Supplementary Data for

Combined blockade of IL-6 and PD-1/PD-L1 signals breaks mutual regulation of their immunosuppressive effects in tumor microenvironment

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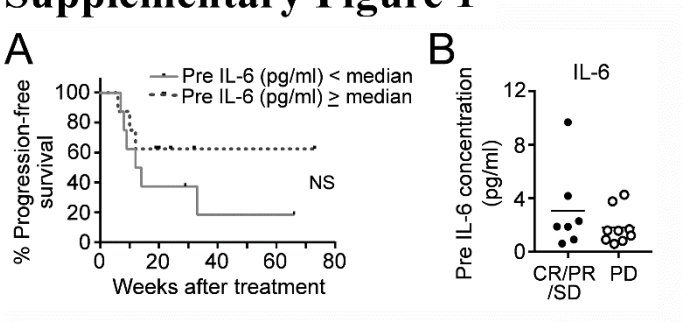
Supplementary Figures 1, 2, 3 and 4.

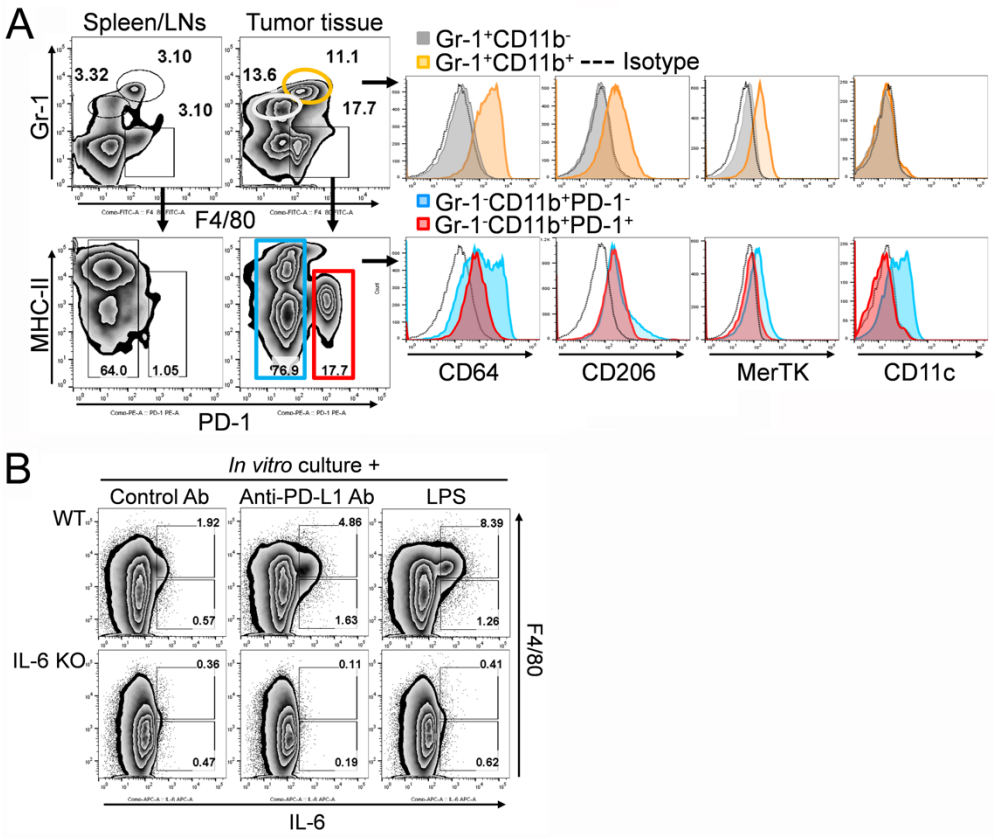
Supplementary Tables 1 and 2.

