressed as tumor index, which is the square root of (length × width) (21). A total of 200 μg of control IgG Ab (Millipore), anti-IL6 (MP5-20F3, Bio X Cell), and/or anti–PD-L1 Abs (10F.9G2, Bio X Cell) were injected intraperitoneally. For *in vivo* depletion, mice were injected with anti-CD4 Ab (100 μg/mouse, GK1.5, TONBO) one day before and 3 or 6 days after tumor inoculation. A total of 200 μg of anti-F4/80 (CI:A3-1, Bio X Cell) was injected twice every other day starting at 7 days after tumor inoculation.

Patients

Inclusion criteria for treatment with the anti–PD-1 Ab nivolumab were patients with unresectable metastasis (stage IV, $n = 16$). Nivolumab was administrated at 3 mg/kg bodyweight every 2 weeks. The evaluable clinical responses with a follow-up period of at least 3 months were indicated as complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) based on the RECIST version 1.1 with some modifications in continuation and response assessment of immunotherapy (29). The cases were collected from January 20, 2016, to August 29, 2017. Clinical data including lactate dehydrogenase (LDH), C-reactive protein (CRP), and treatment outcomes were analyzed and extracted from patient records. Progression-free survival (PFS) was calculated as the time from the start of nivolumab treatment until disease progression determined by imaging and/or clinical observation. Written informed consent was obtained from all the subjects including healthy donors. This study was conducted in accordance with the principles of the Helsinki Declaration and approved by the Institutional Review Board of Kumamoto University (Permit Number: #118 and #103). The detailed characteristics of patients are summarized in Supplementary Tables S1 and S2. Blood samples were obtained from patients with melanoma before and after 6 times administrations. Plasma was collected from blood samples with BD Vacutainer PT tubes (BD Biosciences) according to the manufacturers' instructions, and then cryopreserved until use.

Analysis of tumor-infiltrating cells and isolation of TAMs

Tumor tissues were minced with razors and analyzed for mRNA expression or digested with 2.5 mg/mL collagenase D (Roche) and 0.1 mg/mL DNase I (Sigma) for 30 minutes. Resulting single-cell suspensions were analyzed. For TAM isolation, tumor cells were removed from the above cell suspensions using Lymphoprep (Axis Shield), and then $CD11b^+Gr\text{-}1^-$ macrophages were purified using CD11b microbeads (Miltenyi Biotec) after removing $Gr\text{-}1^+$ cells with Gr-1 microbeads (Miltenyi Biotec). TAMs (5×10^5) were stimulated with plate-coated recombinant PD-L1-Fc or control-Fc protein (3.5 μg/mL; R&D Systems) for 18 hours.

Flow cytometric analysis and cytokine measurements

Cells from spleen, lymph nodes, or tumor tissues were stained with the following Abs for flow cytometric analyses: anti–Gr-1, anti–PD-1, anti-CD11c, anti–MHC-II (BD Biosciences), anti-CD45, anti-Foxp3, anti-CXCR3, anti-CD11b, anti-PD-L1 Abs (eBioscience), anti-CD4, anti-CD8, anti-F4/80 Abs (clone; BM8.1, TONBO), anti-CD64, anti-CD206, and anti-MerTK Abs (Miltenyi Biotec). The $H\text{-}2K^b$/SIINFEKL-tetramer-PE was from MBL. For staining of intracellular cytokines in T cells, the cells were stimulated with PMA/ionomycin, and then stained with anti-IL2 or anti-IFNγ Ab (TONBO) as described previously (8). For the assessment of IL6-producing cells in tumor tissues, cell

suspension from tumors was cultured in the presence of 2 μg/mL control or anti–PD-L1 Ab and Brefeldin A (Sigma) for 18 hours. After cell surface staining, intracellular IL6 was stained with anti-IL6 Ab (eBioscience) using BD Cytofix/Cytoperm Buffer (BD Biosciences). Immunofluorescent images and the data were analyzed using FACSVerse (BD Biosciences) and FlowJo software (Tree Star), respectively. For ELISPOT assay (BD Biosciences), 1×10^5 draining lymph node cells and 3×10^4 bone marrow–derived dendritic cells (DC) pulsed with I-A^b–binding OVA peptide (ISQAVHAAHAEINEAGR; OVA-IIp) were mixed and incubated for 12 hours. IFNγ spots were visualized with ELISPOT Assay Kit (BD Biosciences) and analyzed as described previously (8). ELISA Kits for detecting human and mouse IL6, soluble IL6 receptor (sIL6R), IL1β, and TN-α were purchased from R&D Systems. The levels of the other cytokines were measured using Bio-Plex suspension array system (Bio-Rad).

Real-time PCR

Total RNA was extracted using TRIzol reagent (Ambion) and RNeasy Plus Mini Kit (QIAGEN), and reverse transcribed with ReverTra Ace (TOYOBO). qPCR was performed on ViiA7 or One-Step Real-Time PCR System with Master Mix reagents (Applied Biosystems) and TaqMan probes (*Foxp3*; Mm00475162_m1, *Il4*; Mm00445259_m1, *Ifng*; Mm01168134_m1 *Il10*; Mm01288386_m1, *Il6*; Mm00446190_m1, *Il12b*; Mm01288989_m1, *Tnfa*; Mm00443258_m1, *Il1b*; Mm00434228_m1, *Ccl3*; Mm00441259_m1, and *Gapdh*; Mm99999915_g1). The mRNA levels of the other chemokines were determined by qPCR with Power SYBR Green PCR Master Mix (Life Technologies) using the following primers: *Ccl4* 5′-CCAGGGTTCTCAGCACCA-3′ and 5′-GCTCACTGGGGTTAGCA-CAGA-3′; *Ccl5* 5′-CTCACCATATGGCTCGGACA-3′ and 5′-CTTCTCT-GGGTTGGCACACA-3′; *Cxcl9* 5′-TGGAGTTCGAGGAACCCTAGT-3′ and 5′-AGGCAGGTTTGATCTCCGTT-3′; *Cxcl10* 5′-ACGAACTTAACC-ACCATCT-3′ and 5′-TAAACTTTAACTACCCATTGATACATA-3′; *Cxcl11* 5′-AGGAAGGTCACAGCCATAGC-3′ and 5′-CGATCTCTGCCATTTT-GACG-3′. Expression of each gene was normalized to *Gapdh* expression using the comparative $2[-\Delta\Delta C_t]$ method.

In vitro T-cell differentiation

Mouse naïve T cells were isolated from spleen with Pan T Cell Isolation Kit and CD62L microbeads (Miltenyi Biotec). These cells were stimulated with plate-coated anti–CD3/CD28 Abs (both TONBO) in the presence of IL12 (8 ng/mL; Wako) with or without anti-IL6 Ab (1 μg/mL). After culturing for 7 days, IFNγ production or expansion of effector T cells was analyzed.

Statistical analysis

To ascertain a normal distribution of variables, Shapio–Wilk test was performed. Multiple comparisons were performed by one-way ANOVA followed by Tukey–Kramer *post hoc* tests. A Kruskal–Wallis test was used as a nonparametric alternative to ANOVA. The log-rank test was performed to compare PFS of the two groups in Kaplan–Meier plots. Cox proportional hazards regression was applied to investigate the relationship between IL6 levels and PFS. Data were also analyzed using unpaired Student *t* test when comparing two experimental groups. Correlations between variables were determined by Spearman correlation coefficient. These analyses were performed using the Prism 4.0 (GraphPad) and R version 3.3.1 (The R Foundation for Statistical Computing). *P* values less than 0.05 were considered statistically significant.

