Cancer Science
Japanese Cancer Association JCA
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Intermittent chemotherapy can retain the therapeutic potential of anti-CD137 antibody during the late tumor-bearing state

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Key words

Antibody, CD137, cyclophosphamide, gemcitabine, myeloid-derived suppressor cells

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Funding Information

This study was supported in part by grants from the Ministry of Education, Science, Sport, Culture, and Technology of Japan (no. 25430150 to N. H. and no. 24501331 to M. H.) and from the Shimane University "SUIGANN" Project.

Received August 25, 2014; Revised October 20, 2014; Accepted October 28, 2014

Cancer Sci **106** (2015) 9–17

doi: 10.1111/cas.12568

Immunomodulating monoclonal antibodies (mAb) can evoke antitumor T-cell responses, which are attenuated by regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC). Treatment with cyclophosphamide (CP) and gemcitabine (GEM) can mitigate the immunosuppression by Treg and MDSC, respectively. In the current study, we examined the antitumor effects of a combination of local injection with anti-CD137 mAb and intermittent low-dose chemotherapy using CP and GEM in subcutaneously established CT26 colon carcinoma. Although a significant antitumor effect was observed when local anti-CD137 mAb therapy (5 μg) was started early in the tumor-bearing stage (day 10), no therapeutic efficacy was observed when the mAb therapy was started at a later tumor-bearing stage (day 17). Analyses of the tumor-infiltrating immune cells revealed that the number of Gr-1$^{high/low}$ CD11b^{+} MDSC started to increase 13 days after tumor inoculation, whereas injection with low-dose (50 mg/kg) CP and GEM mitigated this increase. In addition, although intermittent injections with low-dose CP and GEM on days 10 and 18 suppressed tumor growth significantly, additional local injections of anti-CD137 mAb on days 19, 21, and 23 further augmented the therapeutic efficacy. Cytotoxic T lymphocytes reactive to CT26 and a tumor antigen peptide were induced successfully from the spleen cells of tumor-cured or tumor-stable mice. In a bilateral tumor inoculation model, this combination therapy achieved systemic therapeutic effects and suppressed the growth of mAb-untreated tumors. These results suggest that intermittent immunochemotherapy using CP and GEM could retain the therapeutic potential of anti-CD137 mAb that is normally impaired during the late tumor-bearing stage.

Monoclonal antibodies (mAb) that are capable of modulating T-cell function in tumor-bearing hosts have received significant attention as promising anti-cancer therapies. Examples include mAb against immune checkpoint molecules, such as programmed cell death-1 (PD-1), PD-L1 and cytotoxic T-lymphocyte-associated protein 4 (CTLA4).[1] Additional mAb target co-stimulatory molecules such as CD137 (4-1BB), OX40 and glucocorticoid-induced tumor necrosis factor receptor (GITR). The systemic administration of anti-CD137 mAb can induce significant antitumor effects in tumor-bearing hosts.[2,3] CD137 is expressed on T cells, natural killer (NK) cells, dendritic cells and tumor endothelial cells.[4–7] Although NK cells contribute to the anti-CD137 mAb-induced antitumor effect,[4] antitumor T cells are the main effectors of this mAb therapy.[3] The systemic administration of anti-CD137 mAb is accompanied by a risk of liver inflammation.[8,9] However, local treatment with low doses of anti-CD137 mAb could elicit antitumor effects without causing hepatic side effects.[10]

To maximize the therapeutic efficacy of anti-cancer immunotherapies, preventing immunosuppression in the tumor-bearing hosts is essential. CD4^{+} CD25^{+} regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSC) are the two major cell types responsible for immunosuppression in tumor-bearing hosts.[11,12] Tregs can suppress the effects of anti-cancer immunotherapy, and their presence at local tumor sites correlates with an unfavorable prognosis.[13,14] The number of MDSC is increased in cancer-bearing hosts, and can inhibit T-cell responses in cancer patients.[15,16] To overcome the effects of these cells, several methods that use antibodies or reagents have been proposed.[17–22] Alternatively, several chemotherapeutic drugs modulate these immune responses. The administration of low doses of cyclophosphamide (CP) can mitigate Treg-mediated immunosuppression,[23–28] and low-dose gemcitabine (GEM) decreases the number of MDSC selectively in cancer-bearing hosts.[29,30] We reported recently that intermittent chemotherapy (at 8-day intervals) with low-dose CP and GEM could mitigate Treg- and MDSC-mediated immunosuppression, elicit antitumor T-cell immunity, and regress established tumors.[31]

In this study, we used a CT26 colon carcinoma mouse model to demonstrate that low-dose local anti-CD137 mAb therapy was very effective when started at an early

tumor-bearing stage (day 10), but that the therapeutic efficacy was lost when therapy was started at a later tumor-bearing stage (day 17) because of a drastic increase in the number of MDSC at the tumor sites. However, the combination of intermittent low-dose chemotherapy with CP and GEM and subsequent local injections with low-dose anti-CD137 mAb induced a remarkable antitumor effect. In a bilateral tumor inoculation model, this combination therapy suppressed the growth of the tumor on the mAb-untreated side. These results suggest that intermittent immunochemotherapy using CP and GEM could retain the therapeutic potential of anti-CD137 mAb that is normally impaired at the late tumor-bearing stage.