Supplementary Figure 1



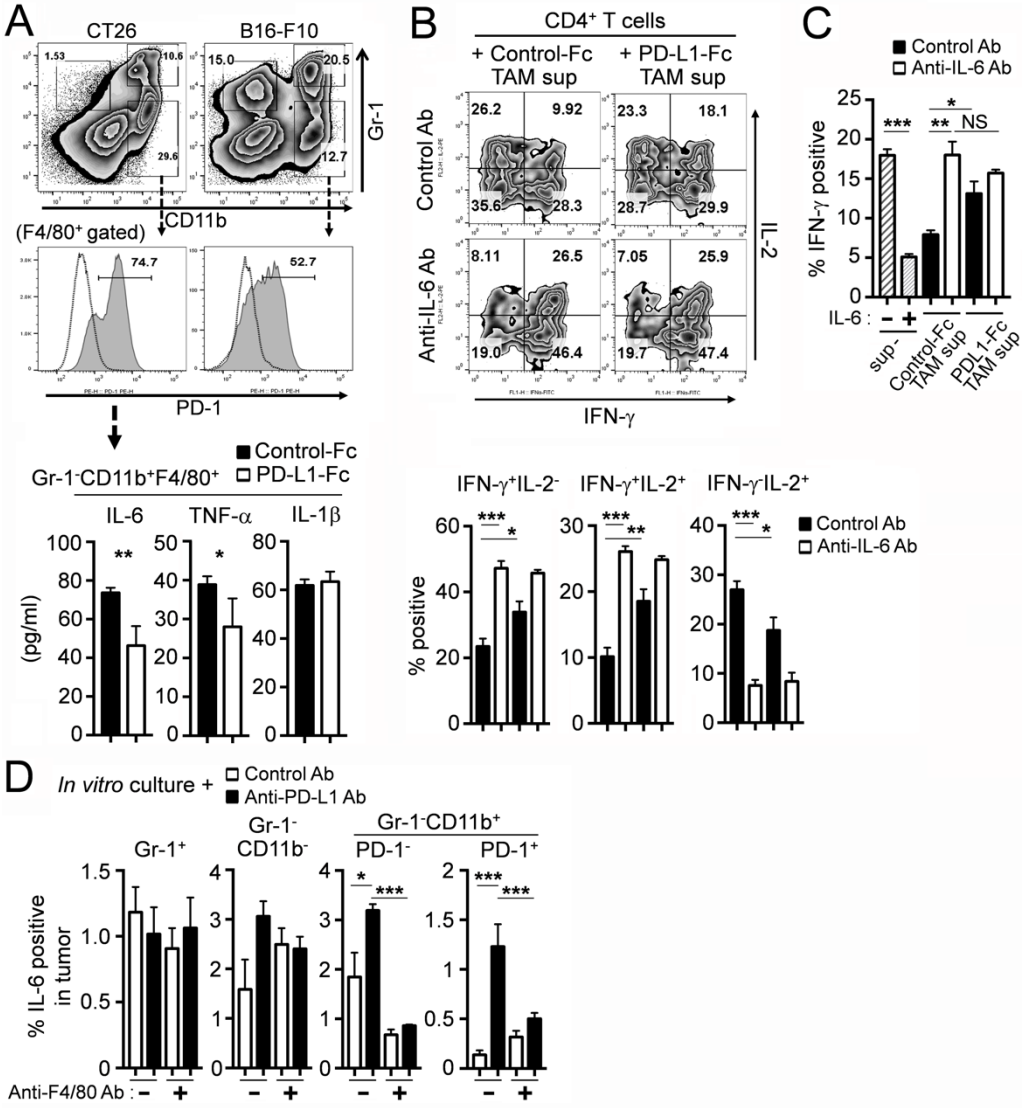
Supplementary Figure 1. Baseline levels of plasma IL-6 before treatment were not associated with responsiveness to Nivolumab in melanoma patients. Concentrations of IL-6 in plasma from the patients before treatment with Nivolumab were analyzed (n = 16). **A**, Based on the median value of IL-6 concentration (less (n = 8) or more (n = 8) than 1.64 pg/mL), the patients were divided into two groups. Left panel shows the ratio of progression-free survival of each group (higher group; median PFS 11 weeks, 95%CI 6–14 weeks, lower group; median PFS NA, 95%CI 8–NA weeks). NS, not significant. **B**, Patients were divided based on their clinical responses (CR, PR, SD versus PD), and the concentrations of IL-6 before treatment were plotted (right panel).

