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Original Article

CRP and soluble CTLA4 are determinants of anti-PD1 resistance in gastrointestinal cancer

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Abstract: Targeting immune inhibitory checkpoint (IC) pathways have attracted great attention as a promising strategy for treating gastrointestinal (GI) cancer. However, the therapeutic efficacy is low in most cases, and little progress has been made in establishing biomarkers that predict the possible responses, and combination regimens that enhance the therapeutic efficacy. As a predictive biomarker, soluble forms of IC molecules have been recently highlighted. However, little is known about which IC molecules is most critically associated with the treatment resistance, and also about the biological and immunological roles of the IC molecules in GI cancer. In this study, we analyzed sera obtained from advanced gastric cancer patients before and one month after treatment with anti-PD1 nivolumab for soluble IC molecules by ELISA. We found that decrease of soluble CTLA4 (sCTLA4) at posttreatment were significantly associated with a better prognosis, and combination with low level of CRP at posttreatment more clearly defined anti-PD1 responders with long-term survival. Indeed, in the in vitro setting, CRP stimulation upregulated CTLA4 expression in tumor cells followed by generation of sCTLA4 that suppressed CTL induction, and simultaneously conferred high self-renewal and invasive abilities on the tumor cells accompanied by increase of EMT-related gene expressions. In the in vivo setting, CRP injection elevated sCTLA4 level in sera of mouse tumor metastasis models, leading to failure of anti-PD1 therapy. However, treatment with anti-CTLA4 mAb or a PPARγ agonist that can reduce in vivo CRP successfully elicited anti-tumor efficacy in the anti-PD1 resistant models. These suggest that targeting CRP and sCTLA4 may be a promising strategy for improving clinical outcomes in the treatments, including anti-PD1 therapy, of GI cancer.

Keywords: Gastrointestinal cancer, gastric cancer, colorectal cancer, anti-PD1 mAb, nivolumab, PD1, PDL1, CTLA4, soluble molecule, CRP

Introduction

Gastric cancer is the third most common cause of cancer-related deaths worldwide [1], and conventional chemotherapy is insufficient for providing long-term survival to patients with unresectable advanced/recurrent gastric cancer (AGC) [2]. Recently, targeting immune inhibitory checkpoint (IC) pathways has attracted attention as a promising strategy for treating AGC because of providing long-lasting durable responses even in advanced and metastatic cancer [3]. However, the clinical responses are limited to a small portion of patients as reported by the ATTRACTION-2 study [4]. Then, biomarkers have been explored to predict the possible responses, and several biomarkers, such as PDL1 expression, mismatch repair deficiency and high microsatellite instability in tumor tissues, have been identified [5, 6]. However, the status is not always correlated with the clinical outcomes, and thus other biomarkers with more accuracy are emergently needed.

As a prognostic biomarker, soluble forms of IC molecules in peripheral blood have been noticed, since the high level is associated with advance stages, metastasis, and a poor prognosis [7, 8]. Soluble PDL1 (sPDL1) is generated by proteolytic cleavage of the membrane-bound

extracellular domain [9], and the high level is correlated with a poor prognosis in AGC after chemotherapy [10]. However, opposite results have been also reported in immunotherapy [11]. Soluble PD1 (sPD1) and soluble CTLA4 (sCTLA4) are splice variants lacking exon 3 encoding the transmembrane domain, and are potentially generated by shedding off the membrane-bound molecules [12, 13]. The sPD1 enhances anti-tumor immune responses by interfering the PDL1-binding to PD1 expressed in anti-tumor effector cells [13]. In contrast, the sCTLA4 suppresses anti-tumor responses by interfering the CD28 activation signaling via competitive binding to the shared costimulatory B7 ligands expressed on APCs [14]. However, the prognostic values are still controversial in clinical settings, and the sources and functions in cancer remain to be fully elucidated.

In this study, we attempted to determine a key soluble IC molecule in the anti-PD1 resistance in gastrointestinal (GI) cancer using sera of AGC patients before and after nivolumab therapy. We also elucidated the molecular mechanisms underlying the anti-PD1 resistance associated with the identified key molecule through in vitro and in vivo experiments using murine and human GI cancer cells.

Materials and methods

Clinical study

We used sera archived at the National Cancer Center Biobank in Japan. Peripheral blood was obtained from AGC patients (n = 36) at the National Cancer Center Hospital (January 2015-March 2019) before and one month after treatment with nivolumab (3 mg/kg or 240 mg/body biweekly) in accordance with the protocol (No. 2019-068) approved by the IRB of the National Cancer Center. Informed consent was obtained from all subjects. Peripheral blood was left at room temperature for 30 min to coagulate, and then serum was separated by high-speed centrifugation (3000 rpm, 10 min). The serum was transferred into several plastic aliquot tubes (0.5-1 ml/tube), and was immediately stored in liquid nitrogen to preserve freshness. On the day of the assay, one tube was taken out, thawed, and centrifuged again at high-speed to remove debris, and the supernatant was used as a test sample for ELISA. The sera were tested for human sPDL1 (Invitrogen), sPD1 (Invitrogen), and sCTLA4 (BioLegend) using the specific ELISA kits, and the relationships between the molecular levels and clinicopathological data were statistically analyzed.

Mice and cell lines

Five-week-old female BALB/c mice were purchased from Charles River Laboratories in Japan, and were maintained under pathogen-free conditions. Mice were used according to the protocol (No. T17-055) approved by the Animal Care and Use Committee at the National Cancer Center Research Institute. Murine colorectal cancer Colon26 cells were purchased from Tohoku University in Japan. Human GC KATO III cells and colorectal cancer Colo201 cells were purchased from ATCC. The cells were expanded and frozen in liquid nitrogen to avoid changes occurred by a long-term culture, and were tested for Mycoplasma negativity using a Hoechst-staining detection kit (MP Biomedicals) before experiments.

Characterization of CRP-treated tumor cells

Tumor cells were stimulated with C-reactive protein (CRP, 30 μg/ml) in the presence or absence of Rosiglitazone (RSG, 10 μM) for 24 hours, and sCTLA4 in the supernatants was measured using the specific ELISA kit (a kit for mouse sCTLA4 was purchased from Invitrogen). Membrane-bound CTLA4 and PDL1 expressions were analyzed by immunostaining with PE-conjugated anti-CTLA4 mAb (BNI3; BioLegend), and FITC-conjugated anti-PDL1 mAb (MIH3; BioLegend). The cells were tested for proliferation (3 days) by counting the number of cells, invasion (4 hours) using a transwell chamber with a matrigel-coated membrane (Corning), and self-renewability (2 days) using a 24-well Ultra-Low Attachment Plate (Corning) as described before [15]. To analyze gene expression in human tumor cells, each cDNA was synthesized with an oligo(dT) primer and Avian Reverse Transcriptase (Takara) from the extracted RNAs using RNeasy kit (Qiagen), and RT-PCR was conducted using the following paired primers (Invitrogen) specific for the ORF region within the targeted human gene: *snail* (CCCCAATCGGAAGCCTAACTAC and GGAGATCCTTGGCCTCAGAGAG), twist (GCAAGCTTAGAGATGATGCAGGACG and GACTCGAGGTGGGACGCGGACATGGA), *cd44* (GAGCATCGGATTTGAGACCTG and AGCTCCATTGCCACTGTTGAT), and

gapdh (CGGAGTCAACGGATTTGGTCG and AGCCTTCTCCATGGTGGTGAA). For CTLA4 knockdown, human tumor cells were transfected with two kinds of siRNAs targeting a different sequence position (Invitrogen) of human *ctla4*, or a non-targeting siRNA (Invitrogen) after making complexes with jetPRIME (PolyPlus). Two days later, the transfection efficacy was validated by RT-PCR using the primers specific for the ORF region of human *ctla4* gene (CATAGCAGTGCTTGATTGCGT and TAAGAATTGGGCCCATCGAAC). CTLA4 protein expression was confirmed by ELISA, flow cytometry, or immunostaining. To assess the immunoregulatory activity of the CRP-treated tumor cells, the tumor supernatants or recombinant CTLA4 (1 μg/mL; BioLegend) as a control were added to the CTL induction system described below. In the in vivo setting, the CRP-treated cells (2×10^5) were intravenously (i.v.) implanted into mice, and tumor nodules in lung were counted on day 15.