Materials and Methods

Mice and tumor cell lines. BALB/c and BALB/c nu/nu female mice (H-2^d: 6–7-weeks old) were purchased from CLEA Japan (Tokyo, Japan) and Japan SLC (Hamamatsu, Japan), respectively. Mice were maintained under specific pathogen-free conditions. Experiments were performed according to the ethical guidelines for animal experiments of the Shimane University Faculty of Medicine (IZ26-5). CT26 and P815 are colon carcinoma and mastocytoma cell lines of BALB/c and DBA/2 mouse origin, respectively.[32] RENCA is a renal cell carcinoma of a BALB/c mouse origin and was provided by Dr Eto, Department of Urology, Kyushu University.[33] All cell lines were maintained in RPMI 1640 supplemented with 10% FBS.

Treatment protocol. BALB/c mice were injected s.c. into the right flank with 5×10^5 CT26 cells. The mice received an i.t. injection with 5 μg of anti-CD137 mAb, clone 2A in a volume of 50 μL on the indicated days.[34] The same volume of rat IgG was injected as an isotype-matched control. Some mice received an i.p. injection of CP (Shionogi, Osaka, Japan) and GEM (Selleck Chemicals, Houston, TX, USA) at a dose of 50 mg/kg at 8-day intervals as indicated. In some experiments, CT26 cells were injected s.c. into the bilateral flanks. These mice were injected i.p. with CP and GEM, followed by an i.t. injection with anti-CD137 mAb or control IgG to the right-flank tumor. Tumor size (mm^2) was measured twice weekly.

Flow cytometry. To assess the expression of CD137 on $CD4^+$ or $CD8^+$ T cells in tumor-bearing mice, BALB/c mice were inoculated s.c. with 5×10^5 CT26 cells into the flank. On day 12, the draining lymph nodes (LN), spleen and tumor tissues were harvested, minced using slide glasses, and passed through gauze mesh. The cells were then stained with the indicated mAb and analyzed using flow cytometry with a FACScaliber (Becton-Dickinson, Fullerton, CA, USA). In some experiments, the effect of chemotherapy on tumor-infiltrating immune cells was assessed after the i.p. injection of CP (50 mg/kg) and GEM (50 mg/kg). Tumor tissues were harvested 3, 6 and 9 days after injection with CP and GEM, stained with the indicated mAb, and analyzed by flow cytometry. In other experiments, the combination effect of chemotherapy with CP/GEM and i.t. injection with anti-CD137 mAb (5 μg) on tumor-infiltrating immune cells was assessed. The following mAb were used for staining: FITC-conjugated anti-CD137mAb (BD Biosciences, San Jose, CA, USA), FITC-conjugated rat IgG1 (BD Biosciences), APC-conjugated anti-CD45 (BioLegend, San Diego, CA, USA), PE-conjugated anti-CD4 (AbD Serotec, Oxford, UK), PE-conjugated anti-CD8 (Southern Biotech, Birmingham, AL, USA), PE-conjugated anti-CD11b (BioLegend) and FITC-conjugated anti-Gr-1 (R&D systems, Minneapolis, MN, USA). To examine Treg in tumor tissues, cell suspension of tumor tissues was stained with APC-conjugated anti-CD45 (BioLegend) and PE-conjugated anti-CD4 (AbD Serotec). After fixing with IntraPrep Permeabilization Reagent (Beckman Coulter, Brea, CA, USA), these cells were satined with anti-Foxp3 mAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The gating strategy is shown in Supplementary Figure S1.

Protective immunity assay. Mice that were CT26-cured after the combination therapy were injected s.c. with 2×10^5 CT26 cells in the right flank and 2×10^5 RENCA cells in the left flank 60 days after the initial tumor inoculation. Tumor size (mm^2) was then measured twice weekly.

Cytotoxicity assays. H-$2L^d$-binding peptide (SPSYVYHQF) is a tumor antigen peptide derived from the envelope protein (gp70) of an endogenous murine leukemia virus. It is a CT26-associated tumor-derived peptide, [35] and is designated AH1 in the current study. Measles virus hemagglutinin (SPGRSFSYF) was used as an H-$2L^d$-binding control peptide. All peptides were >80% pure, and were purchased from Invitrogen (Carlsbad, CA, USA). On day 38 after tumor inoculation, spleen cells were cultured *in vitro* with AH1 peptide (10 μg/mL) in the presence of IL-2 (20 U/mL) for 4 days. Thereafter, their cytotoxicity was measured using a 5 h ^{51}Cr-release assay.

RT-PCR. Total RNA was extracted and first-strand cDNA was generated using the Superscript III First-Strand Synthesis System (Invitrogen) and random primers. Template cDNA were subjected to 28 cycles of PCR using Platinum *Taq* DNA polymerase (Invitrogen). The following primers (sense and antisense, respectively) were used: gp70, 5′-ACCTTGTCCGA AGTGACCG-3′ and 5′- GTACCAATCCTGTGTGGTCG-3′; and β-actin, 5′-TGGAATCCTGTGGCATCCATGAAAC-3′ and 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′. The PCR products were resolved on 1.5% agarose gels, stained with ethidium bromide, and photographed.

Statistical analysis. Data were evaluated using the unpaired two-tailed Student's *t*-test, ANOVA with Bartlett's test, or Fisher's exact probability tests. A *P*-value of <0.05 was considered to indicate statistical significance.

