Combined blockade of IL-6 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 pro-inflammatory cytokine interleukin (IL)-6 enhances tumor-specific Th1 responses and subsequent anti-tumor effects in tumor-bearing mice. IL-6 blockade upregulated expression of the immune checkpoint molecule programmed death-ligand 1 (PD-L1) on melanoma cells. This PD-L1 induction was canceled in IFN-γ-deficient mice or CD4+ T cell-depleted mice, suggesting that CD4+ T cell-derived IFN-γ 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 IL-6, which was associated with poor clinical responses. This PD-L1 blockade-evoked induction of IL-6 was reproducible in melanoma-bearing mice. We found that PD-1/PD-L1 blockade prompted PD-1+ macrophages to produce IL-6 in the tumor microenvironment. Depletion of macrophages in melanoma-bearing mice reduced the levels of IL-6 during PD-L1 blockade, suggesting macrophages are responsible for the defective CD4+ Th1 response. Combined blockade of the mutually regulated immunosuppressive activities of IL-6 and PD-1/PD-L1 signals enhanced expression of T cell-attracting chemokines and promoted infiltration of IFN-γ-producing CD4+ T cells in tumor tissues, exerting a synergistic anti-tumor effect, while PD-L1 blockade alone did not promote Th1 response. Collectively, these findings suggest that IL-6 is a rational immunosuppressive target for overcoming the narrow therapeutic window of anti-PD-1/PD-L1 therapy.
Introduction

Melanoma is one of the leading causes of cancer mortality. Surgery, radiotherapy, and/or systemic therapies including targeted drugs offers a chance for cure in patients with early-staged melanoma, but the vast majority of patients with advanced or metastatic diseases is 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 anti-tumor immune response is relatively weak because of the detrimental effects of immunosuppressive factors or cells such as regulatory T cells (Tregs), tumor-associated macrophages (TAMs), and myeloid-derived suppressor cells (MDSCs) (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 (Abs) that disrupts this interaction has provided dramatic objective response rates ranging from 30 to 40 % in patients with advanced melanoma (4-6). However, while 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 does 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 non-responders.

Many comprehensive studies have shown that IFN-γ-producing CD4+ Th1 cells exert a critical role in anti-tumor responses (7-10) and thus their infiltration into tumor tissue is an indicator of better prognosis (11). In contrast, cancer patients 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 anti-tumor Th1 responses in vivo (14,15), although cytotoxic activity, proliferation, and IFN-γ production in both CD8⁺ and CD4⁺ T cells were recovered by inhibiting the 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 rational-based strategy for improving its therapeutic efficacy.

Inflammation is closely linked to the prognosis of cancer patients. Chronically elevated levels of pro-inflammatory cytokine, interleukin (IL)-6, which promotes tumor cell survival, is a poor prognostic factor in patients with many types of cancer including melanoma (9,19,20). Hence, a therapeutic approach for IL-6 blockade using humanized IL-6/IL-6R Abs has been developed to abrogate its direct effect on tumor growth/survival (20). Additionally, tumor cell-extrinsic effects of IL-6 have been demonstrated in anti-tumor immune responses through myeloid-lineage cells and T cells (8,9,21,22). Furthermore, the higher level of IL-6, 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 anti-tumor immunological relevance of inflammation in such potent immunotherapies remains unclear.

In this study, we found that anti-IL-6 Ab treatment augmented Th1 responses, but in turn, induced up-regulation of PD-L1 expression on melanoma cells through CD4⁺ T-cell-derived IFN-γ. On the other hand, treatment with anti-PD-L1 Ab prompted TAMs to
produce IL-6 counteracting Th1 responses in melanoma-bearing mice. Consistent with this, vigorous increase of circulating IL-6 was observed in a certain population of melanoma patients treated with anti-PD-1 therapy, which was associated with a poor clinical response to this therapy. These findings suggest that combined blockade of IL-6 signaling and PD-1/PDL1 pathways disrupts the mutual “see-saw” interplay between these immunosuppressive events, resulting in synergistic anti-tumor effects.
Materials and Methods