In vivo therapy

Mice were subcutaneously (s.c.) implanted with Colon26 cells (5×10^5), and were intraperitoneally (i.p.) injected with anti-CTLA4 mAb (9H10; BioLegend), anti-PD1 mAb (29F.1A12; BioLegend), or mouse IgG (mIgG, MOPC-21; BioXCell) as a control (10 mg/kg) on days 5 and 9. RSG (0.1 mg/kg; Wako) or PBS as a control was orally administered into mice daily from days 5-11. Mimicking patients with increased CRP, human CRP (200 μg; Wako) was i.p. injected into mice during the therapy (days 5, 7, and 9) based on the report showing that CRP concentration is maintained at 31 μg/ml in sera of mice for 48 hours after i.p. injection with 200 μg of human CRP [16]. Tumor volume was measured ($0.5 \times \text{Length} \times \text{Width}^2$, mm^3). As another tumor model, tumor cells (5×10^5) were i.v. implanted into mice, and tumor nodules in lungs were counted on day 12. Peripheral blood, subcutaneous tumor, and spleen were harvested from the mice 2 weeks after tumor implantation for assays. The sera were tested for sCTLA4 by ELISA. CTLA4 expression in tumor tissues was analyzed by immunostaining with biotin-conjugated anti-CTLA4 mAb (UC10-4B9; BioLegend) or the isotype hamster IgG (BioLegend) followed by visualization with streptavidin-FITC (BioLegend). $CD4^+$ or $CD8^+$ T cells were sorted from spleen cells (SPCs) using a BD IMag system with magnetic particle-conjugated mAbs, and were tested for cell proliferation in response to anti-CD3 mAb (1 μg/ml) using a WST1 assay kit (Takara). To assess cytotoxic activity of splenic $CD8^+$ T cells, bulk SPCs were pre-stimulated with the H-$2L^d$-restricted tumor antigen peptide AH1 (1 μg/mL; MBL) for 6 days, and the sorted $CD8^+$ T cells were cocultured with Colon26 cells for 4 hours as described before [15].

Flow cytometric analysis

After Fc blocking, cells were stained with the following immunofluorescence-conjugated antibodies: anti-CD45-PE-Cy7 (BioLegend), anti-CD3e-BUV496 (BD), anti-CD4-BV785 (BioLegend), anti-CD8a-BUV395 (BD), anti-FOXP3-BV421 (BioLegend), anti-CTLA4-PerCP-Cy5.5 (BioLegend), anti-PD1-BV510 (BioLegend), anti-TIGIT-PE-Cy7 (BioLegend), anti-GZMB-Pacific Blue (BioLegend), anti-Ki67-FITC (BioLegend), and the isotype control. For intracellular staining, cells were treated with Cytofix/Cytoperm solution (BD) before antibody staining. Data were acquired using a BD LSR Fortessa X-20 cytometer (BD), and were analyzed by FlowJo software (BD).

Statistical analysis

Data are presented as means ± SDs unless otherwise specified. Experiments were repeated at least three times to confirm the reproducibility. Significant differences (P value < 0.05) were statistically evaluated using GraphPad Prism 7 software (MDF) or EZR software version 1.53 [17]. To compare between two groups, the data were analyzed by the unpaired two-tailed Student's t test. Non-parametric groups were analyzed by the Mann-Whitney test. In the combination therapy, significance to the single treatment was evaluated using a two-way ANOVA with Bonferroni post-hoc test. Survival was analyzed by the Kaplan-Meier method and the Mantel-Cox Log-Rank test.

Results

Higher increase of sPD1 and decrease of sCTLA4 in sera after nivolumab therapy are associated with a better prognosis in AGC

We tested sera obtained from AGC patients (n = 36; **Table 1**) before and one month after treatment with nivolumab for sPDL1, sPD1, and

Table 1. Patient characteristics

Characteristics (n = 36)		
Median age (range)		64 (40-83)
Sex	Male	20 (56%)
	Female	16 (44%)
ECOG performance status	0	4 (11%)
	1	30 (83%)
	2	2 (6%)
Location of primary tumor	Gastric	32 (89%)
	Gastric esophageal junction	4 (11%)
Histological subtype	Intestinal	19 (53%)
	Diffuse	17 (47%)
HER2 status	Positive	7 (19%)
	Negative	29 (81%)
Disease status	Unresectable	26 (72%)
	Recurrent	10 (28%)
Organ with metastases	< 2	10 (28%)
	≥ 2	26 (72%)
Metastatic site	Liver	14 (39%)
	Lung	3 (8%)
	Peritoneum	23 (64%)
	Lymph node	21 (58%)
Ascites	None - mild	23 (64%)
	Moderate - severe	13 (36%)
Previous gastrectomy	Yes	19 (53%)
	No	17 (47%)

sCTLA4 by ELISA. The median follow-up time of survivors after nivolumab initiation was 10.0 months (2.3-19.3 months). The median progression-free survival (mPFS) was 1.7 months (1.2-2.3 months), and the median overall survival (mOS) was 3.6 months (2.6-4.5 months; **Figure 1A**). No significant differences of PFS and OS were seen between two groups divided by each median concentration at one time point, before (baseline) or after the therapy. We then examined the molecular changes from pretreatment to posttreatment. Serum sPDL1 increased in most patients (27/36 = 75%; **Figure 1B**; **Table 2**), and no significant differences of PFS and OS were seen between increase and decrease groups (**Figure 1C**). Serum sPD1 increased all patients (**Figure 1B**), but the group with higher increase than the median increase rate (7.7-fold) showed significantly longer mOS (6.1 months) as compared to less increase group (3.1 months, hazard ratio [HR] = 0.36, P = 0.008; **Figure 1D**; **Table 3**). Serum sCTLA4 increased in most patients (25/36 = 69%; **Figure 1B**), but the increase group showed significantly shorter mOS (3.2 months) as compared to the decrease group (4.5 months, HR = 2.59, P = 0.032; **Figure 1E**; **Table 4**). These suggest the higher sPD1 increase and the sCTLA4 decrease after nivo-therapy partly defines possible responders with long-term survival. Combination of both factors significantly narrowed down the responders (mPFS = 4.3 months, HR = 0.31, P = 0.037; **Figure 2A**), although combination of sCTLA4, which seemed to be more reliable than sPD1, with the higher sPDL1 increase showed no synergy in PFS and OS as compared to the single factor (**Figure 2B**). This suggests combination of the sCTLA4 decrease and the higher sPD1 increase after nivo-therapy clearly defines possible responders with long-term survival.