Results

Loss of therapeutic efficacy of local anti-CD137 monoclonal antibody (mAb) therapy in the late tumor-bearing stage. We first examined the expression of CD137 on draining LN cells, spleen cells and tumor-infiltrating lymphocytes (TIL) of CT26-bearing mice 12 days after tumor inoculation. Both $CD4^+$ and $CD8^+$ T cells in TIL, but not draining LN or splenic cells, were weakly positive for CD137 (Fig. 1a), consistent with a previous report.[10] We next examined the antitumor effects induced by the intratumoral delivery of low-dose (5 μg) anti-CD137 mAb. The i.t. injection with anti-CD137 mAb on days 10, 12 and 14 after tumor inoculation suppressed tumor growth significantly compared with the injection of control rat IgG (Fig. 1b). This antitumor effect was T-cell-dependent because no antitumor effect was observed in CT26-bearing nude mice (Fig. 1c). We also assessed whether the anti-CD137 mAb therapy was effective when started at a later tumor-bearing stage. Although the tumor in one mouse regressed and another mouse showed continuous suppression of the tumor growth, i.t. injections of anti-CD137 mAb on days 17, 19 and 21 were not effective (Fig. 1d).

Drastic increase in the number of myeloid-derived suppressor cells at the late stage tumor site. To investigate why the

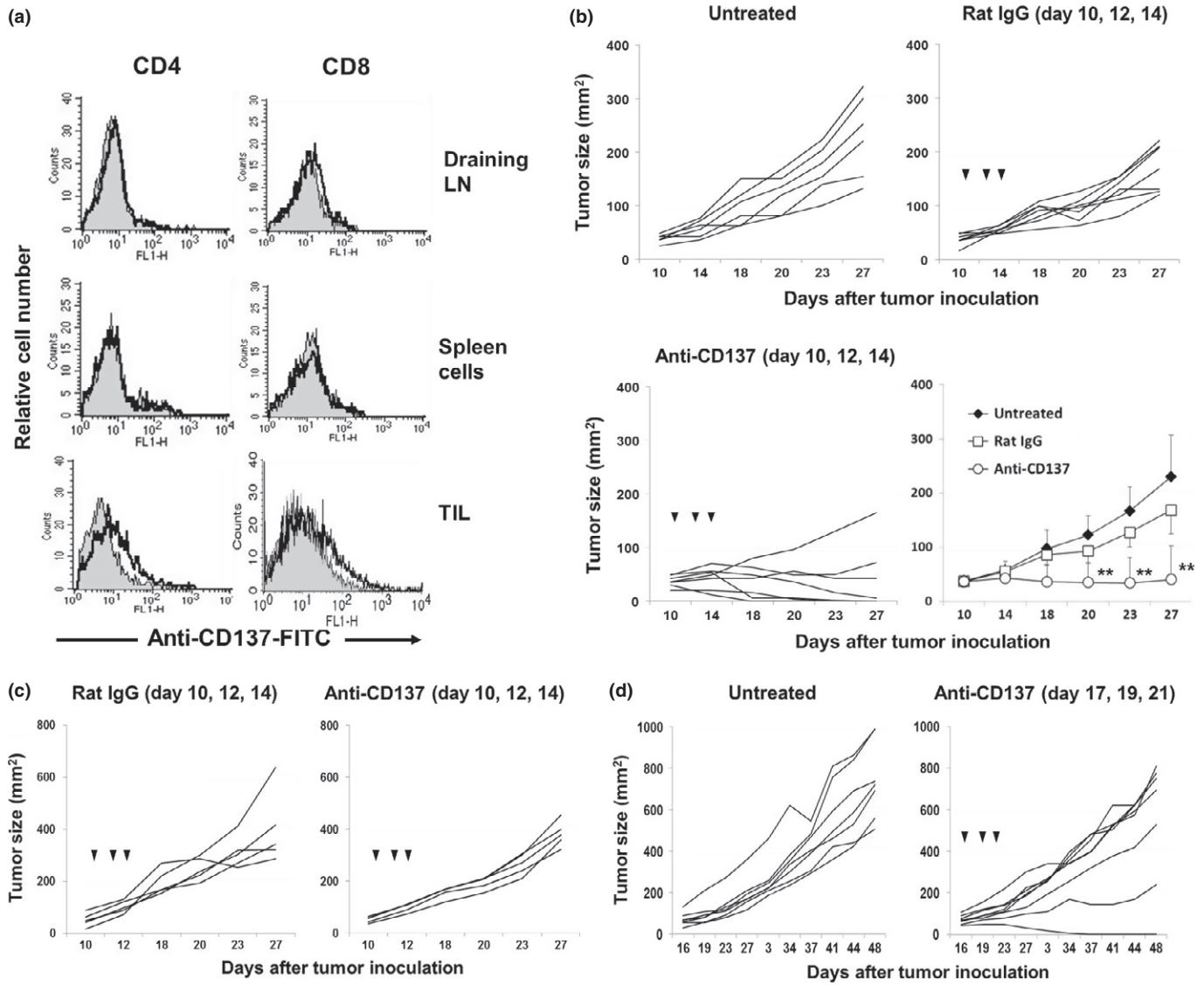


Fig. 1. Antitumor effects of local anti-CD137 mAb therapy in early or late stage tumors. (a) Twelve days after CT26 inoculation, draining LN, spleen and tumor tissues were harvested and their cells were analyzed using flow cytometry. The results of gating for $CD4^+$ or $CD8^+$ cells are shown, and the gray background shows an isotype-matched control. Similar results were obtained from two independent experiments. (b) BALB/c mice were injected s.c. in the flank with 5×10^5 CT26 cells. They were then injected i.t. with anti-CD137 mAb (5 μg) or rat IgG on days 10, 12 and 14. Tumor size (mm^2) was measured twice weekly. Each group contained six or seven mice, and lines represent tumor growth. The arrowheads represent the timing of mAb injection. Data are presented as means ± SD (lower right panel). Similar results were obtained from two independent experiments. $^{**}P < 0.01$ (ANOVA with Bartlett's test). (c) CT26-bearing BALB/c nude mice were treated as described in (b). (d) CT26-bearing BALB/c mice were treated with anti-CD137 mAb (5 μg) on days 17, 19 and 21 after tumor inoculation. Each group contained seven mice, and lines show tumor growth. Arrowheads indicate the timing of mAb injection.