Results

IL6 blockade augmented Th1 responses and retarded melanoma progression

We previously demonstrated that in tumor-bearing mice immunized with tumor-associated cognate antigenic peptide-loaded DCs, Th1 differentiation of adoptively transferred and *in vivo* primed tumor-specific $CD4^+$ T cells was attenuated in an IL6-dependent manner (9, 21). This observation have evoked the possibility that development of IFNγ-producing $CD4^+$ Th1 cells from spontaneously primed endogenous tumor-specific $CD4^+$ T cells is masked by IL6 signal, which is augmented in tumor-bearing animals and patients with cancer (19, 20). To test this possibility, we first evaluated the beneficial effect of IL6 blockade on endogenously primed tumor-specific $CD4^+$ T cells in mice bearing MO4 melanoma cells expressing OVA as a surrogate tumor-associated antigen (27). As shown in Fig. 1A, IFNγ-producing OVA peptide–specific $CD4^+$ T cells were significantly increased by IL6 blockade in tumor-draining lymph nodes.

Consequently, melanoma growth was significantly retarded by IL6 blockade, but not completely rejected (Fig. 1B). Consistent with the efficient induction of Th1 responses, we found a higher frequency of $CD4^+$ T cells expressing CXCR3, which reflects the Th1 responses *in vivo* (30, 31), in the tumor tissue of anti-IL6 Ab-treated mice (Fig. 1C). Furthermore, the recruitment of tumor (OVA)-specific $CD8^+$ T cells at the tumor site was promoted by IL6 blockade, which was constrained by the depletion of $CD4^+$ T cells (Fig. 1C), suggesting a negative impact of IL6 on $CD4^+$ T cell–mediated help for cognate CD8 T-cell induction. In contrast to *Ifng* expression, the mRNA expression of the Th2 cytokine, *Il4* was reciprocally downregulated by IL6 blockade (Fig. 1D). Overall, these results confirmed that the immunosuppressive effect of IL6 had a detrimental effect on spontaneous T cell–mediated antitumor responses by modulating the balance between Th1 and Th2 responses (9, 13). The expression of Treg-associated markers, *Foxp3* and *Il10*, was not modulated in the tumor microenvironment after treatment with anti-IL6 Ab (Fig. 1D).

IL6 blockade induced CD4- and IFNγ-dependent PD-L1 expression on melanoma cells

We focused on the characteristics of melanoma cells and investigated their PD-L1 expression in mice treated with anti-IL6 Ab (Fig. 2A). Surprisingly, IL6 blockade significantly upregulated the PD-L1 expression on MO4 cells, which was completely abrogated in tumor-bearing IFNγ-deficient mice. Given that $CD4^+$ T cells were potent IFNγ producers in response to IL6 blockade (Fig. 1), we explored whether $CD4^+$ T cells contributed to this PD-L1 induction. As shown in Fig. 2B, CD4 depletion with anti-CD4 Ab also prevented anti-IL6 Ab-mediated PD-L1 upregulation on tumor cells. Consistent with the *in vivo* results, *in vitro* stimulation with IFNγ robustly induced PD-L1 upregulation on several tumor cells, B16-F10, MO4, and CT26, but not on the lymphoma, RMA (Fig. 2C). The expression levels of PD-L1 were not altered by IL6 stimulation, excluding the possibility that IL6 directly affected PD-L1 expression. Collectively, these results suggest that IL6 blockade indirectly augments the PD-L1 induction on melanoma via $CD4^+$ T cell–derived IFNγ.

Change in the level of IL6 reflected the therapeutic efficacy of anti–PD-1 Ab treatment in patients with melanoma

As observed in other types of cancers, patients with melanoma exhibited a higher level of IL6 in plasma compared with that in

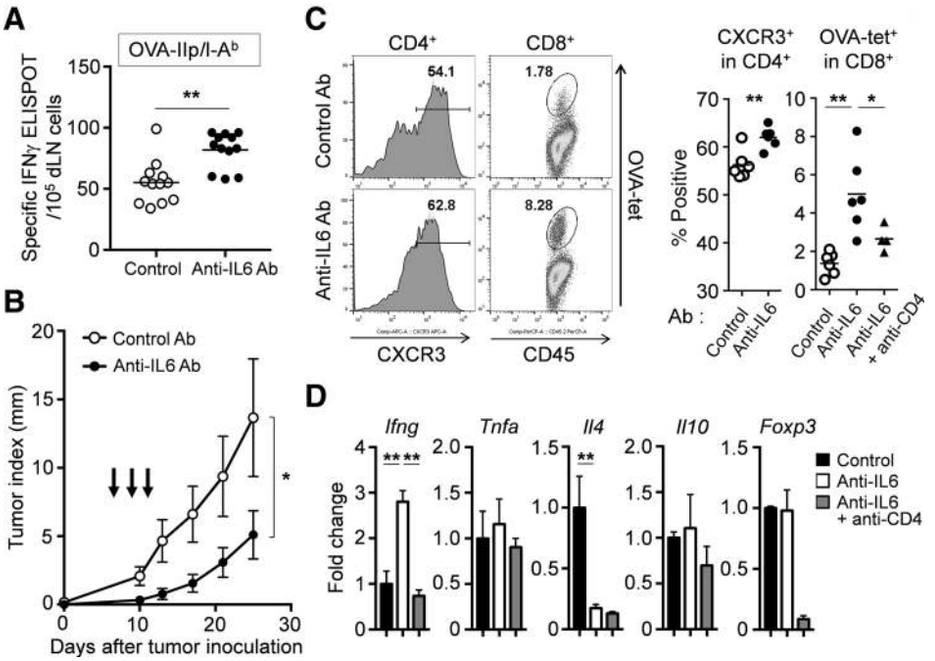

Figure 1.
IL6 blockade promotes Th1 responses and attenuates melanoma progression. **A** and **B,** MO4-bearing mice were treated with anti-IL6 or control Ab three times (indicated by arrows in **B**). Eight days after the first Ab injection, tumor-draining lymph nodes were analyzed for OVA-IIp/I-A^b–specific IFNγ responses by ELISPOT assay (**A**), and tumor outgrowth was monitored over time (**B**). **C** and **D,** Frequencies of $CXCR3^+$ cells in $CD4^+$ T cells and SIINFEKL/H-$2K^b$-$tetramer^+$ cells in $CD8^+$ T cells (**C**), and the indicated mRNA expression (**D**) in tumor tissues were analyzed. Anti-CD4 Ab was injected 1 day before tumor inoculation. Representative histograms and dot plots (**C**, left) and each value of the indicated populations (**C**, right) are shown. The values represent the mean ± SEM with $n = 4$–12/group; *, $P < 0.05$; **, $P < 0.01$. The data are representative of three or more independent experiments.

healthy donors (Fig. 3A). However, the levels of IL6 were decreased after surgical removal of primary melanoma. In contrast, the level of sIL6R, the other component of IL6 signaling, was not altered. We next validated the plasma levels of IL6 during treatment with anti–PD-1, nivolumab for 12 weeks in patients with melanoma for whom sequential blood samples were available. Interestingly, as shown in Fig. 3B, the patients were divided into two groups. Some patients induced a profound increase in IL6 during nivolumab treatment, whereas IL6 levels were not changed or decreased in other patients.