Combination of the sCTLA4 decrease with lower CRP in sera after the nivo-therapy defines responders with long-term survival

However, targeting sPD1 is just same as nivo-therapy. To further improve the diagnostic accuracy of the sCTLA4, we then explored other factors using serological data obtained from the routine blood tests. We found that the group with higher CRP than the median concentration (1.3 mg/dl) at posttreatment showed a significant poor prognosis (mPFS = 1.3 months versus 3.1 months, HR = 5.61, P < 0.001; and mOS = 2.8 months versus 6.4 months, HR = 3.28, P = 0.002) as compared to the lower group (**Figure 3A**, **3B**), although no significant differences in PFS and OS were seen between two groups divided by the median baseline concentration, and between increase and decrease groups after the therapy (P > 0.05). CRP is a polypeptide synthesized in the liver in response to various pro-inflammatory cytokines [18]. Indeed, the CRP increase at posttreatment was significantly associated with liver metastasis (P = 0.015; **Table 5**). Combination of the sCTLA4 decrease with the lower CRP at posttreatment clearly narrowed down the responders with much better prognosis (mPFS = NA versus 1.4 months, HR = 0.12, P = 0.023; and mOS = NA versus

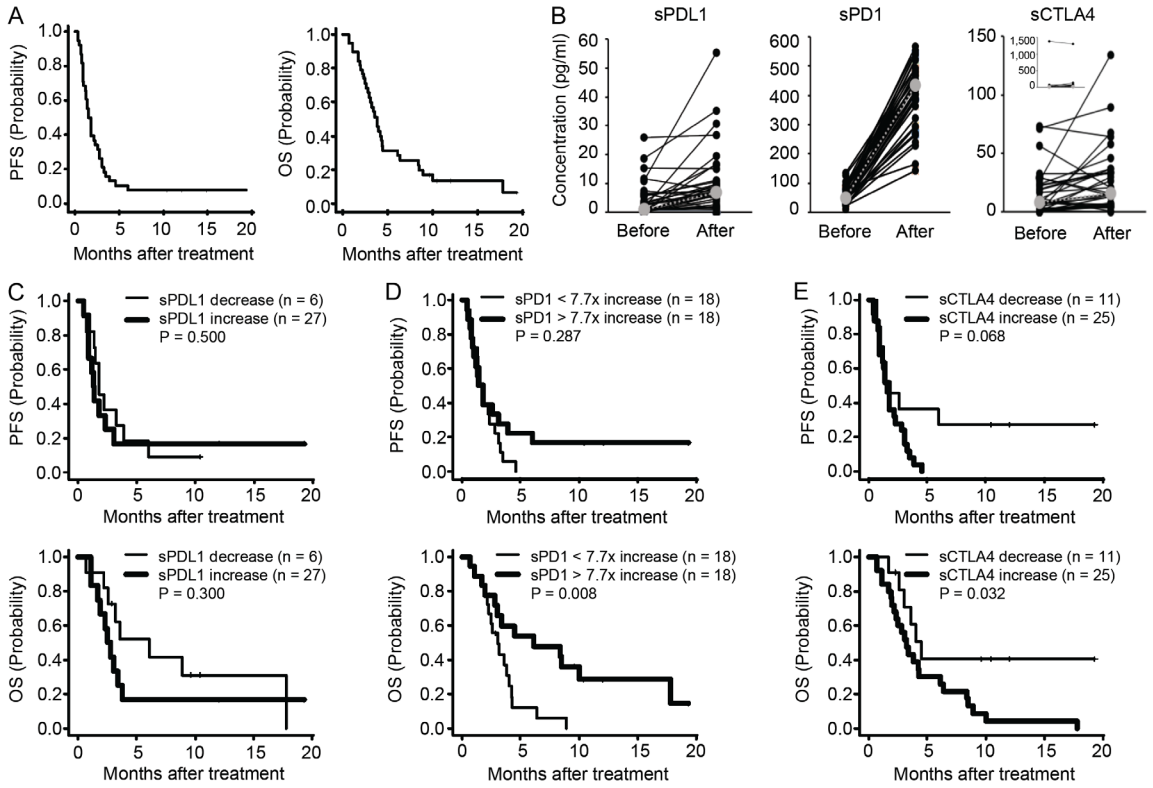


Figure 1. Higher increase of sPD1 and decrease of sCTLA4 after nivolumab therapy are better prognostic factors. A. Progression-free survival (PFS) and overall survival (OS) of patients with advanced gastric cancer (AGC) after nivolumab treatment (n = 36). B. Changes of a soluble form of PDL1 (sPDL1), PD1 (sPD1), or CTLA4 (sCTLA4) in sera at posttreatment (n = 36). Gray dotted lines indicate each median. C. A lack of association of sPDL1 changes. PFS and OS were statistically compared between two groups with increased and decreased sPDL1 at posttreatment, following exclusion of three patients with no changes (0 pg/ml before and after the therapy). D. Higher increase of sPD1 at posttreatment is associated with a favorable prognosis. PFS and OS were statistically compared between two groups divided by the median increase rate (7.7-fold). E. Decrease of sCTLA4 at posttreatment is associated with a favorable prognosis. PFS and OS were statistically compared between two groups with increased and decreased sCTLA4 at posttreatment by the Kaplan-Meier method and the Mantel-Cox Log-Rank test.

3.6 months, HR = 0.12, P = 0.030), and 80% of the patients who fit the criteria survived until the end of the follow-up time (**Figure 3C**). Combination of the higher sPD1 increase with the lower CRP at posttreatment showed no synergy as compared to the single factor (**Figure 3D**). These suggest the combination of CRP and sCTLA4 levels is a good biomarker for predicting possible anti-PD1 responses.

CRP injection increases sCTLA4 in the sera of mouse tumor models, leading to failure of anti-PD1 therapy

We next investigated the underlying mechanisms using mouse tumor models implanted with murine colorectal cancer Colon26 cells (because murine GC cell lines were unavailable). Mimicking the AGC patients (mean CRP concentration was 34.2 μg/ml at posttreatment), we intraperitoneally (i.p.) injected human CRP (200 μg) in mice on days 5, 7, and 9 after tumor implantation based on the previous report [16]. Despite no impact in naive mice, CRP injection significantly elevated the serum sCTLA4 level in tumor-implanted mice (**Figure 4A**). CTLA4 expression was also upregulated in the tumor tissues of the mice, implying a source of the sCTLA4. Tumor growth was significantly promoted, particularly 10 days after CRP injection (P = 0.014; **Figure 4B**). CRP is known to suppress T-cell proliferation and CTL induction [19]. However, no significant differences of immunological factors were seen between the CRP-injected group and PBS-injected control group in the in vivo setting when evaluated at only one time point: tumor-infiltrating cells (TILs; **Figure 4C**), including potentially cytotoxic

Table 2. Patient characteristics divided by the sPDL1 changes after nivolumab treatment