therapeutic efficacy of local anti-CD137 mAb therapy was lost at the late tumor-bearing stage, we quantified the number of tumor-infiltrating immune cells (Fig. 2a). The percentage of $CD45^+$ immune cells, particularly $CD11b^+$ $Gr\text{-}1^{high/low}$ MDSC, in the tumor tissues increased significantly 13 days after tumor inoculation. Although the percentage of $CD4^+$ T cells in TIL decreased gradually, the number of $CD8^+$ T cells increased slightly. In addition, the percentage of Tregs increased in TIL on days 13 and 18 after tumor inoculation. However, given the low percentages of total $CD4^+$ T cells in TIL, MDSC appeared to be responsible for the impaired efficacy of local anti-CD137 mAb therapy in the late tumor-bearing stage. Representative results of the number of $CD45^+$ immune cells and MDSC on days 10 and 18 after tumor inoculation are shown in Figure 2(b).

We recently reported that intermittent (8-day intervals) chemotherapy using low-dose CP and GEM (50 mg/kg) could induce antitumor effects against subcutaneously established CT26 tumors by inhibiting the accumulation of MDSC and Tregs at tumor sites.[31] Therefore, we quantified the number of $CD45^+$ immune cells and MDSC at the tumor sites when low doses of CP and GEM were injected i.p. into mice 10 days after tumor inoculation. This immunochemotherapy could mitigate the increase in $CD45^+$ cells at the tumor sites, especially $CD11b^+$ $Gr\text{-}1^{high/low}$ MDSC (Fig. 2c).

Antitumor effect of intermittent chemotherapy followed by local anti-CD137 monoclonal antibody (mAb) therapy. We next examined the antitumor effect of the combination of anti-CD137 mAb and intermittent chemotherapy with low-dose CP