To examine whether the elevated IL6 levels were associated with tumor progression in individual patients, PFS was assessed on the basis of stratification by the fold change in IL6 levels during nivolumab treatment (on-/pretreatment; median value, 1.516). As shown in Fig. 3C, the patients with increased IL6 level (on-/pretreatment IL6 $\geq$ 1.516) exhibited a shorter PFS compared with patients whose IL6 levels were not increased [higher group; median PFS 11 weeks; 95% confidence interval (CI), 6–14 weeks, lower group; median PFS NA; 95% CI, 8–NA weeks]. In contrast, there was no significant difference in the duration of PFS when patients were grouped according to the baseline of IL6 concentration (median value; 1.64 pg/mL, Supplementary Fig. S1A and S1B). Consistent with the result of PFS, poor clinical responses were associated with greater increase in IL6 levels, whereas the change in IL6 level was modest (<1.516) in patients achieving disease control (Fig. 3D). Cox regression analysis indicated that patients with large increases in IL6 were at high risk for poor clinical responses (HR = 13.6; 95% CI, 1.67–110.8), suggesting that an increased IL6 level serves as a predictive factor for poor PFS and clinical response in patients with melanoma treated with nivolumab. On the other hand, the level of LDH, an indicator for the malignancy and rapid progression of melanoma (32), was not altered for 12 weeks after initial nivolumab treatment (Fig. 3E), and changes in the LDH level were not associated with those of IL6 (Fig. 3F), suggesting that the increased IL6 in nonresponders was not simply reflected by the tumor burden. The levels of CRP or IL8, which are both clinical and blood parameters for inflammatory responses, tended to be increased in patients with poor clinical responses, but their changes were not drastic, similarly to IL10 or TNFα (Fig. 3G). Taken together, these results imply that an increase in IL6 during PD-1/PD-L1 blockade is correlated with the therapeutic responsiveness of patients with melanoma.

Blockade of PD-1–PD-L1 interaction led to IL6 production by TAMs

We further investigated the mechanistic action of IL6 upregulation during anti–PD-L1 Ab treatment in melanoma-bearing mice. Although a large increase in IL6 levels in the serum was not detected in control Ab-treated MO4-bearing mice as compared with that in tumor-free mice, anti–PD-L1 Ab treatment prominently augmented the IL6 levels in wild-type (WT) mice (Fig. 4A). This model recapitulated some of the anti–PD-1 Ab-treated patients with melanoma (Fig. 3B). However, such IL6 induction was not observed in IL6-deficient counterparts, suggesting that IL6 was produced by host-derived cells but not by melanoma cells in response to PD-L1 blockade. PD-L1 blockade–induced IL6 upregulation was reproducibly detected in isolated TAMs (Fig. 4B), suggesting that TAMs are one of the possible cellular source of IL6 in response to PD-L1 blockade. Therefore, we next analyzed the PD-1 expression on TAMs localized at the tumor site and found that PD-1 was substantially expressed on $Gr\text{-}1^-F4/80^+CD11b^+$TAMs during melanoma progression, whereas tumor-infiltrated $Gr\text{-}1^+CD11b^+$MDSCs or splenic $Gr\text{-}1^-F4/80^+$ macrophages did not express PD-1 (Fig. 4C; Supplementary Fig. S2A). $PD\text{-}1^+$TAMs expressed the macrophage markers CD64 and CD206, and the lower levels of MHC-II molecules, but not CD11c or scavenger receptor MerTK (Supplementary Fig. S2A).

To explore the mechanistic basis of the interconnection between PD-1/PD-L1 and IL6 pathway in TAMs, the level of IL6 was assessed in TAMs when PD-1/PD-L1 interaction was blocked

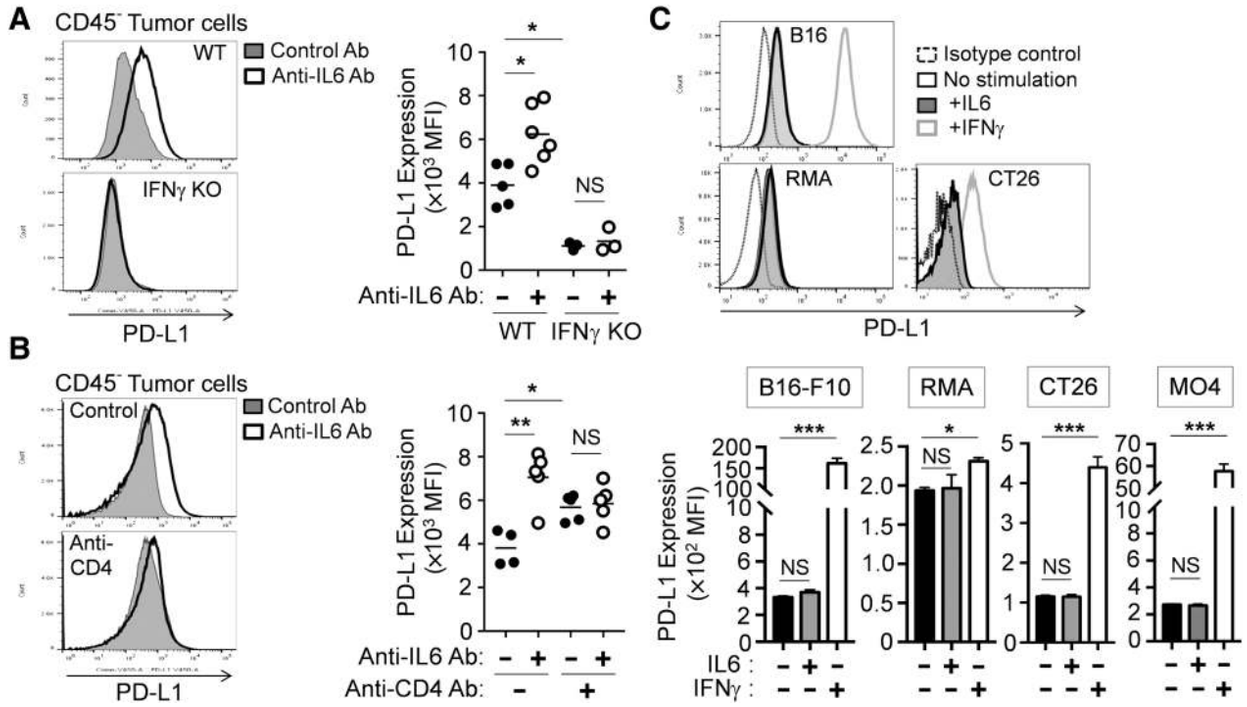

Figure 2.