Characteristics	Decrease (n = 6)	Increase (n = 27)	*P* values
Median age (range)	70 (52-76)	65 (40-83)	0.434
Sex			0.241
Male	4 (67%)	15 (56%)	
Female	2 (33%)	12 (44%)	
Location of primary tumor			1.000
Gastric	6 (100%)	23 (85%)	
GEJ	0 (0%)	4 (15%)	
Histological subtype			0.665
Intestinal	4 (67%)	14 (52%)	
Diffuse	2 (33%)	13 (48%)	
HER2 status			0.563
Positive	0 (0%)	6 (22%)	
Negative	6 (100%)	21 (78%)	
Disease status			0.336
Unresectable	3 (50%)	20 (74%)	
Recurrent	3 (50%)	7 (26%)	
Organ with metastases			1.000
< 2	2 (33%)	7 (26%)	
≥ 2	4 (67%)	20 (74%)	
Metastatic site			
Liver	1 (17%)	13 (43%)	0.371
Lung	0 (0%)	3 (100%)	1.000
Peritoneum	3 (50%)	20 (67%)	0.645
Lymph node	6 (100%)	15 (50%)	0.030

Table 3. Patient characteristics divided by the median rate of sPD1 increase after nivolumab treatment

Characteristics	< 7.7-fold increase (n = 18)	> 7.7-fold increase (n = 18)	*P* values
Median age (range)	66 (40-83)	62 (43-83)	0.586
Sex			0.500
Male	9 (50%)	12 (67%)	
Female	9 (50%)	6 (33%)	
Location of primary tumor			0.603
Gastric	17 (94%)	15 (83%)	
GEJ	1 (6%)	3 (17%)	
Histological subtype			1.000
Intestinal	10 (56%)	10 (56%)	
Diffuse	8 (44%)	8 (44%)	
HER2 status			0.471
Positive	3 (17%)	4 (22%)	
Negative	15 (83%)	14 (78%)	
Disease status			0.471
Unresectable	11 (61%)	14 (78%)	
Recurrent	7 (39%)	4 (22%)	
Organ with metastases			1.000
< 2	5 (28%)	6 (33%)	
≥ 2	13 (72%)	12 (67%)	

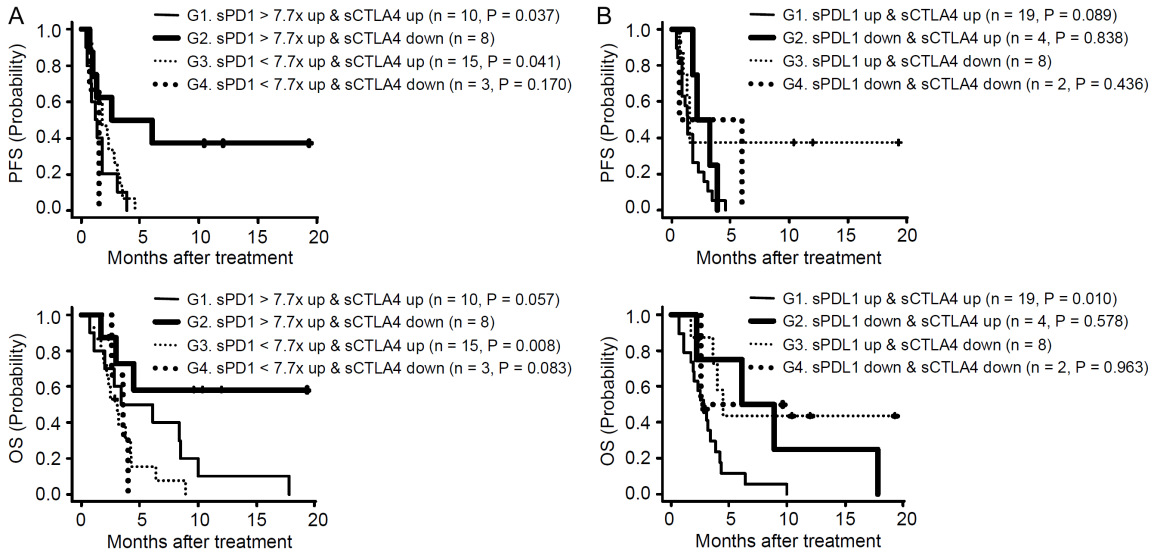
Metastatic site			
Liver	4 (22%)	10 (56%)	0.086
Lung	1 (5%)	2 (11%)	1.000
Peritoneum	14 (78%)	9 (50%)	0.164
Lymph node	9 (50%)	12 (67%)	0.500

Table 4. Patient characteristics divided by the sCTLA4 changes after nivolumab treatment

Characteristics	Decrease (n = 11)	Increase (n = 25)	*P* values
Median age (range)	66 (43-79)	64 (40-83)	0.608
Sex			0.077
Male	9 (82%)	12 (48%)	
Female	2 (18%)	13 (52%)	
Location of primary tumor			1.000
Gastric	10 (91%)	22 (88%)	
GEJ	1 (9%)	3 (12%)	
Histological subtype			0.718
Intestinal	7 (64%)	13 (52%)	
Diffuse	4 (36%)	12 (48%)	
HER2 status			1.000
Positive	2 (18%)	5 (20%)	
Negative	9 (82%)	20 (80%)	
Disease status			0.703
Unresectable	7 (64%)	18 (72%)	
Recurrent	4 (36%)	7 (28%)	
Organ with metastases			0.252
< 2	5 (46%)	6 (24%)	
≥ 2	6 (55%)	19 (76%)	
Metastatic site			
Liver	5 (46%)	9 (36%)	0.716
Lung	0 (0%)	3 (12%)	0.538
Peritoneum	6 (55%)	17 (68%)	0.475
Lymph node	8 (73%)	13 (52%)	0.295

$CD3^+CD8^+Ki67^+GZMB^+$ T cells, potentially immunosuppressive regulatory $CD3^+CD4^+FOXP3^+CTLA4^+$ T cells (Tregs), and potentially exhausted $CD3^+CD8^+PD1^+TIGIT^+$ T cells (Texs), splenic T-cell proliferation (**Figure 4D**), and splenic CTL cytotoxicity (**Figure 4E**).

This raised a question whether CRP would directly affect tumors. Indeed, in vitro stimulation with CRP upregulated CTLA4 expression in tumor cells accompanied by sCTLA4 increase in the culture (**Figure 4F**). When the CRP-stimulated tumor supernatant was added to the CTL induction system, the cytotoxicity was extremely reduced, but this was significantly rescued by adding anti-CTLA4 mAb to the culture ($P < 0.05$; **Figure 4G**). This suggests CRP suppresses CTL induction indirectly via generation of sCTLA4 from tumor cells, probably in the middle of the tumor progression process. CRP injection significantly reduced anti-PD1 therapeutic efficacy ($P < 0.001$; **Figure 4B**), and rather increased Tregs and Texs in the tumors (**Figure 4C**). In the mice, proliferative and cytotoxic activities of splenic T cells were significantly reduced as compared to those of the CRP-noninjected group ($P < 0.003$; **Figure 4D**, **4E**). However, regardless of whether CRP was injected or not, anti-CTLA4 therapy significantly suppressed tumor growth ($P = 0.003$; **Figure 4B**) following significant increase of cytotoxic T cells in the tumors ($P = 0.008$), although Tregs



PFS	P values
G1 vs G2	0.037
G1 vs G3	0.350
G1 vs G4	0.628
G2 vs G3	0.041
G2 vs G4	0.170
G3 vs G4	0.129

OS	P values
G1 vs G2	0.057
G1 vs G3	0.146
G1 vs G4	0.400
G2 vs G3	0.008
G2 vs G4	0.083
G3 vs G4	0.905

PFS	P values
G1 vs G2	0.213
G1 vs G3	0.089
G1 vs G4	0.213
G2 vs G3	0.838
G2 vs G4	0.546
G3 vs G4	0.436

OS	P values
G1 vs G2	0.063
G1 vs G3	0.010
G1 vs G4	0.261
G2 vs G3	0.578
G2 vs G4	0.735
G3 vs G4	0.963

Figure 2. Combination of sCTLA4 decrease and sPD1 increase after nivolumab therapy improves the diagnostic accuracy. A. Combination of sPD1 and sCTLA4 in sera after nivolumab therapy (n = 36). B. Combination of sPDL1 and sCTLA4 in sera after nivolumab therapy (n = 33). *P* values were analyzed by the Kaplan-Meier method and the Mantel-Cox Log-Rank test.