Mice, tumor cells, and Ab treatment

Male C57BL/6NCrSlc and Balb/cCrSlc mice were purchased from Japan SLC, Inc. IL-6-deficient mice were obtained from The Jackson Laboratory. All the mice including IFN-γ-deficient mice (26) were housed at the Center for Animal Resources and Development, Kumamoto University, 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 (SSLP) or isozyme analysis and provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer (Tohoku University), and RIKEN BRC Cell Bank, respectively. Ovalbumin (OVA)-expressing melanoma MO4 (27) were kindly provided from Dr. Kenneth L. Rock (Department of Pathology, UMass Medical School). RMA lymphoma (9,28) was kindly provided from Dr. Akira Shibuya (Department of Immunology, University of Tsukuba). 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, tumor formation in syngeneic mouse strain provide evidence of correct cell identity. Mice were inoculated subcutaneously with $3 \times 10^5$ B16-F10, CT26, MO4, or RMA. Tumor size was expressed as tumor index, which is the square root of (length × width) (21). Two hundred micrograms of control IgG Ab (Millipore), anti-IL-6 (MP5-20F3, BioXCell),
and/or anti-PD-L1 Abs (10F.9G2, BioXCell) 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. Two hundred micrograms of anti-F4/80 (CI:A3-1, BioXCell) 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 body weight 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 Response Evaluation Criteria in Solid Tumors 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 Table 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 min. Resulting single cell suspensions were analyzed. For TAMs isolation, tumor cells were removed from the above cell suspensions using Lymphoprep (Axis Shield), and then CD11b⁺Gr-1⁻ macrophages were purified using CD11b microbeads (Miltenyi Biotec) after removing Gr-1⁺ cells with Gr-1 microbeads (Miltenyi Biotec). TAMs (5 × 10⁵) 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 (LNs), 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-2Kᵇ/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-IL-2 or anti-IFN-γ Ab (TONBO) as described previously (8). For the assessment of IL-6-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 Breferdin A (Sigma) for 18 hours. After cell surface staining, intracellular IL-6 was stained with anti-IL-6 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 LN cells and 3 × 10^4 bone marrow-derived dendritic cells (DC) pulsed with I-A^b-binding OVA peptide (ISQAVHAAHAEEAGR; OVA-IIp) were mixed and incubated for 12 hours. IFN-γ spots were visualized with ELISPOT assay kit (BD Biosciences) and analyzed as previously described (8). ELISA kits for detecting human and mouse IL-6, soluble IL-6 receptor (sIL-6R), IL-1β, and TNF-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 (QIGEN), and reverse-transcribed with ReverTra Ace (TOYOBO). Real-time quantitative PCR (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: \( \text{Ccl4} \)

5‘-CCAGGGTTCTCAGCACCA-3’ and 5‘-GCTCACTGGGTTAGCACAGA-3’; 

\( \text{Ccl5} \)

5‘-CTCACCATATGGCTCGGACA-3’ and 5‘-CTTCTCTGGGTTGCGACACA-3’; 

\( \text{Cxcl9} \)

5‘-TGGAGTTCGAGAACCCTAGT-3’ and 5‘-AGGCAGGTTTGATCTCCGTG-3’; 

\( \text{Cxcl10} \)

5‘-ACGAACTTAACCACCACATCT-3’ and 5’-TAAACTTTAACTACCATTGACATA-3’; 

\( \text{Cxcl11} \)

5‘-AGGAAGGTACAGCCCATAGC-3’ and 5’-CGATCTCTGCGATTTTGACG-3’.

Expression of each gene was normalized to \( \text{Gapdh} \) expression using the comparative 2\([\Delta\DeltaCT]\) 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 IL-12 (8 ng/mL; Wako) with or without anti-IL-6 Ab (1 μg/mL). After the culture for 7 days, IFN-γ production or expansion of effector T cells was analyzed.