IL6 blockade augments PD-L1 expression on tumor cells through $CD4^+$ T cell–derived IFNγ. **A** and **B,** Anti-IL6 Ab was injected twice into MO4-bearing WT, IFNγ-deficient (KO; **A**), or CD4-depleted mice (**B**) as in Fig. 1. Seven days after the first Ab injection, PD-L1 expression on $CD45^-$ tumor cells was analyzed. Representative histograms (left) and mean fluorescence intensity (MFI) from each mouse are shown. The values represent the mean with $n = 3$–6/group. **C,** B16-F10, RMA, CT26, and MO4 were cultured with or without recombinant IL6 (25 ng/mL) or IFNγ (50 ng/mL) for 48 hours. The expression of PD-L1 was analyzed. Representative histograms (top) and MFI (bottom) are shown. $n = 3$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. NS, not significant. The data are representative of three independent experiments.

or stimulated *in vitro*. PD-L1 blockade under *in vitro* culture of tumor tissues elicited IL6 production in $Gr\text{-}1^-$ cells, but not in $Gr\text{-}1^+$ populations (Fig. 4D). A large part of these IL6-producing cells were $F4/80^+$ cells, which were not detected in tumor-bearing IL6-deficient mice even when stimulated with LPS (Supplementary Fig. S2B). Although a substantial frequency of $IL6^+$ cells was spontaneously detected in $PD\text{-}1^-Gr\text{-}1^-CD11b^+$ cells, the augmentation of IL6 production in response to PD-L1 blockade was more pronounced in $PD\text{-}1^+Gr\text{-}1^-CD11b^+$TAMs, suggesting this population was the major responder to PD-L1 blockade in the tumor microenvironment. Conversely, as shown in Fig. 4E, stimulation of PD-1 on $Gr\text{-}1^-CD11b^+$TAMs with recombinant PD-L1 significantly downregulated the expression of IL6, but did not alter the expression of other inflammatory cytokine, TNFα. The PD-1 ligation-mediated suppression of IL6 production was reproducible in TAMs from CT26-bearing mice (Supplementary Fig. S3A). PD-1 stimulation seemed to decrease *Il1b* mRNA expression in TAMs from MO4, but not significantly reduced its production in TAMs from MO4 or CT26. Furthermore, we examined the functional consequence of PD-1 ligation in TAMs, particularly on $CD4^+$ T-cell responses. When the culture supernatant of PD1-stimulated TAMs was added to the culture of $CD4^+$ T cells stimulated with anti–CD3/CD28 Abs *in vitro*, the development of IFNγ-producing T cells and IFNγ/IL2-double producers was significantly improved, compared with $CD4^+$ T cells treated with the supernatant from control TAMs (Supplementary Fig. S3B and S3C). This impaired Th1 differentiation was rescued by IL6 blockade *in vitro*, suggesting that PD-1 ligation modulates TAM-derived IL6 that suppresses the Th1 development.

To more precisely evaluate the *in vivo* role of TAMs in PD-L1 blockade–induced upregulation of IL6, the IL6 levels were assessed when tumor-infiltrating $Gr\text{-}1^-CD11b^+$ macrophages including $PD\text{-}1^+$TAMs were depleted by anti-F4/80 Ab (Fig. 5A). Depletion of macrophages constrained PD-L1 blockade–induced upregulation of IL6 in the tumor microenvironment (Fig. 5B; Supplementary Fig. S3D), supporting the result that IL6 production from TAMs was suppressed by PD-1 ligation. The expression of *Il4* and *Il1b* but not *Tnfa* or *Il10* induced by anti–PD-L1 therapy was also diminished by macrophage depletion. Focusing on T-cell responses, the number and function of tumor-infiltrating $CD8^+$ T cells enhanced by anti–PD-L1 therapy were not affected when macrophages were depleted in MO4 model (Fig. 5C). On the other hand, in CT26-bearing mice, PD-L1 blockade augmented the function of $CD8^+$ T cells only when macrophage was depleted (Fig. 5D). The difference in the responses of $CD8^+$ T cells between these two tumor models might be reflective of their distinct susceptibilities to the PD-L1 blockade (Supplementary Fig. S4A). Notably, although treatment with anti–PD-L1 Ab alone did not efficiently elicit the IFNγ-producing $CD4^+$ T cells, depletion of macrophages increased IFNγ-producing $CD4^+$ T cells in response to PD-L1 blockade in both models (Fig. 5C–E), which was consistent with *Ifng* expression in the tumor tissues (Fig. 5B) and *in vitro* Th1 inhibition mediated by TAM-derived IL6 (Supplementary Fig. S3B and S3C). In such

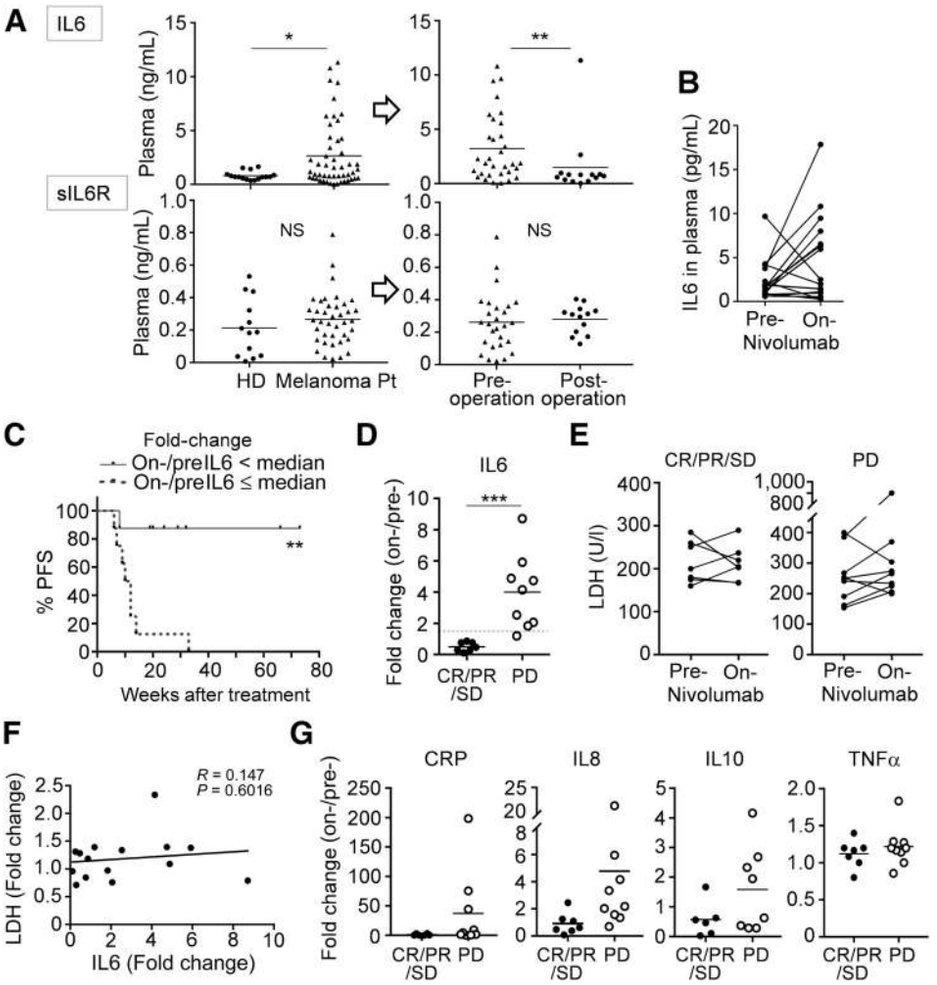

Figure 3.