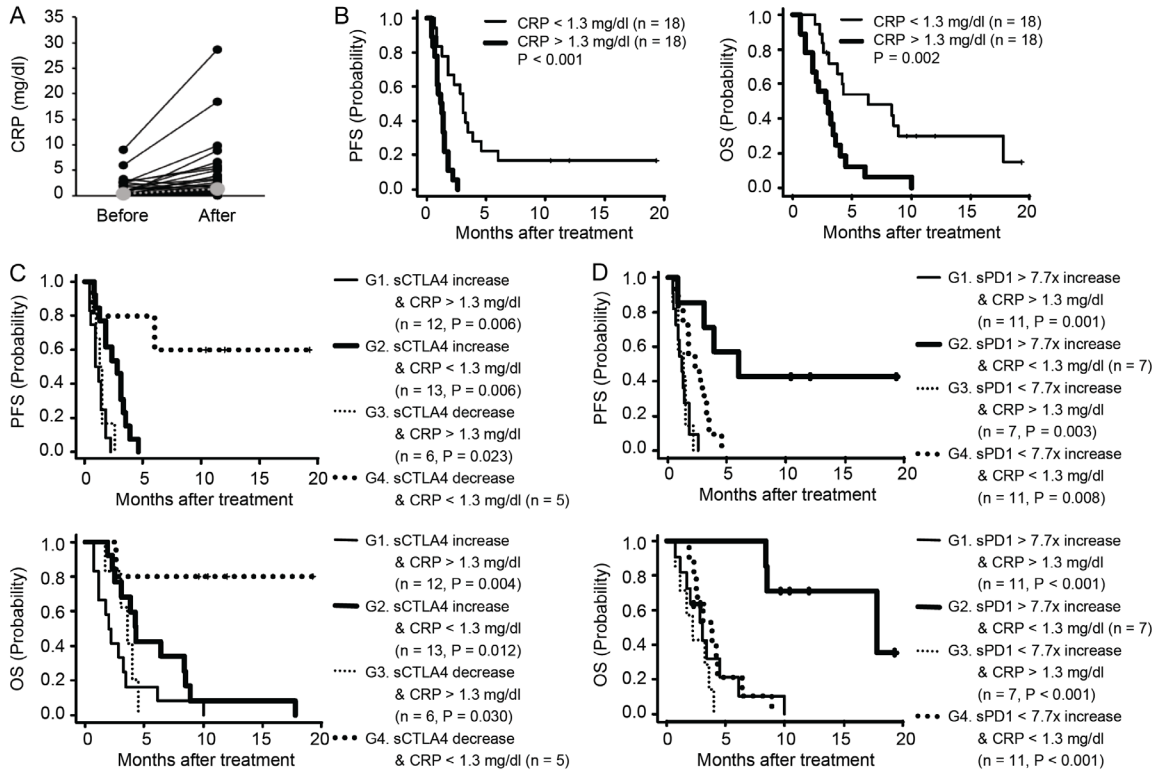
and Texs also increased (**Figure 4C**). Splenic CTL cytotoxicity was also significantly enhanced in the mice (P = 0.010 versus mIgG control; **Figure 4E**). These suggest blocking CTLA4 is effective in eliciting potent anti-tumor immunity in the host with increased CRP and sCTLA4.

CRP stimulation directly promotes tumor progression via CTLA4 upregulation

We further examined CRP effects on tumor cells. CRP stimulation suppressed tumor proliferation, but remarkably enhanced tumor invasion and sphere colony formation accompanied by increase of gene expressions of EMT/cancer stem-governing transcriptional factors (P < 0.001; **Figure 5A**). The size of the CRP-treated tumor colonies was much larger than that of the untreated control, suggesting a higher self-renewability. When the CRP-treated tumor cells were intravenously (i.v.) implanted in mice, tumor nodules significantly increased in lung (P < 0.001), and mouse survival was significantly shortened (P = 0.003) as compared to those of the control group implanted with untreated tumors (**Figure 5B**). These suggest CRP stimulation directly facilitates tumor progression and metastasis by conferring EMT/cancer stem-like malignant properties [20]. In the mice implanted with the CRP-treated tumors, anti-CTLA4 therapy, but not anti-PD1 therapy, significantly suppressed tumor dissemination to lung (P < 0.001 versus mIgG), and significantly prolonged mouse survival (P = 0.002). CTLA4 knockdown with the specific siRNAs in tumor cells abrogated the CRP-caused events (**Figure 5C**). These suggest blocking CTLA4 expressed on tumor cells is effective in impeding the CRP-caused tumor aggravation.

Treatment with rosiglitazone synergizes with anti-PD1 therapy in the CRP-injected tumor models

Anti-diabetic drugs have been shown to reduce CRP in diabetic patients [21, 22]. To enhance the anti-PD1 therapeutic efficacy, we chose to combine rosiglitazone (RSG), which is a highly



PFS	P values
G1 vs G2	0.001
G1 vs G3	0.405
G1 vs G4	0.006
G2 vs G3	0.014
G2 vs G4	0.006
G3 vs G4	0.023

OS	P values
G1 vs G2	0.042
G1 vs G3	0.438
G1 vs G4	0.004
G2 vs G3	0.138
G2 vs G4	0.012
G3 vs G4	0.030

PFS	P values
G1 vs G2	0.001
G1 vs G3	0.980
G1 vs G4	0.007
G2 vs G3	0.003
G2 vs G4	0.008
G3 vs G4	0.015

OS	P values
G1 vs G2	0.001
G1 vs G3	0.271
G1 vs G4	0.827
G2 vs G3	0.000
G2 vs G4	0.000
G3 vs G4	0.042

Figure 3. Combination of sCTLA4 decrease with lower CRP after nivolumab therapy defines responders with long-term survival. A. Changes of C-reactive protein (CRP) in sera after nivolumab therapy (n = 36). Gray dotted lines indicate each median. B. Lower CRP at posttreatment is associated with a favorable prognosis. PFS and OS were statistically compared between two groups divided by the median concentration (1.3 mg/dl) at posttreatment. C. Combination of sCTLA4 and CRP in sera after nivolumab therapy (n = 36). D. Combination of sPD1 and CRP in sera after nivolumab therapy (n = 36). *P* values were analyzed by the Kaplan-Meier method and the Mantel-Cox Log-Rank test.

selective agonist of PPARγ that is critical for adipogenesis and glucose metabolism, because other drugs directly damage liver [22]. RSG addition to the tumor culture rarely affected the CRP-induced sCTLA4 generation (**Figure 6A**). However, in vivo treatment with RSG partly but significantly suppressed the sCTLA4 increase in the CRP-injected mice (P < 0.001; **Figure 6B**), although the therapeutic efficacy on tumor growth was only partial (but significant, P = 0.035; **Figure 6C**). These results imply that RSG treatment may have lowered CRP in the in vivo setting, thereby indirectly suppressing the sCTLA4 production from tumor cells. RSG combination with anti-PD1 therapy significantly elicited great anti-tumor efficacy in the CRP-injected mice (P < 0.001 versus anti-PD1 monotherapy; **Figure 6C**). This combination regimen was also significantly effective in another tumor model that Colon26 cells were i.v. implanted in mice followed by CRP injection, providing a significant better prognosis (P = 0.005; **Figure 6D**). These suggest RSG therapy is an alternative strategy to overcome anti-PD1 resistance under CRP increase. Collectively, these results suggest CRP and sCTLA4 are diagnostic and therapeutic determinants in GI cancer. Targeting these molecules may be a promising strategy for improving clinical outcomes in the treatment of GI cancer.