**Statistical analysis**

To ascertain a normal distribution of variables, Shapio-Wilk’s 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 group in Kaplan-Meier plots. Cox proportional hazards regression was applied to investigate the relationship between IL-6 levels and PFS. Data were also analyzed using unpaired Student’s t-test when comparing two experimental groups. Correlations between variables were determined by Spearman’s 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 significant.
Results

IL-6 blockade augmented Th1 responses and retarded melanoma progression

We previously demonstrated that in tumor-bearing mice immunized with tumor-associated cognate antigenic peptide-loaded DC, Th1 differentiation of adoptively transferred and in vivo primed tumor-specific CD4+ T cells were attenuated in an IL-6-dependent manner (9,21). This observation have evoked the possibility that development of IFN-γ-producing CD4+ Th1 cells from spontaneously primed endogenous tumor-specific CD4+ T cells is masked by IL-6 signal, which is augmented in tumor-bearing animals and cancer patients (19,20). To test this possibility, we first evaluated the beneficial effect of IL-6 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, IFN-γ-producing OVA peptide-specific CD4+ T cells were significantly increased by IL-6 blockade in tumor-draining LNs.

Consequently, melanoma growth was significantly retarded by IL-6 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-IL-6 Ab-treated mice (Fig. 1C). Furthermore, the recruitment of tumor (OVA)-specific CD8+ T cells at the tumor site was promoted by IL-6 blockade, which was constrained by the depletion of CD4+ T cells (Fig. 1C), suggesting a negative impact of IL-6 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 down-regulated by IL-6 blockade (Fig. 1D). Overall, these results confirmed that the immunosuppressive effect of IL-6 had a detrimental effect on spontaneous T cell-mediated anti-tumor responses by modulating the balance between Th1 and Th2 responses (9,13). The expression of Treg-associated markers, Foxp3 and Il10, were not modulated in the tumor microenvironment after treatment with anti-IL-6 Ab (Fig. 1D).

**IL-6 blockade induced CD4- and IFN-γ-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-IL-6 Ab (Fig. 2A). Surprisingly, IL-6 blockade significantly up-regulated the PD-L1 expression on MO4 cells, which was completely abrogated in tumor-bearing IFN-γ-deficient mice. Given that CD4+ T cells were potent IFN-γ producers in response to IL-6 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-IL-6 Ab-mediated PD-L1 up-regulation on tumor cells. Consistent with the in vivo results, in vitro stimulation with IFN-γ robustly induced PD-L1 up-regulation 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 IL-6 stimulation, excluding the possibility that IL-6 directly affected PD-L1 expression. Collectively, these results suggest that IL-6 blockade indirectly augments the PD-L1 induction on melanoma via CD4+ T cell-derived IFN-γ.

**Change in the level of IL-6 reflected the therapeutic efficacy of anti-PD-1 Ab treatment**


As observed in other types of cancer, patients with melanoma exhibited a higher level of IL-6 in plasma compared to that in healthy donors (Fig. 3A). However, the levels of IL-6 were decreased after surgical removal of primary melanoma. In contrast, the level of soluble IL-6 receptor (sIL-6R), the other component of IL-6 signaling was not altered. We next validated the plasma levels of IL-6 during treatment with anti-PD-1, Nivolumab for 12 weeks in melanoma patients 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 IL-6 during Nivolumab treatment, while IL-6 levels were not changed or decreased in other patients.

To examine whether the elevated IL-6 levels were associated with tumor progression in individual patients, PFS was assessed based on stratification by the fold-change in IL-6 levels during Nivolumab treatment (On-/Pre-treatment, median value; 1.516). As shown in Fig. 3C, the patients with increased IL-6 level (On-/Pre-treatment IL-6 ≥ 1.516) exhibited a shorter PFS compared to patients whose IL-6 levels were not increased (higher group; median PFS 11 weeks, 95% 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 IL-6 concentration (median value; 1.64 pg/mL, Supplementary Fig. S1A and B). Consistent with the result of PFS, poor clinical responses were associated with greater increase in IL-6 levels, while the change in IL-6 level was modest (< 1.516) in patients achieving disease control (Fig. 3D). Cox regression analysis indicated that patients with large increases in IL-6 were at high risk for poor clinical
responses (Hazard ratio; 13.6, 95% confidence intervals; lower 1.67, upper 110.8), suggesting that an increased IL-6 level serves as a predictive factor for poor PFS and clinical response in melanoma patients 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 IL-6 (Fig. 3F), suggesting that the increased IL-6 in non-responders was not simply reflected by the tumor burden. The levels of CRP or IL-8, 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 IL-10 or TNF-α (Fig. 3G). Taken together, these results imply that an increase in IL-6 during PD-1/PD-L1 blockade is correlated with the therapeutic responsiveness of melanoma patients.