Changes in plasma IL6 level during the treatment are associated with responsiveness to nivolumab in patients with melanoma. **A,** Levels of IL6 and sIL6R in plasma from patients with melanoma ($n = 42$–46) or healthy donors (HD) older than 50 years ($n = 16$) were analyzed (left). IL6 levels were further analyzed before ($n = 34$) and after ($n = 18$) surgical resection of tumor mass (right). **B,** Changes of IL6 level in plasma from the patients before and 12 weeks after initial treatment of nivolumab were analyzed ($n = 16$). **C,** Fold changes in IL6 levels (on-/pre-IL6) were analyzed and the patients were divided into two groups according to their median value [less ($n = 8$) or more ($n = 8$) than 1.51]. PFS of each group was analyzed over time. **D,** Patients were divided on the basis of their clinical responses (CR, PR, SD, vs. PD), and the fold changes in IL6 were plotted. **E** and **F,** LDH levels were measured before and during nivolumab treatment (**E**). The correlation between the changes in IL6 and LDH is shown (**F**). **G,** Fold changes of the indicated factors were analyzed and the values were divided into two groups based on the clinical responses. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. NS, not significant.

situation, exogenous administration of IL6 largely diminished this Th1 induction, but did not alter the frequency of tumor-infiltrating $CD4^+$ T cells. Furthermore, the responses of $CD8^+$ T cells had a propensity to be decreased by additional IL6 stimulation, which was emphasized in CT26-bearing mice (Fig. 5D). This effect also might be due to, in part, the depletion of immunosuppressive $F4/80^+$ monocytic MDSCs (33), although this possibility was not addressed in these models. Nonetheless, these data suggest that PD-L1 blockade attenuates Th1 response partly through enhancing the production of IL6 from TAMs.

Combined blockade of IL6 and PD-L1 signalings exerted synergistic antitumor effects

IL6 blockade might facilitate PD-1/PD-L1–mediated immunosuppression as an adaptive immune-resistant mechanism for tumor cells through contradictorily promoting Th1 responses (Fig. 2). In contrast, PD-L1 blockade reinforced the attenuation of Th1 responses through TAM-derived IL6 (Fig. 5). On the basis of these findings, we hypothesized that anti-IL6 Ab treatment combined with PD-L1 blockade elicited synergistic antitumor effects. Consistent with this hypothesis, the combination of IL6 and PD-L1 blockade achieved a significant reduction in growth of MO4 and CT26 compared with the single treatment (Fig. 6A; Supplementary Fig. S4A). The synergistic effect of IL6/PD-L1 blockades on MO4 progression was abrogated when $CD4^+$ T cells were depleted (Fig. 6B), suggesting a substantive contribution of $CD4^+$ T cells to this synergistic effect. On the other hand, the effect of anti–PD-L1 Ab alone was not abrogated by CD4 depletion. In contrast to the results from MO4 and CT26, RMA- or B16-F10–bearing mice were refractory to these therapies (Supplementary Fig. S4B and S4C), which might be due to the resistance to PD-1/PD-L1 blockade with their less immunogenicity and hypoxic environment (15, 34).

We also explored whether the combination therapy altered the responsiveness of tumor-infiltrating T cells in MO4 (Fig. 6C and D) and CT26 (Supplementary Fig. S4D)-bearing mice. PD-L1 blockade alone promoted infiltration and IFNγ production of $CD8^+$ T cells within the tumor. However, this was not observed for $CD4^+$ T cells, as demonstrated previously (14, 15). The combined therapy did not increase the frequency of infiltrating $CD4^+$ T cells, but elicited the qualitative change into IFNγ-producing Th1 cells (Fig. 6C and D). Efficient induction of $CXCR3^+CD4^+$ T cells in tumor-draining lymph nodes was reconciled by the enhanced Th1 response, whereas the frequencies of $Foxp3^+$Tregs were not alerted by the combined therapy (Fig. 6E).

Furthermore, we analyzed the intratumoral expression of T cell–attracting chemokines and found in both MO4 and CT26 models that expression of *Ccl3/4/5* and *Cxcl9/10* were

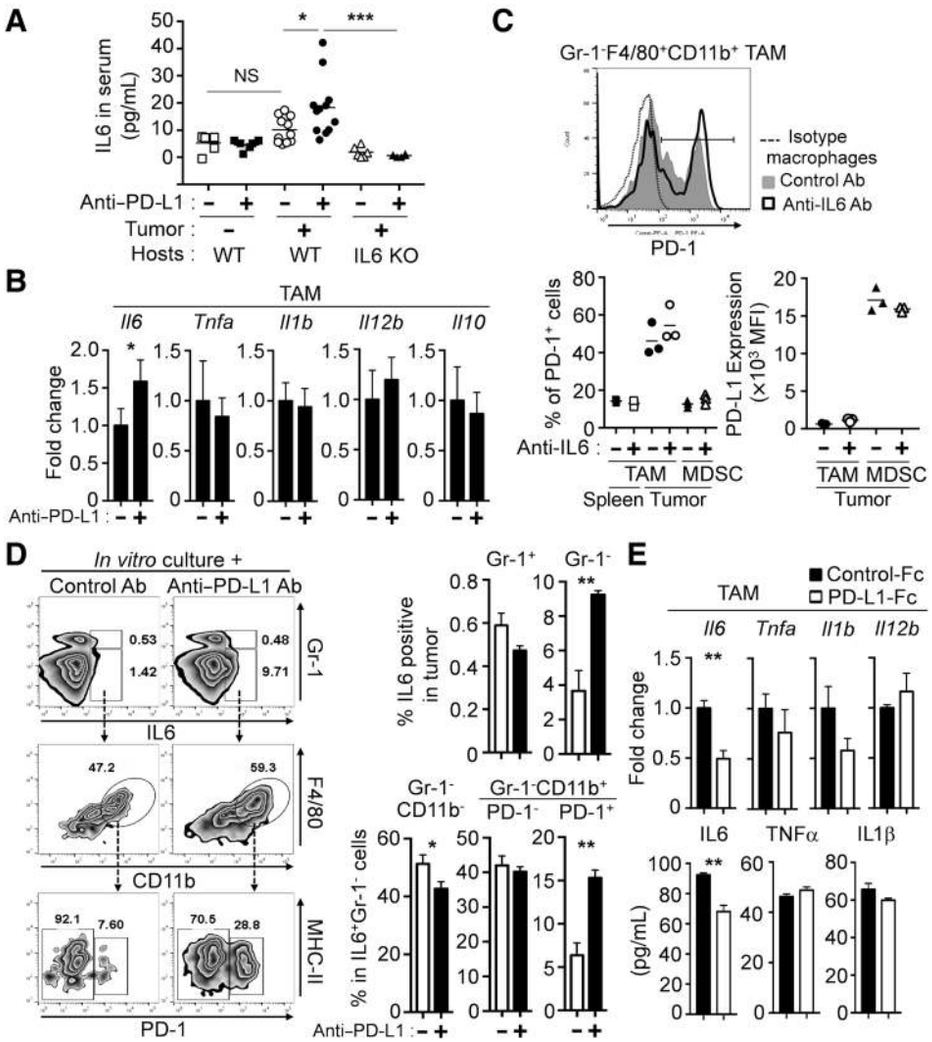

Figure 4.