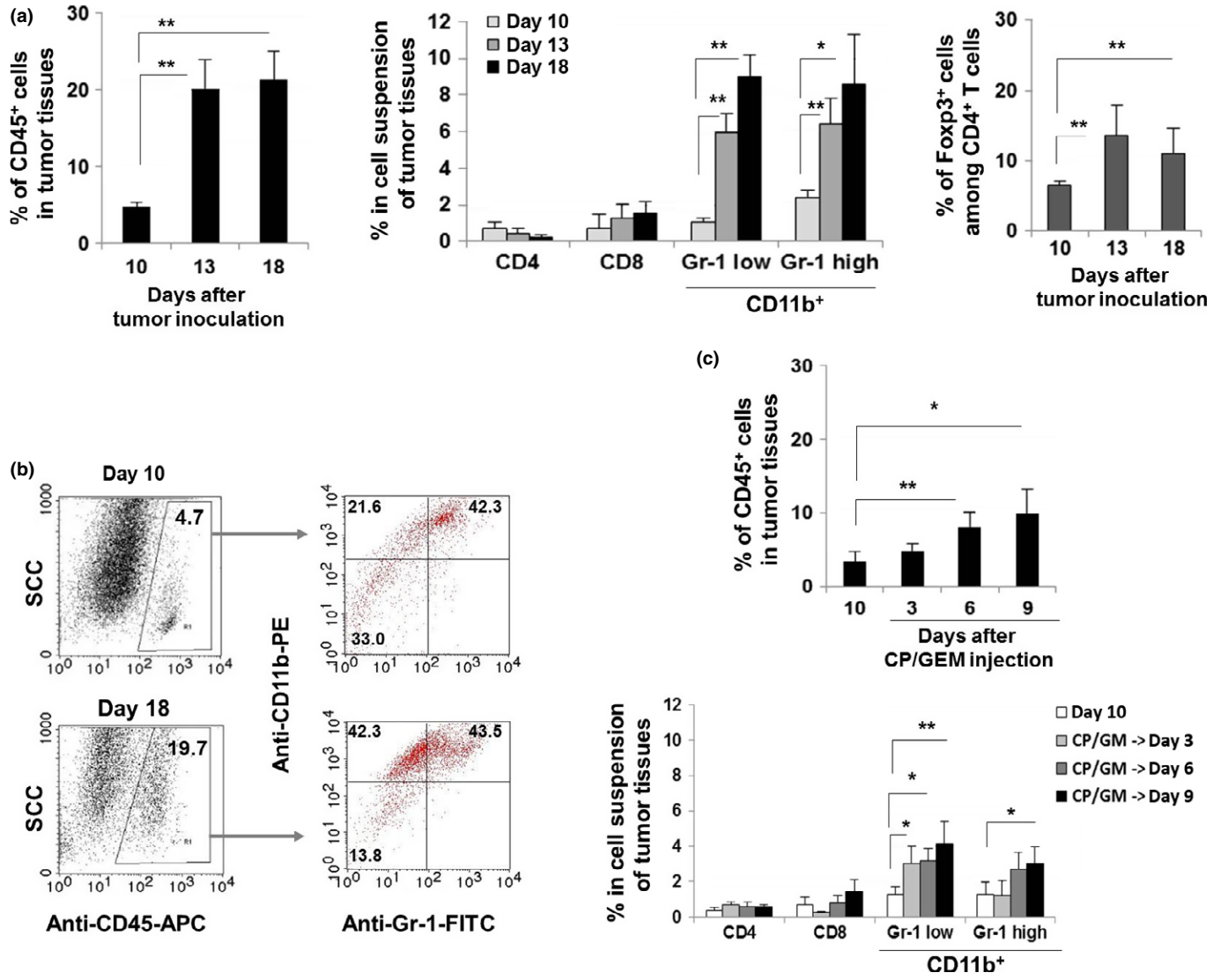


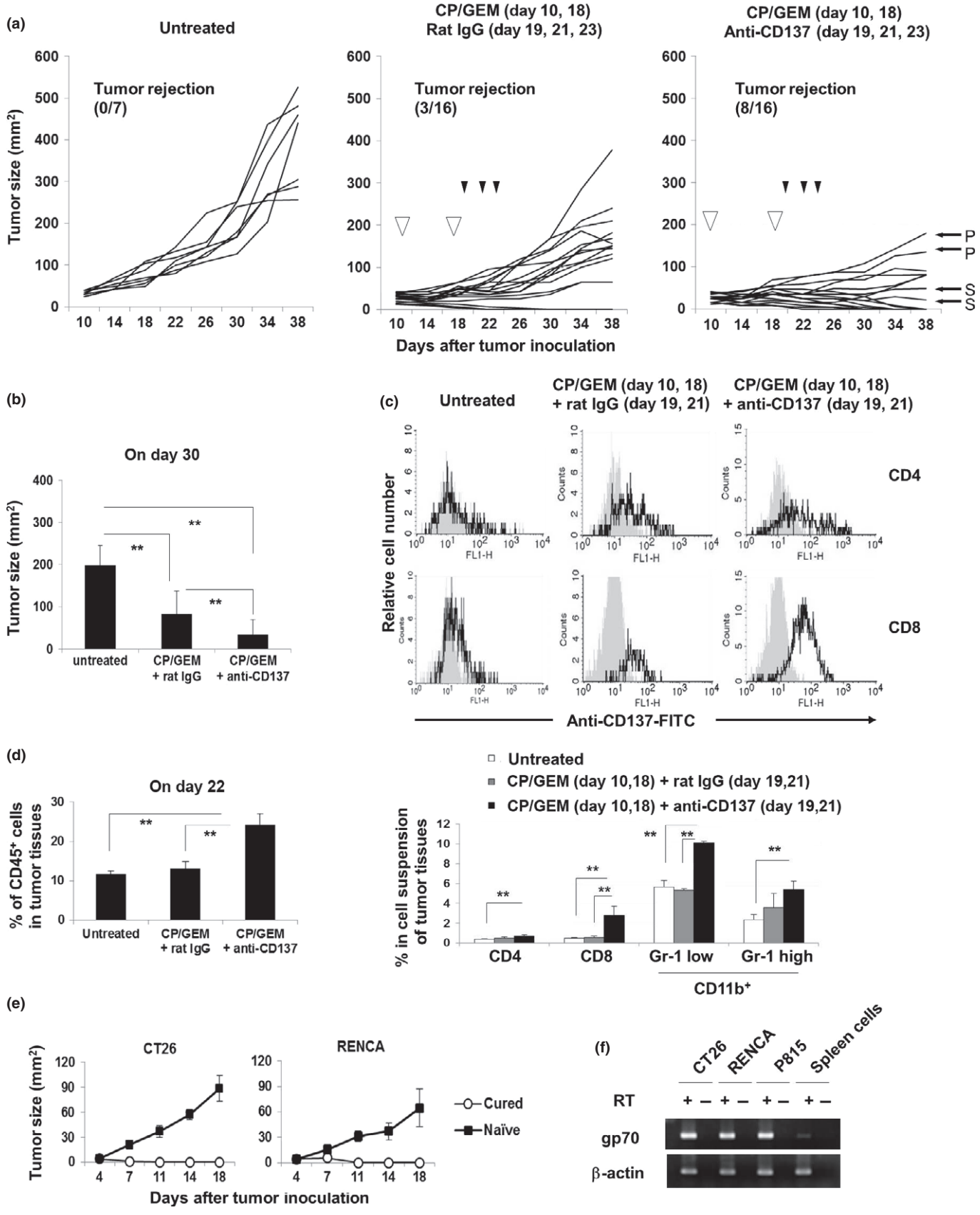
Fig. 2. Increased myeloid-derived suppressor cells at the sites of late-stage tumors. (a) BALB/c mice were injected s.c. in the flank with 5×10^5 CT26 cells. Tumor tissues were harvested on the indicated days, and tumor tissue cell suspensions were analyzed using flow cytometry. Each data point shows the means ± SD of four samples. Similar results were obtained from two independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ (Student's *t*-test). (b) Representative data from tumor-infiltrating immune cells 10 or 18 days after CT26 inoculation; numbers represent the percentage of each cellular subset. (c) Ten days after CT26 inoculation, CP (50 mg/kg) and GEM (50 mg/kg) were injected i.p. On days 3, 6 and 9 after injection of CP and GEM, the tumor-infiltrating immune cells were analyzed using flow cytometry. Data show the means ± SD of three samples. Similar results were obtained from two independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ (Student's *t*-test).

and GEM (Fig. 3a,b). Intermittent chemotherapy on days 10 and 18 suppressed tumor growth significantly, and 3 of the 16 mice were cured by chemotherapy alone. When the intermittent chemotherapy was followed by local anti-CD137 mAb therapy, more significant tumor regression was observed; 8 of the 16 mice were cured. Although some mice were not cured, they showed continuous growth suppression, so-called "stable disease," for more than 2 weeks after the last Ab therapy.