**Blockade of PD-1-PD-L1 interaction led to IL-6 production by tumor-associated macrophages**

We further investigated the mechanistic action of IL-6 up-regulation during anti-PD-L1 Ab treatment in melanoma-bearing mice. Although a large increase in IL-6 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 IL-6 levels in wild-type (WT) mice (Fig. 4A). This model recapitulated some of the anti-PD-1 Ab-treated melanoma patients (Fig. 3B). However, such IL-6 induction was not observed in IL-6-deficient counterparts, suggesting that IL-6 was produced by host-derived cells but not
by melanoma cells in response to PD-L1 blockade. PD-L1 blockade-induced IL-6 up-regulation was reproducibly detected in isolated TAMs (Fig. 4B), suggesting that TAMs are one of the possible cellular source of IL-6 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-1^{-}F4/80^{+}CD11b^{+}TAMs during melanoma progression, while tumor-infiltrated Gr-1^{+}CD11b^{+}MDSCs or splenic Gr-1^{-}F4/80^{+}macrophages did not express PD-1 (Fig. 4C and Supplementary Fig. S2A). PD-1^{+}TAMs expressed the macrophage marker 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 IL-6 pathway in TAMs, the level of IL-6 was assessed in TAMs when PD-1/PD-L1 interaction was blocked or stimulated in vitro. PD-L1 blockade under in vitro culture of tumor tissues elicited IL-6 production in Gr-1^{-} cells, but not in Gr-1^{+} populations (Fig. 4D). A large part of these IL-6-producing cells were F4/80^{+} cells, which were not detected in tumor-bearing IL-6-deficient mice even when stimulated with LPS (Supplementary Fig. S2B). While a substantial frequency of IL-6^{+} cells was spontaneously detected in PD-1^{-}Gr-1^{-}CD11b^{+} cells, the augmentation of IL-6 production in response to PD-L1 blockade was more pronounced in PD-1^{+}Gr-1^{-}CD11b^{+}TAMs, suggesting this population was the major responder to PD-L1 blockade in tumor microenvironment. Conversely, as shown in Fig. 4E, stimulation of PD-1 on Gr-1^{-}CD11b^{+}TAMs with recombinant PD-L1 significantly down-regulated the expression of IL-6, but did not alter the expression of other inflammatory cytokine, TNF-α. The PD-1 ligation-mediated suppression of IL-6 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 PD-1-stimulated TAMs were added to the culture of CD4+ T cells stimulated with anti-CD3/CD28 Abs \textit{in vitro}, the development of IFN-\(\gamma\)-producing T cells and IFN-\(\gamma\)/IL-2-double producers were significantly improved, compared to CD4+ T cells treated with the supernatant from control TAMs (Supplementary Fig. S3B and C). This impaired Th1 differentiation was rescued by IL-6 blockade \textit{in vitro}, suggesting that PD-1 ligation modulates TAMs-derived IL-6 that suppresses the Th1 development.

To more precisely evaluate the \textit{in vivo} role of TAMs in PD-L1 blockade-induced up-regulation of IL-6, the IL-6 levels were assessed when tumor-infiltrating Gr-1-CD11b+ macrophages including PD-1+TAMs were depleted by anti-F4/80 Ab (Fig. 5A). Depletion of macrophages constrained PD-L1 blockade-induced up-regulation of IL-6 in tumor microenvironment (Fig. 5B and Supplementary Fig. S3D), supporting the result that IL-6 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 IFN-γ-producing CD4+ T cells, depletion of macrophages increased IFN-γ-producing CD4+ T cells in response to PD-L1 blockade in both model (Fig. 5C-E), which was consistent with $\text{Ifng}$ expression in the tumor tissues (Fig. 5B) and in vitro Th1 inhibition mediated by TAM-derived IL-6 (Supplementary Fig. S3B and C). In such situation, exogenous administration of IL-6 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 IL-6 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 MDSC (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 IL-6 from TAMs.