PD-L1 blockade elicits IL6 production from tumor-associated macrophages. **A** and **B,** Tumor-free, MO4-bearing WT, or IL6-deficient (KO) mice were treated with anti–PD-L1 Ab. Two days later, IL6 concentration in serum was measured (**A**). Expression of the indicated mRNA in isolated TAMs was analyzed (**B**). **C,** PD-1 expression on $CD11b^+F4/80^+Gr\text{-}1^-$ TAMs or $CD11b^+Gr\text{-}1^+$ MDSCs from tumor tissues or spleen was assessed 10 days after melanoma inoculation. Representative histograms are shown (top). PD-L1 expression was also analyzed (bottom right). **D,** Cell suspension from MO4 tumors was cultured *in vitro* in the presence or absence of anti–PD-L1 Ab for 18 hours. IL6-producing cells were analyzed by flow cytometric analysis. Representative plots (left) and the frequencies of indicated $IL6^+$ population in the culture (top right) and percentage of indicated population in $IL6^+Gr\text{-}1^-$ fraction (bottom right) are shown. **E,** $CD11b^+Gr\text{-}1^-$ TAMs were isolated from tumor tissues and stimulated with plate-coated control-Fc or PD-L1-Fc *in vitro*. Expression of the indicated mRNA (top) and cytokines in the supernatants (bottom) was assessed by qPCR and ELISA, respectively. Three independent experiments were performed, and the values represent the mean with $n = 5$–12 per group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. NS, not significant.

preferentially enriched in tumors by the treatment with anti-IL6 Ab and anti–PD-L1 Ab, respectively (Fig. 6F; Supplementary Fig. S4E). Of note, the combined therapy induced vigorous increases in all of them. These chemokine expressions were closely correlated with the optimal T-cell recruitment and the synergistic antitumor effects of combined blockade of IL6 and PD-1/PD-L1 signaling. In addition, as shown in Fig. 6G, the combined therapy–induced expression of Ccl4/5 was significantly impaired by CD4 depletion, supporting the importance of Th1 responses in the therapeutic benefits of this combined therapy. Expression of *Cxcl10* was conversely upregulated by CD4 depletion, which might be due to the abolishment of Treg-mediated inhibition.

Discussion

Coherent immunologic biomarkers for predicting the efficacy of anti–PD-1/PD-L1 therapy are needed even during the treatment because some cases show delayed responses and pseudoprogression of the tumor mass (29). In this initial study involving a limited number of patients, increased IL6 levels were associated with decreased susceptibility to PD-1 blockade in patients with melanoma. Thus, we proposed the possibility that augmentation of circulating IL6 levels during anti–PD-1 therapy could help estimate whether patients with melanoma are at high risk of disease progression. Similar to this, lower levels of IL6 were associated with longer survival of patients with melanoma treated with anti–CTLA-4 Ab (35). CRP, a signature of inflammation and direct target of IL6 signaling (36), has been reported to be associated with the clinical outcomes in patients with melanoma (37) as well as LDH (32). However, in nivolumab-treated patients, a strict correlation between their clinical responses and the levels of CRP or LDH was not observed. Thus, it is anticipated that the prognostic value of the change in plasma IL6 levels for predicting the susceptibility to PD-1/PD-L1 blockade reflects immunosuppressive status rather than mere inflammatory environment or tumor burden.

Intriguingly, an alteration of IL6 during treatment, rather than its baseline level was correlated with the poor clinical response to PD-1 blockade. It is rather conceivable that, as compared with the quiescent "cold" situation with little spontaneous antitumor immune responses in nontreated tumors, the efficacy of anti–PD-1/PD-L1 therapy more strongly mirrors the immunologic (immunostimulatory vs. immunosuppressive) status at the "hot"

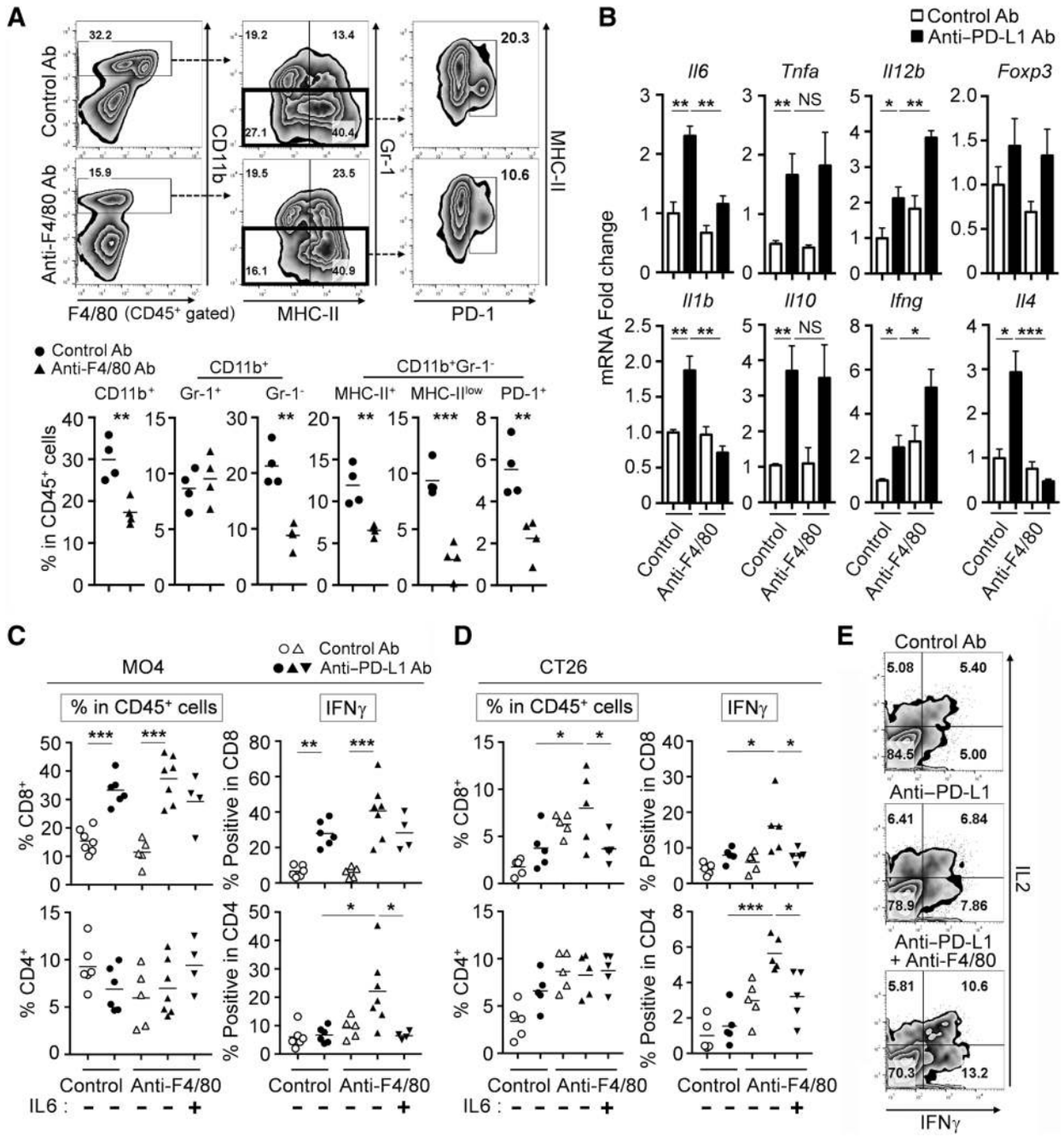

Figure 5.