Supplementary Figure 2



Supplementary Figure 2. Characterization of PD-1-expressing macrophages from tumor tissues. **A**, Tumor-infiltrating macrophages were segregated with SSC/FSC parameters, and then were discriminated from Gr-1^{+}CD11b^{+} MDSCs by their Gr-1 and F4/80 expression. Gr-1^{-}F4/80^{+} macrophages expressed CD11b, and were further divided by their PD-1 and MHC-II expression (lower left panels). Histograms show the expression levels of indicated molecules in each myeloid fraction. **B**, Cell suspension from tumor tissues of MO4-bearing WT or IL-6-deficient (KO) mice were cultured *in vitro* in the presence or absence of anti-PD-L1 Ab for 18 hours, or were stimulated with LPS for 10 hours. IL-6-producing cells were determined by flow cytometric analysis with intracellular IL-6 staining. The representative dot plots of CD45^{+}F4/80^{+} IL-6 producing cells are shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. The data are representative of 2 independent experiments, and the values represent the mean with n = 3–5 per group.

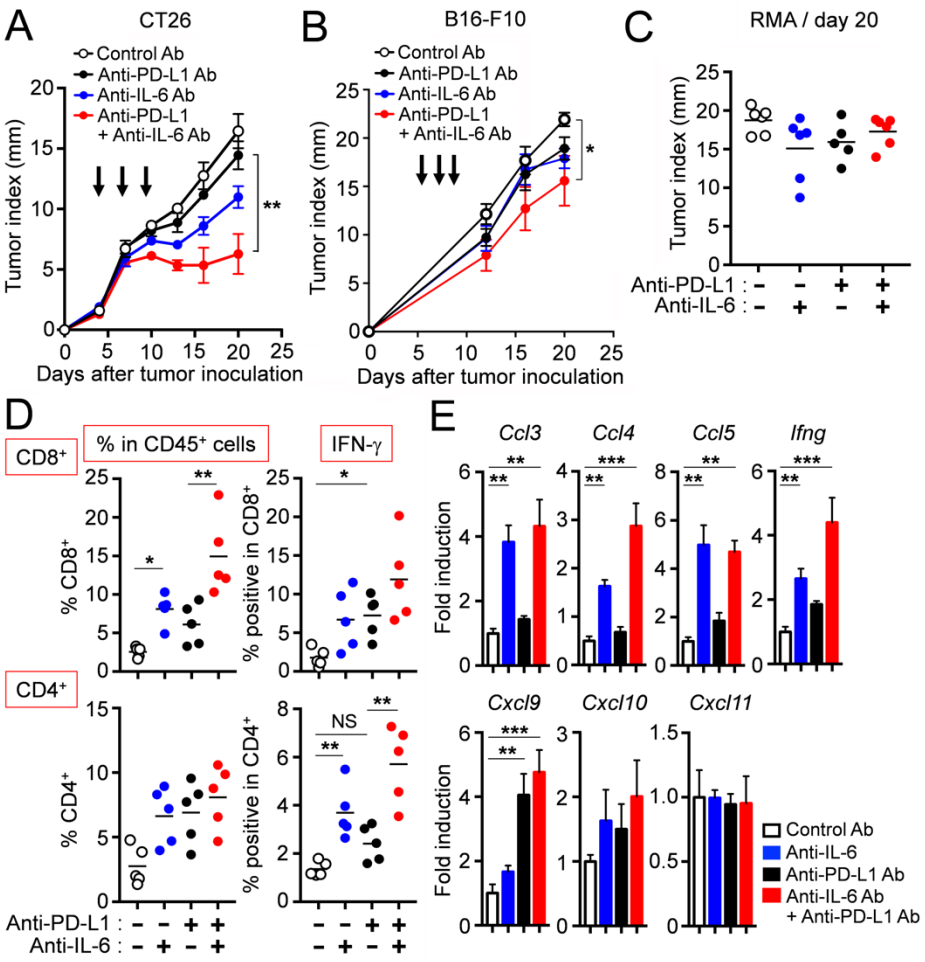
Supplementary Figure 3



Supplementary Figure 3. Characterization of IL-6-producing TAMs. **A**, $CD11b^+Gr\text{-}1^-F4/80^+$ TAMs from CT26- or B16-F10-bearing mice were analyzed for PD-1 expression (upper panels). TAMs from CT26-bearing mice were stimulated with control-Fc or PD-L1-Fc *in vitro*. Concentration of the indicated cytokines in their culture supernatants were assessed. **B** and **C**, TAMs were isolated from B16-F10 (B) or CT26 (C)-bearing mice, and were stimulated with control-Fc or PD-L1-Fc *in vitro*. Supernatant from the culture of TAMs or B16-F10, or recombinant IL-6 was then added into T-cell culture, in which C57BL/6 (B) or Balb/c (C) $CD4^+$ T cells were stimulated with anti-CD3/CD28 Abs in the presence of IL-12 (under Th1-skewed condition). Seven (B) or 5 (C) days later, IFN-γ- and IL-2-producing T cells were analyzed. Representative dot plots (upper panels of B) and their frequencies in each condition (lower panels of B, and C) are shown. **D**, Tumor tissues from MO4-bearing mice that were injected with control or anti-F4/80 Ab *in vivo*, were treated with control or anti-PD-L1 Ab for 18 hours *in vitro*, and then were stained with indicated cell surface markers and intracellular IL-6 as in Supplementary Fig. S2B. The frequencies of $IL\text{-}6^+$ cells of indicated populations in the culture (tumor) are shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. The data are representative of 2

independent experiments, and the values represent the mean with n = 3–5 per group.

Supplementary Figure 4



Supplementary Figure 4. Therapeutic effects of combined treatment with anti-IL-6 and anti-PD-L1 Abs in tumor-bearing mice. **A**–**C**, CT26 (A, D, and E)-, B16-F10 (B), or RMA (C)-bearing mice were treated with anti-IL-6 and/or anti-PD-L1 Ab. Arrows in A and B indicates the time when the Abs were injected in mice. Tumor outgrowth was monitored over time (A and B). Tumor sizes of RMA at day 20 are shown (C). **D** and **E**, Three days after second Abs injection, tumor tissues were harvested from CT26-bearing mice, and analyzed for the frequencies of indicated population (D) and the expression of indicated mRNA (E). The data are representative of 2 or 3 independent experiments (n = 5–10). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. NS, not significant.

Supplementary Table 1

Demographics and disease characteristics of melanoma patients treated with Nivolumab.