We examined tumor-infiltrating immune cells when treated with low-dose chemotherapy (twice) with or without local

Fig. 3. Tumor regression after combination therapy. (a) BALB/c mice were injected s.c. in the flank with 5×10^5 CT26 cells. CP (50 mg/kg) and GEM (50 mg/kg) were then injected i.p. on days 10 and 18, followed by the i.t. injection of anti-CD137 mAb (5 μg) or rat IgG on days 19, 21 and 23. White arrows indicate the injection of CP and GEM, and black arrows indicate injections of Ab. Tumor size (mm^2) was measured twice weekly. The untreated group contained 7 mice, and the treated groups contained 16 mice. Similar results were obtained in two experiments. The letters "P" and "S" in the right figure are representative of tumor-progressing and tumor-stable mice, respectively. These mice were used in the experiment shown in Figure 4. (b) Means ± SD of the results on day 30 after tumor inoculation. $^{**}P < 0.01$ (ANOVA with Bartlett's test). (c) CT26-bearing BALB/c mice were injected i.p. with CP (50 mg/kg) and GEM (50 mg/kg) on days 10 and 18, followed by the i.t. injection of anti-CD137 mAb (5 μg) or rat IgG on days 19 and 21. On day 22, the tumor tissues were harvested and the CD137 expression of $CD4^+$ or $CD8^+$ T cells was examined by flow cytometry. The gray background shows an isotype-matched control. (d) Similarly, the tumor-infiltrating immune cells were analyzed. Data show the means ± SD of three samples. $^{**}P < 0.01$ (Student's *t*-test). (e) CT26 and RENCA (2×10^5 cells) were inoculated s.c. and bilaterally on day 60 after the initial inoculation. Naïve BALB/c mice were also inoculated with CT26 and RENCA cells. Tumor growth was measured twice weekly. Each group contained six mice, and data are presented as means ± SD. (f) Total RNA was extracted from three tumor cell lines and normal spleen cells, and *pg70* mRNA expression was examined using RT-PCR. RT, reverse transcriptase. β-actin was used as a control.

injections with anti-CD137 mAb. On day 22 after tumor inoculation, low expression of CD137 was observed on tumor-infiltrating $CD8^+$ T cells, but not $CD4^+$ T cells, from untreated mice (Fig. 3c). In contrast, the expression of CD137 was clearly detected on CT26-bearing mice that were treated with chemotherapy with CP/GEM with or without CD137 therapy,



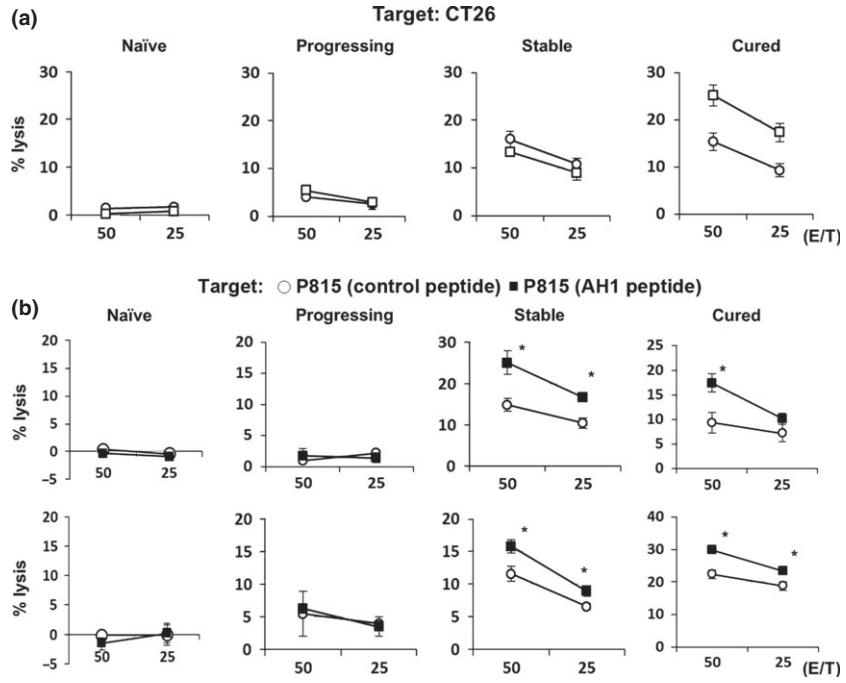


Fig. 4. Tumor-reactive and AH1 peptide-recognizing CTL in CT26-cured or CT26-stable mice after combination therapy. On day 38 after tumor inoculation, spleen cells from naïve mice and CT26-progressing, CT26-stable or CT26-cured mice after combination therapy were cultured *in vitro* with AH1 peptide in the presence of IL-2 (20 U/mL) for 4 days. (a) The cytotoxicity against CT26 cells was examined using a 5 h ^{51}Cr-release assay. Each group contained two mice. CT26-progressing and -stable mice correspond to "P" and "S" in Figure 3(a), respectively. (b) The cytotoxicity against P815 cells pre-treated with control or AH1 peptide was examined. *$P < 0.05$ (Student's *t*-test).

suggesting that this intermittent low-dose chemotherapy can increase/keep the expression of CD137 on TIL. In addition, the combination therapy significantly increased the percentages of tumor-infiltrating CD45$^+$ immune cells compared with the other two groups, and infiltrating CD8$^+$ T cells and MDSC were responsible for this increase (Fig. 3d).