Table 5. Patient characteristics divided by the median CRP after nivolumab treatment

Characteristics	< 1.3 mg/dl (n = 18)	> 1.3 mg/dl (n = 18)	*P* values
Median age (range)	66 (40-83)	64 (43-83)	0.807
Sex			0.500
Male	9 (50%)	12 (67%)	
Female	9 (50%)	6 (33%)	
Location of primary tumor			0.104
Gastric	18 (100%)	14 (78%)	
GEJ	0 (0%)	4 (22%)	
Histological subtype			0.738
Intestinal	9 (50%)	11 (61%)	
Diffuse	9 (50%)	7 (39%)	
HER2 status			1.000
Positive	3 (17%)	4 (22%)	
Negative	15 (83%)	14 (78%)	
Disease status			0.146
Unresectable	10 (56%)	15 (83%)	
Recurrent	8 (44%)	3 (17%)	
Organ with metastases			0.146
< 2	8 (44%)	3 (17%)	
≥ 2	10 (56%)	15 (83%)	
Metastatic site			
Liver	3 (17%)	11 (61%)	0.015
Lung	1 (6%)	2 (11%)	1.000
Peritoneum	13 (72%)	10 (56%)	0.489
Lymph node	8 (44%)	13 (72%)	0.176

Discussion

Our clinical study demonstrated that sCTLA4 decrease and low CRP level after nivo-therapy are good biomarkers to predict possible responders in AGC. The subsequent basic research revealed molecular mechanisms underlying the anti-PD1 resistance: CRP stimulation promotes tumor progression and metastasis directly and indirectly via sCTLA4 generation that suppresses CTL induction. We finally established treatment regimens with anti-CTLA4 mAb and RSG to overcome the CRP-induced anti-PD1 resistance. These regimens may be practically useful in the treatment of GI cancer.

CRP increase in sera is frequently seen in patients with inflammatory diseases, including cancer [18], and it is associated with advanced stages, metastasis, and a poor prognosis in various types of cancers, including GI cancer, in clinical settings [23]. CRP is known to promote tumor progression directly and indirectly via inducing immune suppression and exhaustion [19, 24]. However, immune suppression induced by CRP has not been observed in our in vivo setting, although CRP-induced sCTLA4 suppresses CTL induction in the in vitro setting. CRP stimulation directly conferred low proliferative but high proliferative and metastatic properties on tumor cells via upregulation of CTLA4/sCTLA4. Cancer stemness is represented by abnormal cell division, including both slow-cycling hypoproliferative and hyperproliferative properties, and the hypoproliferative tumor cells can convert to hyperproliferative upon stimulation due to its high plasticity [25, 26]. Probably, CRP injection in mice may induce a large amount of pro-inflammatory molecules, which may easily trigger trans-differentiation into a hyperproliferative type leading to outgrowth in the mice. CTLA4 expression in tumors has been reported in various types of cancers, including GC [27]. However, its clinical significance is still controversial. For example, CTLA4 positivity in tumors is associated with better survival [28], or meaningless in the prognosis [27]. The results may vary dependent