No.	Age / Gender	Prior systemic therapies	The day when the treatment started	Pre IL-6 (ng/ml), Date[b]	On IL-6 (ng/ml), Date[b]	On /Pre IL-6	Clinical response
1	75 / F[a]	BRAF inhibitors/ MEK inhibitor/ IDO inhibitor	2017/03/09	1.68 (2017/03/09)	6.36 (2017/05/23)	3.78	PD[a]
2	53 / F	-	2016/03/17	1.60 (2016/03/17)	9.46 (2016/06/16)	5.92	PD
3	62 / M	Ipilimumab/ carbon-ion radiotherapy	2016/04/04	4.17 (2016/04/04)	1.97 (2016/07/14)	0.47	SD
4	81 / M	Interferon-β	2016/05/19	1.22 (2016/05/19)	5.95 (2016/08/18)	4.88	PD
5	74 / M	-	2017/02/16	0.81 (2017/01/30)	0.97 (2017/05/08)	1.19	PD
6	64 / M	carbon-ion radiotherapy	2016/08/18	3.76 (2016/08/18)	17.8 (2016/11/08)	4.75	PD
7	53 / F	carbon-ion radiotherapy	2016/08/22	0.60 (2016/08/22)	1.10 (2016/11/28)	1.84	PD
8	66 / M	-	2016/09/01	0.62 (2016/09/01)	0.54 (2016/10/14)	1.87	SD
9	76 / M	-	2016/11/10	0.92 (2016/11/10)	8.00 (2017/01/26)	8.72	PD
10	31 / M	Ipilimumab	2016/12/26	4.27 (2016/12/26)	10.8 (2017/04/12)	2.53	PD
11	79 / F	carbon-ion radiotherapy	2017/04/06	1.89 (2017/04/06)	1.46 (2017/07/10)	0.77	CR
12	63 / M	-	2016/09/28	1.58 (2016/09/28)	6.54 (2016/12/27)	4.15	PD
13	70 / M	Ipilimumab/ carbon-ion radiotherapy	2017/01/12	1.88 (2017/01/05)	1.43 (2017/03/13)	0.76	PR
14	71 / M	BRAF inhibitors/ MEK inhibitor	2017/02/02	0.91 (2017/02/02)	0.27 (2017/04/27)	0.30	PR
15	77 / M	-	2017/03/09	2.29 (2017/03/09)	0.25 (2017/06/08)	0.11	PR
16	58/ M	carbon-ion radiotherapy	2017/04/13	9.68 (2017/04/13)	2.50 (2017/07/28)	0.26	SD

[a]Abbreviations: CR; complete response, PR; partial response, SD; stable disease, PD; progressive disease, M; male, F; female

[b]Blood sampling was performed before and during Nivolumab treatment.

Supplementary Table 2

Demographics and disease characteristics of melanoma patients receiving surgery.

No.	Age / Gender	The day of surgery	Pre IL-6 (ng/ml), Date[b]	Post IL-6 (ng/ml), Date[b]	Post /Pre IL-6
1	79 / F[a]	2016/01/21	8.64 (2016/01/21)	2.99 (2016/05/02)	0.35
2	65 / F	2016/01/27	1.77 (2016/01/27)	1.08 (2016/05/02)	0.61
3	66 / M	2016/02/18	2.28 (2016/02/18)	0.62 (2016/05/20)	0.27
4	68 / M	2016/02/24	1.14 (2016/02/24)	1.30 (2016/06/02)	1.15
5	71 / M	2016/04/20	0.60 (2016/04/20)	0.78 (2017/07/11)	1.29
6	91 / M	2016/05/25	1.71 (2016/05/25)	3.66 (2016/08/31)	2.15
7	85 / F	2016/06/22	1.22 (2016/06/22)	0.58 (2016/10/06)	0.48
8	54 / F	2016/06/22	0.64 (2016/06/22)	0.18 (2016/10/13)	0.29
9	77 / F	2016/07/14	0.30 (2016/07/14)	0.82 (2016/11/09)	2.78
10	75 / M	2016/07/14	0.85 (2016/07/14)	0.55 (2017/10/13)	0.64
11	93 / F	2016/07/22	2.17 (2016/07/22)	2.64 (2016/12/07)	1.22
12	86 / F	2016/08/03	0.78 (2016/08/03)	0.84 (2016/11/30)	1.09
13	56 / F	2017/08/17	0.30 (2017/08/17)	0.70 (2016/12/05)	2.35
14	72 / M	2016/06/29	0.35 (2016/06/29)	0.35 (2016/09/15)	0.99
15	68 / F	2017/01/25	2.32 (2017/01/25)	0.03 (2017/05/08)	0.01
16	54/ F	2016/10/12	2.94 (2016/10/12)	0.22 (2017/03/06)	0.07
17	86 / M	2016/10/26	1.19 (2016/10/26)	11.3 (2017/02/20)	9.51
18	72 / M	2016/11/24	1.79 (2016/11/24)	0.65 (2017/02/20)	0.37

[a]Abbreviations: M; male, F; female

[b]Blood sampling was performed before and after surgery.