PD-L1 blockade-stimulated IL6 induction was mediated through TAMs in melanoma-bearing mice. **A,** MO4-bearing mice were treated with control or anti-F4/80 Ab. Representative plots (top) and the frequencies of indicated tumor-infiltrating populations in $CD45^+$ cells (bottom) are shown. **B,** MO4-bearing mice were treated with anti-F4/80 and anti–PD-L1 Abs. Three days after injecting Abs twice a week, expression of the indicated mRNA in tumor tissues was assessed. **C–E,** Ab injections were performed and MO4 (**C** and **E**) or CT26 (**D**) tumor tissues were analyzed as in **B**. Recombinant IL6 was injected 1 day before anti–PD-L1 treatment. Frequencies of tumor-infiltrating T cells and their IFNγ production were analyzed (**C** and **D**). Representative plots for IFNγ-producing cells in gated $CD4^+$ T cells are shown (**E**). Two or three independent experiments were performed, and the values represent mean with $n = 4$–7 per group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. NS, not significant.

circumstance when dramatic immune reactions such as tumor killing through effector CTLs recovered from exhaustion, an increase in tumor antigen–engulfing DCs, and further priming of tumor-specific T cells are elicited (4). Therefore, in such situations, the immunosuppressive effect of IL6 induced by various immune reactions on $CD4^+$ T cells is likely to become underscored. In addition to a requirement of further analysis of the IL6 levels in patients treated with other PD-1/PD-L1 blockade

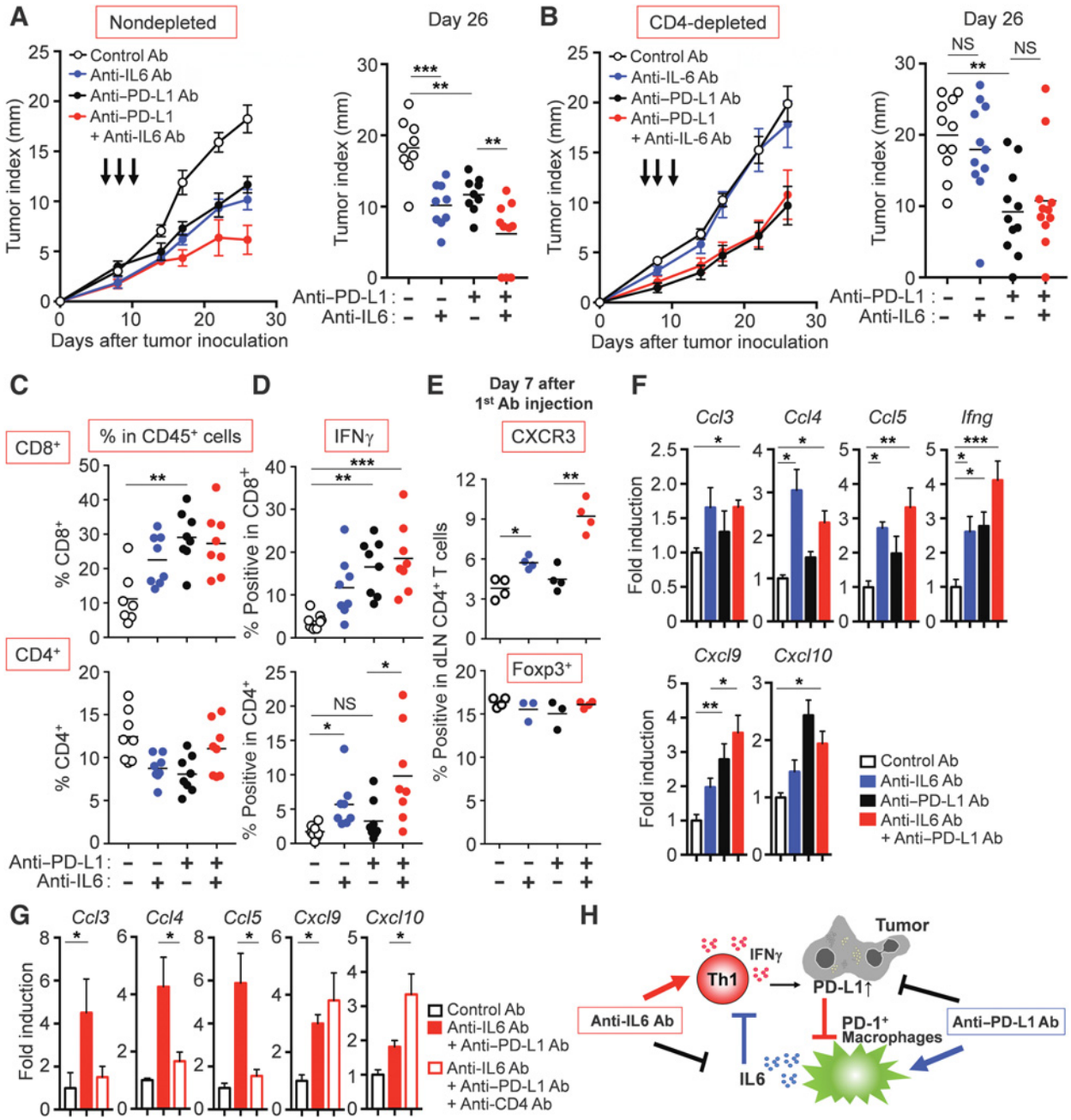

Figure 6.

Combined blockade of IL6 and PD-L1 signals elicits synergistic antitumor immune responses. **A** and **B,** Control (**A**) or CD4-depleted (**B**) MO4-bearing mice were treated with anti-IL6 and/or anti–PD-L1 Abs three times (arrows). Tumor progression was monitored over time (left). Tumor sizes at the endpoint are also shown (right). **C–F,** Seven days after the first Ab injection, frequencies of $CD8^+$ and $CD4^+$ T cells in tumor-infiltrating $CD45^+$ cells (**C**), their IFNγ-producing cells (**D**), and the mRNA expression of indicated chemokines in tumors (**F**) were analyzed. CXCR3 and Foxp3 expression in $CD4^+$ T cells from tumor-draining lymph node cells was also analyzed (**E**). **G,** Tumors from CD4-depleted tumor-bearing mice with combined therapy were analyzed for the mRNA expression of indicated chemokines. The values represent the mean with $n = 3$–9/group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. NS, not significant. The data are representative of two independent experiments. **H,** Schematic representation of reciprocal interaction between IL6-mediated attenuation of Th1 response and PD-1/PD-L1 ligation on TAMs.

reagents such as atezolizumab, it remains to be investigated the optimal and earliest time point for detecting the upregulation of IL6 levels in patients with cancer after starting treatment and before 12 weeks of anti–PD-1 therapy. An earlier evaluation of treatment efficacy and prompt identification of treatment-sensitive patients can help to avoid unnecessary prolonged

treatment, thus limiting the costs and giving the other treatment options.

We demonstrated here that in tumor-bearing hosts, targeting immunosuppressive effects of IL6 potentiated the qualitative but not quantitative changes of $CD4^+$ T cells, particularly in the context of Th1 response–mediated antitumor immunity. Considering the differentiation from naïve into effector T cells, newly generated neoantigen-specific $CD4^+$ T cells against mutated melanoma may be more sensitive to the suppressive effect of IL6 (38). However, IL6 blockade alone did not efficiently control the tumor growth, as observed for DC immunization combined with IL6 blockade (Fig. 1; refs. 8, 21). Consistent with our mouse model, a large randomized clinical trial with single use of anti-IL6 Ab, CNTO328 showed few clinical benefits in patients despite full inhibition of CRP levels (20). One possible mechanism that limited the effectiveness of IL6 blockade was the immunosuppression via upregulation of PD-L1 on tumor cells. Although IFNγ expression is associated with better prognosis (10), IL6 blockade–induced Th1 skewing of tumor-specific $CD4^+$ T cells and their IFNγ production caused a contradicting effect of PD-1/PD-L1–mediated immunosuppression, which is considered to be an adaptive resistant mechanism of tumor cells in response to immune activation including IFNγ production (39). In such a situation without exogenous strong interventions such as active immunization with DCs, IL6 blockade appeared to be insufficient for inducing functional antitumor immunity.