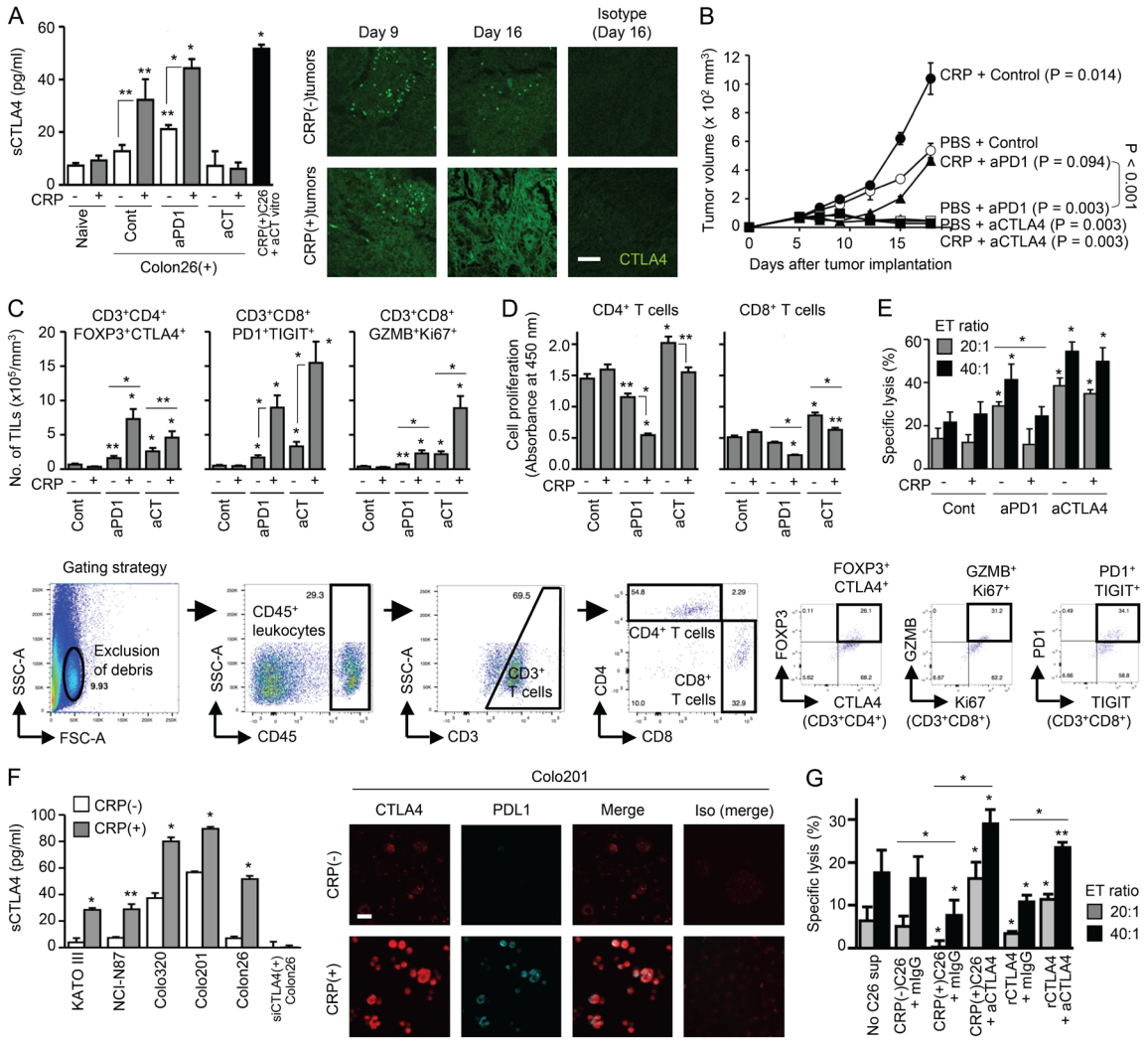


Figure 4. CRP injection increases sCTLA4 in the sera of mouse tumor models, leading to failure of anti-PD1 therapy. BALB/c mice were subcutaneously (s.c.) implanted with murine colorectal cancer Colon26 cells (5×10^5), and were intraperitoneally (i.p.) injected with anti-CTLA4 mAb, anti-PD1 mAb, or mouse IgG (mIgG) as a control at 10 mg/kg on days 5 and 9 after tumor implantation. CRP (200 μg) or PBS as a control was i.p. injected into the mice during the therapy (days 5, 7, and 9). Data on day 15. A. Increase of sCTLA4 in tumor-implanted mice after CRP injection. Isolated sera were tested for mouse sCTLA4 by ELISA (n = 3). Photos show CTLA4 expression in tumor tissues (scale = 50 μm). B. Anti-PD1 therapy fails in the CRP-injected mice (n = 5). C. Immune cells infiltrating in the tumor tissues (n = 5). The gating strategy for flow cytometric analysis is shown below. D. Splenic T-cell proliferation (n = 3). E. Cytotoxic activity of splenic cytotoxic T cells (n = 3). F. CRP stimulation upregulates CTLA4/sCTLA4 production in tumor cells. Tumor supernatants cultured with CRP (30 μg/ml) or PBS (24 hours) as a control were tested for sCTLA4 by ELISA (n = 3). Photos show membrane-bound CTLA4 (PE) and PDL1 (FITC) expressions on Colo201 cells (scale = 100 μm). G. Tumor-derived sCTLA4 suppresses CTL induction. Tumor supernatants or recombinant CTLA4 (1 μg/mL) were added to the CTL induction system, and the sorted $CD8^+$ T cells were tested for the cytotoxic activity (n = 3). Graphs show means ± SDs. *P < 0.01, **P < 0.05 versus control without CRP by the Mann-Whitney test. Representative data of an experiment out of three independent experiments with consistent results.

on the degree of CRP and CTLA4 shedding, or the antibody (epitope) used for the immunostaining.

The CRP-induced sCTLA4 suppressed CTL induction probably via interference of the CD28 activation signaling as reported elsewhere [14, 29], albeit further investigation is needed. Tregs have been considered as a prominent source of sCTLA4 so far [30]. However, at least in our study, the number of Tregs was very few in the tumor models, and therefore tumor-derived sCTLA4 is likely explainable for the sCTLA4. Myeloid cells, which are the majority of

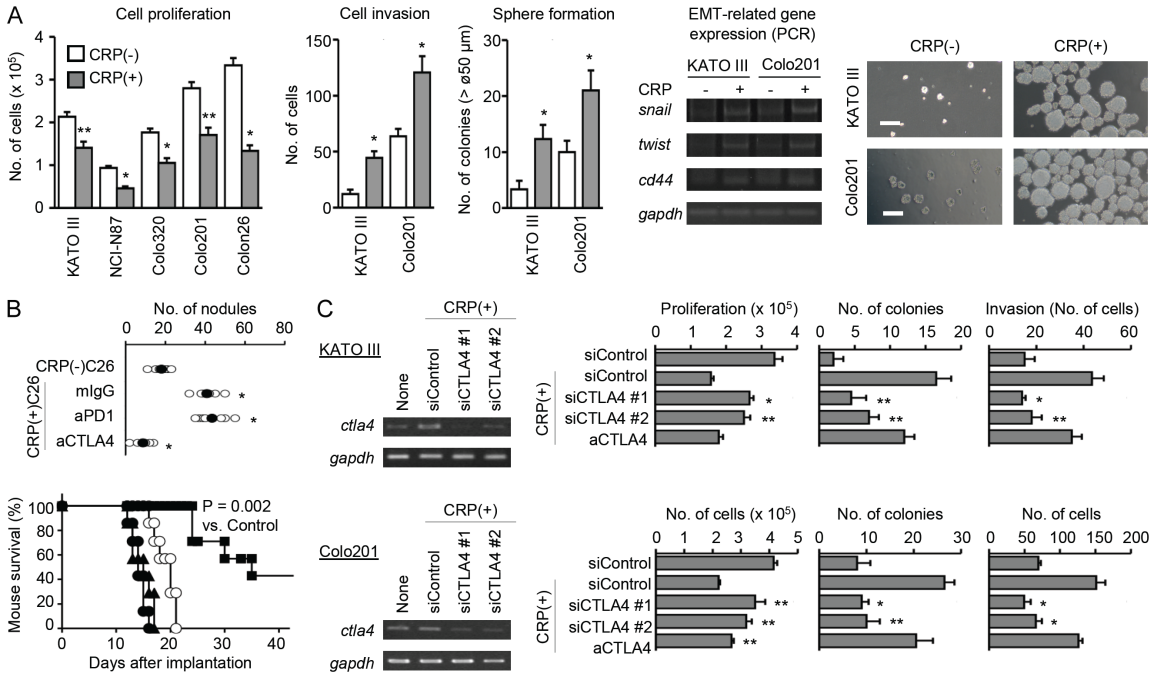


Figure 5. CRP stimulation directly promotes tumor progression via CTLA4 upregulation. A. CRP stimulation suppresses proliferation, but enhances invasive and self-renewal abilities of tumor cells accompanied by increase of EMT/cancer stem-related gene expressions. Tumor cells were stimulated with CRP (30 μg/ml) or PBS as a control for 24 hours before assays (n = 3). Right photos show the appearance of sphere colony formation (scale = 100 μm). *P < 0.01, **P < 0.05 versus CRP(-) group by the unpaired two-tailed Student's t test. B. CRP stimulation enhances in vivo metastatic ability of tumor cells. Mice were intravenously (i.v.) implanted with the CRP-treated Colon26 cells (2×10^5) into mice, and were i.p. injected with anti-CTLA4 mAb, anti-PD1 mAb, or mIgG as a control at 10 mg/kg on days 5 and 9 after tumor implantation (n = 7). Tumor nodules in lung were counted on day 15 (open circle, individual data; and closed circle, mean), or mouse survival was observed (open circles, untreated tumors + mIgG; closed circles, CRP-treated tumors + mIgG; closed triangles, CRP-treated tumors + anti-PD1 mAb; and closed squares, CRP-treated tumors + anti-CTLA4 mAb). *P < 0.01, **P < 0.05 versus CRP(-) group. Lung metastasis was analyzed by the Mann-Whitney test. Mouse survival was analyzed by the Kaplan-Meier method and the Mantel-Cox Log-Rank test. C. CTLA4 is a functional molecule regulating the CRP-induced malignant properties of tumor cells. Tumor cells were transfected with *ctla4*-specific siRNAs (siCTLA4) or non-targeting siRNA as a control (siControl), and were stimulated with CRP for 24 hours. In a group, anti-CTLA4 mAb (aCTLA4, 10 μg/ml) was added to the culture instead of siRNAs. Graphs show means ± SDs. *P < 0.01, **P < 0.05 versus CRP(+) siControl group by the unpaired two-tailed Student's t test. Representative data of an experiment out of three independent experiments with consistent results.

cellular components in the human immune system, also express IC molecules [31], $CTLA4^+$ myeloid cells may be another part of the sCTLA4 source. Regardless of the source, the specific protease to cleave CTLA4 remains unclear, and further investigation is needed. ADAM10 and/or ADAM17, sheddases to cleave PDL1 [9], may be involved because of pathogenic factors in GI cancer [32].