**Combined blockade of IL-6 and PD-L1 signalings exerted synergistic anti-tumor effects**

IL-6 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 IL-6 (Fig. 5). Based on these findings, we hypothesized that anti-IL-6 Ab treatment combined with PD-L1 blockade elicited synergistic anti-tumor effects. Consistent with this hypothesis, the combination of IL-6 and PD-L1 blockade achieved a significant reduction in growth of MO4 and CT26 compared to the single
treatment (Fig. 6A and Supplementary Fig. S4A). The synergistic effect of IL-6/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 was refractory to these therapies (Supplementary Fig. S4B and C), 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 IFN-γ 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 IFN-γ-producing Th1 cells (Fig. 6C and D). Efficient induction of CXCR3$^+$CD4$^+$ T cells in tumor-draining LNs was reconciled by the enhanced Th1 response, whereas the frequencies of Foxp3$^+$Tregs was 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 preferentially enriched in tumors by the treatment with anti-IL-6 Ab and anti-PD-L1 Ab, respectively (Fig. 6F and 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 anti-tumor effects of combined
blockade of IL-6 and PD-1/PD-L1 signaling. In addition, as shown in Fig. 6G, the combined therapy-induced expression of Ccl4/5 significantly impaired by CD4 depletion, supporting the importance of Th1 responses in the therapeutic benefits of this combined therapy. Expression of *Cxcl10* was conversely up-regulated by CD4 depletion, which might be due to the abolishment of Treg-mediated inhibition.
Discussion

Coherent immunological 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 pseudo-progression of the tumor mass (29). In this initial study involving a limited number of patients, increased IL-6 levels were associated with decreased susceptibility to PD-1 blockade in melanoma patients. Thus, we proposed the possibility that augmentation of circulating IL-6 levels during anti-PD-1 therapy could help estimate whether melanoma patients are at high risk of disease progression. Similar to this, lower levels of IL-6 were associated with longer survival of melanoma patients treated with anti-CTLA-4 Ab (35). CRP, a signature of inflammation and direct target of IL-6 signaling (36), has been reported to be associated with the clinical outcomes in melanoma patients (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 were not observed. Thus, it is anticipated that the prognostic value of the change in plasma IL-6 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 IL-6 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 to the quiescent “cold” situation with little spontaneous anti-tumor immune responses in non-treated tumors, the efficacy of anti-PD-1/PD-L1 therapy more strongly mirror the immunological (immune-stimulatory vs immunosuppressive) status at the “hot” circumstance when dramatic immune reactions such as tumor killing through effector CTL
recovered from exhaustion, an increase in tumor antigen-engulfing DC, and further priming of tumor-specific T cells are elicited (4). Therefore, in such situations, the immunosuppressive effect of IL-6 induced by various immune reactions on CD4+ T cells likely to become underscored. In addition to a requirement of further analysis of the IL-6 levels in patients treated with other PD-1/PD-L1 blockade reagents such as Atezolizumab, it remains to be investigated the optimal and earliest time point for detecting the up-regulation of IL-6 levels in cancer patients 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 IL-6 potentiated the qualitative but not quantitative changes of CD4+ T cells, particularly in the context of Th1 response-mediated anti-tumor immunity. Considering the differentiation from naive into effector T cells, newly generated neo-antigen-specific CD4+ T cells against mutated melanoma may be more sensitive to suppressive effect of IL-6 (38). However, IL-6 blockade alone did not efficiently control the tumor growth, as observed for DC immunization combined with IL-6 blockade ((8,21) and Fig. 1). Consistent with our mouse model, a large randomized clinical trial with single use of anti-IL-6 Ab, CNTO328 showed few clinical benefits in patients despite a full inhibition of CRP levels (20). One possible mechanism that limited the effectiveness of IL-6 blockade was the immunosuppression via up-regulation of PD-L1 on tumor cells. While IFN-γ expression is associated with better prognosis (10), IL-6 blockade-induced Th1 skewing of
tumor-specific CD4$^+$ T cells and their IFN-$\gamma$ 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 IFN-$\gamma$ production (39). In such situation without exogenous strong interventions such as active immunization with DC, IL-6 blockade appeared to be insufficient for inducing functional anti-tumor 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 IL-6 blockade fitted with these observations, because pre-conditioning of IL-6 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. Furthermore, 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 IL-6 was associated with the development of pathological 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-IL-6/R Ab not only to provide better management of anti-PD-1 therapy-associated irAE, 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-IL-6 Ab treatment along with PD-L1 blockade triggered the synergistic anti-tumor activity (22,41,42). However, the detailed mechanistic actions were not fully elucidated. Here, we proposed that IL-6-mediated immunosuppression functioned as a rheostat modulating anti-tumor 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 IL-6 production in tumor microenvironment, because the depletion of macrophages allowed the PD-L1 blockade to stimulate local Th1 responses in an IL-6-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, IL-1β and IL-17 (45), which can render TAMs to produce inflammatory mediators including IL-6. However, our data suggested the possibility that an ectopic expression of PD-1 on TAMs and its ligation with PD-L1 directly suppressed their IL-6 production in tumor microenvironment. In addition to the direct effect, PD-1/PD-L1 blockade might indirectly dampen the IL-6 up-regulation through modification of the property to produce IL-6 not only in PD-1+TAMs but also in PD-1- TAMs with unknown mechanism(s), because the total frequency of IL-6-producing PD-1+ TAMs was also increased upon PD-L1 blockade (Supplementary Fig. S3D). Thus, the depletion of both PD-1+ and PD-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 IL-6 levels during Nivolumab treatment, it should be assessed whether IL-6 production in human PD-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-1⁺TAMs expressed M2 macrophage marker CD206 (Supplementary Fig. S2A and Ref. (18)). Therefore, detailed characterization of IL-6-producing human TAMs may help to explain the poor prognostic role of M2-like macrophages in melanoma patients (46).