Across multiple cancer types, clinical benefits from PD-1/PD-L1 blockade are frequently observed in patients with high PD-L1 expression during the course of cancer progression (5, 6). PD-L1 induction in tumor cells by IL6 blockade fitted with these observations, because preconditioning of IL6 in tumor-bearing mice boosted the better responsiveness to the PD-1/PD-L1 blockade and facilitated Th1 differentiation, leading to a significant delay in tumor growth. In addition, recent finding that higher MHC-II expression on melanoma cells was correlated with the better effectiveness of anti–PD-1 therapy (40) is reminiscent of an important role of MHC-II–mediated $CD4^+$ T-cell activation in increasing the susceptibility to anti–PD-1/PD-L1 therapy. Furthermore, a reproducible increase in circulating IL6 was associated with the development of pathologic immune-related adverse events (irAE) in anti–PD-1 therapy (24, 25). Thus, this study may pave the way for a promising rational treatment with anti-IL6/R Ab not only to provide better management of anti–PD-1 therapy-associated irAEs, but also to properly recover from immunosuppressive status in patients with anti–PD-1/PD-L1 therapy-resistant cancers.

Monotherapy with anti–PD-1 Ab is not sufficient for enhancing the $CD4^+$ T cell–mediated Th1 response *in vivo* (14, 15), while PD-1 blockade was reported to promote the Th1 response *in vitro* (16, 17). On the other hand, a recent study, as well as our results, demonstrated that combination of anti-IL6 Ab treatment along with PD-L1 blockade triggered the synergistic antitumor activity (22, 41, 42). However, the detailed mechanistic actions were not fully elucidated. Here, we proposed that IL6-mediated immunosuppression functioned as a rheostat modulating antitumor Th1 responses in tumor-bearing hosts during anti–PD-1/PD-L1 therapy (Fig. 6H). The limitation of anti–PD-1 therapy in eliciting Th1 response was accounted for by macrophage-derived IL6 production in tumor microenvironment, because the depletion of macrophages allowed the PD-L1 blockade to stimulate local Th1 responses in an IL6-dependent manner. In general, macrophages are exposed to various stimuli from the tumor microenvironment such as tumor-derived ligands for Toll-like receptors (43, 44) or other inflammatory cytokines, IL1β and IL17 (45), which can render TAMs to produce inflammatory mediators including IL6. However, our data suggested the possibility that an ectopic expression of PD-1 on TAMs and its ligation with PD-L1 directly suppressed their IL6 production in tumor microenvironment. In addition to the direct effect, PD-1/PD-L1 blockade might indirectly dampen the IL6 upregulation through modification of the property to produce IL6 not only in $PD\text{-}1^+$TAMs but also in $PD\text{-}1^-$TAMs with unknown mechanism(s), because the total frequency of IL6-producing $PD\text{-}1^-$TAMs was also increased upon PD-L1 blockade (Supplementary Fig. S3D). Thus, the depletion of both $PD\text{-}1^+$ and $PD\text{-}1^-$TAMs could contribute to the amelioration in T-cell function in tumor microenvironment. These ideas propose a novel function of PD-1/PD-L1 signal in TAMs and provide a possible explanation for the mechanistic action of PD-L1 blockade to mobilize macrophages for immunosuppression. Although this possible mechanism was supported by the escalation of IL6 levels during nivolumab treatment, it should be assessed whether IL6 production in human $PD\text{-}1^+$TAMs is liberated from the suppression via PD-1–PD-L1 interaction in cancer specimens in further investigation. It is interesting to note that $PD\text{-}1^+$TAMs expressed M2 macrophage marker CD206 (Supplementary Fig. S2A; ref. 18). Therefore, a detailed characterization of IL6-producing human TAMs may help to explain the poor prognostic role of M2-like macrophages in patients with melanoma (46).

An increase in IL6 is often observed at baseline in patients with cancer and tumor-bearing mice (9, 20, 21). As demonstrated in Fig. 4D, $PD\text{-}1^-$TAMs also appeared to contribute to spontaneous production of IL6 in tumor tissues. This idea was supported by the observation that depletion of macrophages reduced the baseline level of IL6. Hence, it is reasonable to assume that in contrast to the therapy-induced inflammation, other types of tumor-associated cells, such as MDSCs (21), cancer-associated fibroblasts (42), and pericytes (47), are responsible for the steady-state measurable level of IL6. Therefore, these cells are likely candidates for preconditioning of the tumor microenvironment through amelioration of baseline immunosuppression before therapeutic approaches including immune checkpoint blockade (48).

Although Th1 response mediated the interplay between tumor cells and TAMs, the fundamental mechanism(s) underlying how Th1 cells can contribute to antitumor responses during anti–PD-1/PD-L1 therapy is worth considering. Although anti–PD-1 therapy alone seemed to be sufficient to potentiate the recruitment of $CD8^+$ T cells in early phase of the therapy in MO4 but not in CT26 model, the restoration of defective Th1 development via additional IL6 blockade or macrophage depletion led to a synergistic enhancement of $CD8^+$ T-cell response to a greater or lesser extent in both models. Thus, it was likely possible that the combined blockade of IL6 and PD-1/PD-L1 signals provided the synergistic effects not only on $CD4^+$ Th1 response but also on the recruitment and function of $CD8^+$ T cells in the tumor microenvironment. This idea was also supported by IL6 blockade–mediated and CD4-dependent upregulation of *Ccl3/4/5* expression in the combined therapy, and the previous report demonstrating that $CD4^+$ T-cell/DC interaction–induced CCL3/4 promoted the recruitment and priming of cognate $CD8^+$ T cells (49). $CD4^+$ Th1 cell–mediated enhancement of memory

$CD8^+$ T-cell formation and their durable response (10, 49) or counteracting the IL4 (Th2)-skewed immunosuppressive environment (9, 50) are the other possible targets of Th1 cells in the synergistic antitumor effects.

In conclusion, PD-1/PD-L1 blockade fostered vigorous IFNγ-producing T-cell responses when IL6 blockade was given, and ameliorated the immunosuppressive environment governed by tumor cells and TAMs, providing an optimal immunologic window for the treatment. These findings shed light on the complexity of the modes of action of anti–PD-1/PD-L1 therapy and suggest a promising and feasible combined therapeutic approach targeting the mutually immunosuppressive cross-talk between PD-1/PD-L1 and IL6 signals.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Tsukamoto, K. Fujieda, A. Miyashita, S. Fukushima, T. Ikeda, Y. Kubo, S. Senju, H. Ihn
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Tsukamoto, K. Fujieda, S. Fukushima, T. Ikeda, H. Oshiumi
Writing, review, and/or revision of the manuscript: H. Tsukamoto, K. Fujieda, S. Fukushima, T. Ikeda, S. Senju, H. Ihn, Y. Nishimura, H. Oshiumi
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Tsukamoto
Study supervision: H. Tsukamoto, H. Oshiumi

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