CT26-cured mice exhibited protective immunity against re-challenge with CT26 60 days after the initial tumor inoculation (Fig. 3e). Unexpectedly, CT26-cured mice also rejected RENCA cells, which is a renal carcinoma of BALB/c origin. Therefore, we assessed the possibility that CT26 and RENCA shared tumor antigens, and found that both CT26 and RENCA cells express *gp70* mRNA, which encodes the envelope protein of an endogenous murine leukemia virus that is a known CT26 tumor antigen (Fig. 3f).[35] *gp70* mRNA was also expressed in P815 mastocytoma cells, but not in normal spleen cells.

Tumor-reactive cytotoxic T-lymphocytes (CTL) in CT26-cured or CT26-stable mice after combination therapy. We next analyzed the tumor-reactive cytotoxic T-lymphocytes (CTL) in CT26-progresssing, CT26-stable or CT26-cured mice after combination therapy. The spleen cells from these three groups and naïve mice were stimulated with AH1 peptide *in vitro* and their cytotoxicity against CT26 cells was examined (Fig. 4a). CT26-progresssing and CT26-stable mice were designated "P" and "S," respectively in Figure 3(a). Each group contained two mice. The means of tumor size (mm^2) of "P" and "S" were 157.5 and 35.8, respectively. Cytotoxicity against CT26 was observed in the spleen cells of CT26-stable and CT26-cured, but not naïve, mice. In addition, a low level of cytotoxicity was observed in the spleen cells of CT26-progressing mice. We also assessed the cytotoxicity against P815 (H-2^d) cells that had been pulsed with either control or AH1 peptide (Fig. 4b). Some cytotoxicity against P815 was induced in the spleen cells of CT26-stable and CT26-cured mice, likely because P815 cells express gp70 (Fig. 3f). In addition, spleen cells from CT26-stable and CT26-cured mice showed higher cytotoxicity against AH1 peptide-pulsed P815 cells than against control peptide-pulsed P815 cells, providing indirect evidence that AH1 peptide-specific CTL were induced in these mice.

Antitumor effect of the combination therapy on monoclonal antibody-untreated tumors. Finally, we assessed whether combination treatment with intermittent chemotherapy and anti-CD137 mAb exerted an antitumor effect on tumors not treated with mAb on the opposite flank of the mice. Mice were injected s.c. and bilaterally with CT26 cells, and anti-CD137 mAb therapy was administered locally to the right-side tumor (Fig. 5a,b). Although there was no statistically significant tumor growth of the right-side tumors between the mice treated with chemotherapy and control Ab and those with chemotherapy with anti-CD137 mAb, the combination therapy suppressed the growth of mAb-untreated tumors significantly (Fig. 5a,b). In addition, the tumor regression rate after combination therapy was significantly higher than that after the combination of chemotherapy and control Ab (Table 1).

Discussion

Before examining the effect of the combination of intermittent immunochemotherapy and local anti-CD137 mAb therapy, we first confirmed that CD137 molecules were expressed on tumor-infiltrating CD8$^+$ or CD4$^+$ T cells, but not on T cells from the draining LN and spleen of tumor-bearing mice (Fig. 1a). Palazon *et al.*[10] reveal that the selective expression of CD137 is the result of the response of T cells to hypoxia in the tumor sites. Given that the systemic administration of anti-CD137 mAb is accompanied by a risk of liver inflammation,[8,9] the local delivery of immunostimulatory anti-CD137 mAb to tumor sites is a reasonable method for eliciting potent