RSG therapy significantly reduced serum sCTLA4 in mouse tumor models, albeit no impact on the CRP-induced sCTLA4 increase in the tumor culture. In vivo RSG therapy may suppress de novo CRP induction caused by many other factors induced by CRP, resulting in decrease of sCTLA4. Several studies reported direct anti-tumor effects of RSG on tumor proliferation [33] and metastasis [34]. RSG therapy may help blunt the CRP-induced vicious cycle leading to treatment resistance.

Our study showed that anti-CTLA4 therapy was effective in the anti-PD1-resistant tumor models with increased CRP and sCTLA4. Several studies also reported CTLA4 expression in tumors [35] and high levels of sCTLA4 in sera [36] of melanoma patients, and they demonstrated that these markers are significantly correlated with anti-CTLA4 therapeutic efficacy. However, anti-CTLA4 ipilimumab monotherapy showed no significant benefits in the phase II

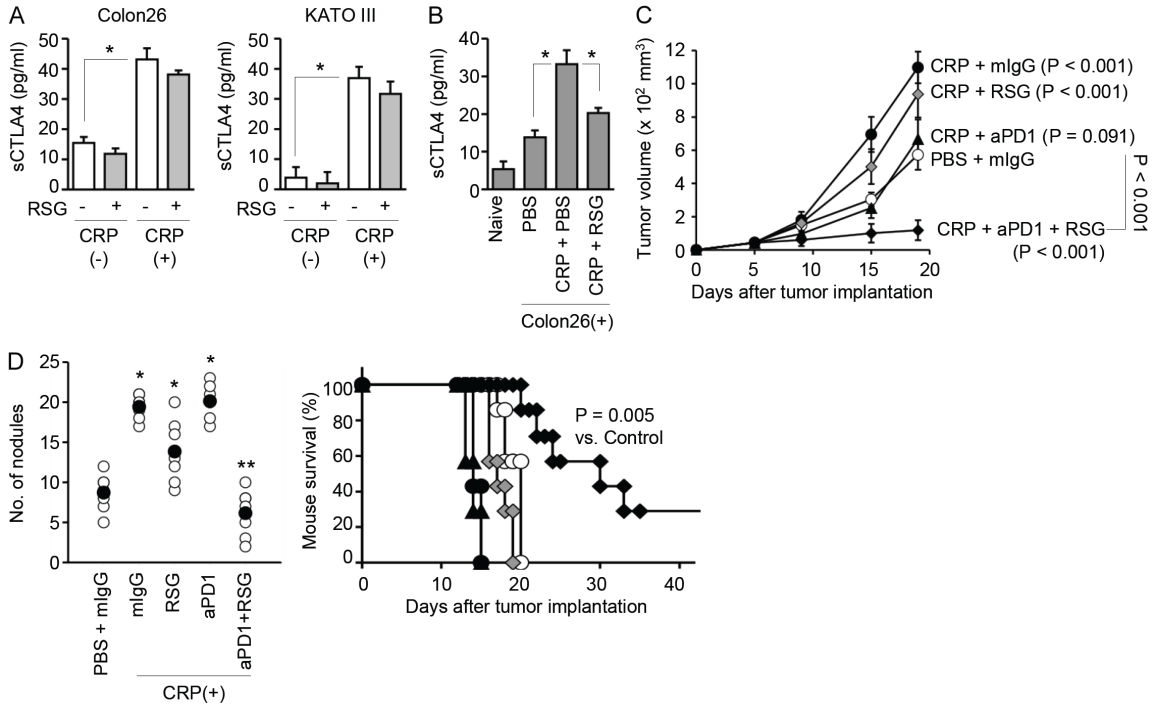


Figure 6. Treatment with rosiglitazone synergizes with anti-PD1 therapy in the CRP-injected tumor models. A. No direct effect of rosiglitazone (RSG) on the sCTLA4 production in tumor cells. Tumor cells were stimulated with CRP in the presence of RSG (10 μM) or PBS as a control for 24 hours, and the cultured supernatants were tested for sCTLA4 by ELISA (n = 3; means ± SDs). *P < 0.01 by the unpaired two-tailed Student's t test. B. Treatment with RSG reduces sCTLA4 increase in sera of the CRP-injected mice. Mice were i.p. injected with CRP (200 μg) on days 5, 7, and 9 after tumor implantation, and were orally administered with RSG (0.1 mg/kg) daily on days 5-11. The isolated sera were tested for sCTLA4 by ELISA on day 15 (n = 3; means ± SDs). *P < 0.01 by the unpaired two-tailed Student's t test. C. RSG therapy improves anti-PD1 therapeutic efficacy in the CRP-injected mice (n = 5; means ± SDs). Mice were s.c. implanted with Colon26 cells (5×10^5), and received i.p. injection with anti-PD1 mAb or mIgG (10 mg/kg) on days 5 and 9, and/or oral administration with RSG or PBS daily on days 5-11. CRP (200 μg) was i.p. injected into the mice during the therapy (days 5, 7, and 9). *P* values were analyzed by the Mann-Whitney test. D. Therapeutic efficacy induced by the anti-PD1/RSG combination therapy in another tumor model (n = 7). Mice were i.v. implanted with Colon26 cells (2×10^5), and received the same treatments described above. Tumor nodules in lung were counted on day 15 (open circle, individual data; and closed circle, mean), or mouse survival was observed (open circles, PBS + mIgG; closed circles, CRP + mIgG; gray diamonds, CRP + RSG; closed triangles, CRP + anti-PD1 mAb; and closed diamonds, CRP + RSG + anti-PD1 mAb). *P < 0.01, **P < 0.05 versus PBS + mIgG. Lung metastasis was analyzed by the Mann-Whitney test. Mouse survival was analyzed by the Kaplan-Meier method and the Mantel-Cox Log-Rank test. Significance of aPD1/RSG combination to the single treatment was also evaluated by a two-way ANOVA with Bonferroni post-hoc test. Representative data of an experiment out of three independent experiments with consistent results.

study for AGC as compared to the best support care [37]. Also, in the Checkmate649 study, nivolumab plus ipilimumab did not improve OS as compared to chemotherapy alone in gastro-esophageal cancer [38]. One possible reason may be due to high rates of adverse events and early death in the nivo/ipi-treated patients. Probably, double blockade of the immune brakes may intensely spark immune responses, and may expand Tregs to maintain self-tolerance, exhausted T cells due to over-activation, and immature T cells due to quick generation without education/activation. These adverse effects may permit tumor progression and metastasis leading to a poor prognosis. Therefore, ipilimumab may be better used after anti-PD1 therapy as a follow-up treatment based on the serum CRP/sCTLA4 levels.

In conclusion, this study provides a strong rationale of targeting CRP and CTLA4 for overcoming anti-PD1 resistance in GI cancer, and may facilitate practical implementation of the established regimens for improving the clinical outcomes.

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Disclosure of conflict of interest

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