An increase in IL-6 is often observed at baseline in cancer patients and tumor-bearing mice (9,20,21). As demonstrated in Fig. 4D, PD-1⁺TAMs also appeared to contribute to spontaneous production of IL-6 in tumor tissues. This idea was supported by the observation that depletion of macrophages reduced the baseline level of IL-6. 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 IL-6. 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 anti-tumor 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 IL-6 blockade or macrophage depletion led to a synergistic enhancement of CD8\(^+\) T-cell response to a greater or lesser extent in both model. Thus, it was likely possible that the combined blockade of IL-6 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 tumor microenvironment. This idea was also supported by IL-6 blockade-mediated and CD4-dependnet up-regulation 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 IL-4 (Th2)-skewed immunosuppressive environment (9,50) are the other possible targets of Th1 cells in the synergistic anti-tumor effects.

In conclusion, PD-1/PD-L1 blockade fostered vigorous IFN-\(\gamma\)-producing T-cell responses when IL-6 blockade was given, and ameliorated the immunosuppressive environment governed by tumor cells and TAMs, providing an optimal immunological 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 crosstalk between PD-1/PD-L1 and IL-6 signals.
Acknowledgements

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References

by NY-ESO-1 have negative impacts on survival of patients with stage IV melanoma. Clin Cancer Res 2014;20:4390-9


**Figure legends**

**Figure 1.** IL-6 blockade promotes Th1 responses and attenuates the melanoma progression. 

A and B, MO4-bearing mice were treated with anti-IL-6 or control Ab three times (indicated by arrows in B). Eight days after the first Ab injection, tumor-draining LNs were analyzed for OVA-IIp/I-A<sup>b</sup>-specific IFN-γ responses by ELISPOT assay (A), and tumor outgrowth was monitored over time (B). C and D, Frequencies of CXCR3<sup>+</sup> cells in CD4<sup>+</sup> T cells and SIINFEKL/H-2K<sup>b</sup>-tetramer<sup>+</sup> cells in CD8<sup>+</sup> 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 3 or more independent experiments.

**Figure 2.** IL-6 blockade augments PD-L1 expression on tumor cells through CD4<sup>+</sup> T cell-derived IFN-γ. A and B, Anti-IL-6 Ab was injected twice into MO4-bearing WT, IFN-γ-deficient (KO: A), or CD4-depleted mice (B) as in Fig. 1. Seven days after the first Ab injection, PD-L1 expression on CD4<sup>+</sup> 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 IL-6 (25 ng/mL) or IFN-γ (50 ng/mL) for 48 hours. The expression of PD-L1 was analyzed. Representative histograms (upper panels) and MFI
(lower panels) are shown. n = 3. * P < 0.05, ** P < 0.01, *** P < 0.001. NS, not significant. The data are representative of 3 independent experiments.

**Figure 3.** Changes in plasma IL-6 level during the treatment are associated with responsiveness to Nivolumab in melanoma patients. **A,** Levels of IL-6 and sIL-6R in plasma from melanoma patients (n = 42-46) or healthy donors (HD) older than 50 years (n = 16) were analyzed (left panels). IL-6 levels were further analyzed before (n = 34) and after (n = 18) surgical resection of tumor mass (right panels). **B,** Changes of IL-6 level in plasma from the patients before and 12 weeks after initial treatment of Nivolumab were analyzed (n = 16). **C,** Fold-changes in IL-6 levels (On-/Pre-IL-6) 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). Progression-free survival of each group was analyzed over time. **D,** Patients were divided based on their clinical responses (CR, PR, SD versus PD), and the fold-changes in IL-6 were plotted. **E** and **F,** LDH levels were measured before and during Nivolumab treatment (E). The correlation between the changes in IL-6 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.

**Figure 4.** PD-L1 blockade elicits IL-6 production from tumor-associated macrophages. **A** and **B,** Tumor-free, MO4-bearing wild-type (WT), or IL-6-deficient (KO) mice were treated with anti-PD-L1 Ab. Two days later, IL-6 concentration in serum was measured (A). Expression of the indicated mRNA in isolated TAMs were analyzed (B). **C,** PD-1
expression on CD11b^F4/80^Gr-1^- TAMs or CD11b^Gr-1^+ MDSCs from tumor tissues or spleen were assessed 10 days after melanoma inoculation. Representative histograms are shown (upper panel). PD-L1 expression were also analyzed (lower right panel). D, Cell suspension from MO4 tumors was cultured in vitro in the presence or absence of anti-PD-L1 Ab for 18 hours. IL-6-producing cells were analyzed by flow cytometric analysis. Representative plots (left panels) and the frequencies of indicated IL-6^+ population in the culture (right upper panels) and % of indicated population in IL-6^Gr-1^- fraction (right lower panels) are shown. E, CD11b^Gr-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 (upper) and cytokines in the supernatants (lower) were 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.

**Figure 5.** PD-L1 blockade-stimulated IL-6 induction was mediated through TAMs in melanoma-bearing mice. A, MO4-bearing mice were treated with control or anti-F4/80 Ab. Representative plots (upper panels) and the frequencies of indicated tumor-infiltrating populations in CD45^+ cells (lower panels) 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 IL-6 was injected 1 day before anti-PD-L1 treatment. Frequencies of
tumor-infiltrating T cells and their IFN-γ production were analyzed (C and D). Representative plots for IFN-γ-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.

**Figure 6.** Combined blockade of IL-6 and PD-L1 signals elicits synergistic anti-tumor immune responses. A and B, Control (A) or CD4-depleted (B) MO4-bearing mice were treated with anti-IL-6 and/or anti-PD-L1 Abs three times (indicated by arrows). Tumor progression was monitored over time (left panels). Tumor sizes at the endpoint is also shown (right panels). C-F, Seven days after the first Ab injection, frequencies of CD8+ and CD4+ T cells in tumor-infiltrating CD45+ cells (C), their IFN-γ-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 LNs cells were 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 2 independent experiments. H, Schematic representation of reciprocal interaction between IL-6-mediated attenuation of Th1 response and PD-1/PD-L1 ligation on TAMs.
Figure 1

A

Specific IFN-γ ELISPOT /10^5 dLN cells

OVA-IIP / I-A^b

Control Anti-IL-6 Ab

**

B

Tumor index (mm)

Control Ab Anti-IL-6 Ab

C

CD4^+ CD8^+

Control Ab Anti-IL-6 Ab

CXCR3 CD45

% positive

D

Fold change

Ifng Tnfa Il4 Il10 Foxp3

Control Anti-IL-6 Anti-IL-6 + anti-CD4

* ** ****
Figure 2

A. CD45^-tumor cells

- Control Ab
- Anti-IL-6 Ab

IFN-γ KO

PD-L1

B. CD45^-tumor cells

- Control Ab
- Anti-IL-6 Ab
- Anti-CD4 Ab

Anti-IL-6 Ab: - + +
Anti-CD4 Ab: - +

PD-L1

C. B16, RMA, CT26

- Isotype control
- No stimulation
- + IL-6
- + IFN-γ

B16-F10, RMA, CT26, MO4

PD-L1 expression (× 10^3 MFI)

* NS

IL-6

IFN-γ
Figure 3

A

IL-6

sIL-6R

plasma (pg/ml)

plasma (ng/ml)

HD Melanoma Pt

Pre-operation Post-operation

B

IL-6 in plasma (pg/ml)

Pre-Nivolumab

C

Fold change

On-/Pre IL-6 ≤ median

On-/Pre IL-6 ≥ median

% Progression-free survival

Weeks after treatment

D

Fold change (On-/Pre-)

IL-6

***

E

CR/PR/SD

On-Nivolumab

PD

F

R = 0.147

p = 0.6016

LDH (fold change)

IL-6 (fold change)

G

CRP

IL-8

IL-10

TNF-α

Fold change (On-/Pre-)

CR/PR PD

CR/PR PD

CR/PR PD

CR/PR PD
Figure 4

A

![Graph showing IL-6 in serum with Anti-PD-L1 and Tumor status with Hosts: WT and IL-6 KO.](image)

B

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<th>II1b</th>
<th>II12b</th>
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Anti-PD-L1: - + - + - +
Anti-IL-6: - - - + - +

C

![Graph showing Gr-1-F4/80+CD11b+ TAM with PD-1 expression and Isotype Macrophages.](image)

D

![In vitro culture with Control Ab and Anti-PD-L1 Ab showing Gr-1 and IL-6 in tumor.](image)

E

<table>
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<th>II1b</th>
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Anti-PD-L1: - + - + - +

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Figure 5

A

Control Ab
Anti-F4/80 Ab

F4/80 (CD45+ gated) MHC-II PD-1

CD11b+ Gr-1+ CD11b+Gr-1- MHC-II+ MHC-IIlow PD-1+

% in CD45+ cells

B

Il6 Tnfa Il12b Foxp3

mRNA fold change

Control Ab Anti-PD-L1 Ab

Il1b Il10 Ifng Il4


C

MO4

Control Ab Anti-PD-L1 Ab

% in CD45+ cells IFN-γ

% positive in CD8+ % positive in CD4+

% positive in CD4+

D

CT26

Control Anti-F4/80

IL-6

% in CD45+ cells IFN-γ

% positive in CD8+

% positive in CD4+

E

Control Ab Anti-PD-L1 Anti-PD-L1 + Anti-F4/80

IFN-γ

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Figure 6

A Non-depleted

Day 26

B CD4-depleted

Day 26

C

CD8+

% in CD45+ cells

% positive in CD8+

% positive in dLN CD8+ T cells

CD4+

% in CD45+ cells

% positive in CD4+

% positive in dLN CD4+ T cells

D

IFN-γ

CXCR3

E

F

Fold induction

G

Ccl3

Ccl4

Ccl5

Cxcl9

Cxcl10

H

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Combined blockade of IL-6 and PD-1/PD-L1 signaling abrogates mutual regulation of their immunosuppressive effects in the tumor microenvironment

Hirotake Tsukamoto, Koji Fujieda, Azusa Miyashita, et al.

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