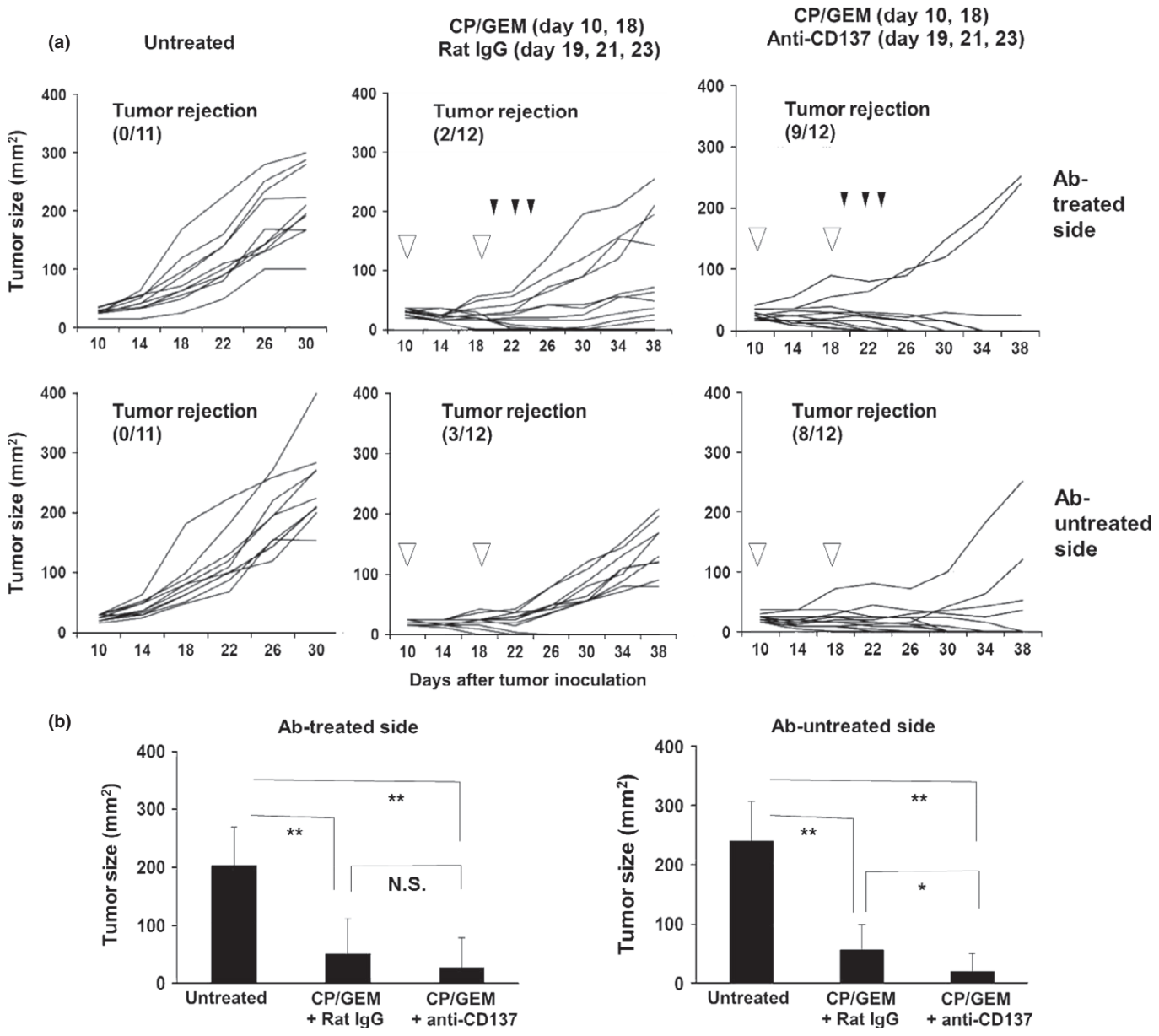


Fig. 5. Systemic antitumor effects of combination therapy. (a) BALB/c *mice* were injected s.c. and bilaterally with CT26 (right flank, 5×10^5 cells; left flank, 2.5×10^5 cells). CP (50 mg/kg) and GEM (50 mg/kg) were injected i.p. on days 10 and 18. Subsequently, anti-CD137 mAb (5 μg) or rat IgG was injected i.t. into the CT26 tumor on the right flank on days 19, 21 and 23. White arrows indicate the injection of CP and GEM, and black arrows indicate the local injections of Ab. Tumor size (mm^2) was measured twice weekly. There were 11 mice in the control group, and 12 mice in the treatment group. (b) The statistical significance of tumor size was evaluated on day 30 after tumor inoculation. $^*P < 0.05$, $^{**}P < 0.01$ (ANOVA with Bartlett's test) N.S., not significant.

Table 1. Combination therapy suppressed the growth of the tumor of the flank not treated with anti-CD137 mAb

Side	Mice were treated with	Number of mice		*P*-value
		Not cured	Cured	
Ab-treated side	CP/GEM + rat IgG	10	2	<0.01
	CP/GEM + α-CD137	3	9	
Ab-untreated side	CP/GEM + rat IgG	9	3	<0.05
	CP/GEM + α-CD137	4	8	

CT26-bearing mice were treated as described in Figure 5(a). The numbers of uncured and cured mice are shown. The statistical significance was evaluated by Fisher's exact test.

antitumor effects while sparing liver damage.[10] In addition, several reports reveal that the antitumor effects of anti-CD137 mAb could be augmented through the use of combination treatment with other immunotherapies or chemotherapeutic agents.[36–39] Therefore, the current study assessed the potential of combining two distinct therapies.

T cells are suppressed by immunosuppressive cells such as Tregs and MDSC, particularly in the tumor microenvironment. In this study, we examined the antitumor effects of local anti-CD137 mAb therapy in both the early and late tumor-bearing stages. The result revealed a loss of the therapeutic efficacy of local anti-CD137 mAb therapy in late stage tumors, which could be attributed to a drastic increase in the number of

MDSC at the tumor sites on day 17. We also tested another possibility that CD137 expression was lost in late stage tumors. The results were that at least $CD8^+$ TIL were positive for the CD137 expression on day 22 after tumor inoculation (Fig. 3c). Interestingly, $CD4^+$ or $CD8^+$ T cells in TIL from the mice treated with low-dose CP/GEM chemotherapy were clearly positive for CD137 expression. Intermittent chemotherapy with low-dose CP/GEM might have the potential to increase/keep the CD137 expression on T cells in TIL even at late tumor-bearing stages.

We recently reported a unique protocol for chemotherapy: intermittent injections of low-dose CP and GEM at 8-day intervals could elicit antitumor T-cell immunity effectively without impairing the immune competency of the tumor-bearing hosts.[31] The intermittent chemotherapy can suppress the tumor growth of CT26 significantly and the "plateau" of tumor growth, so-called stable disease, can be observed in wild type mice. In contrast, this intermittent chemotherapy can delay the tumor growth of CT26 in nude mice significantly but the antitumor effect is slight. These results indicate that CT26 is sensitive to low-dose CP/GEM to some degree *in vivo*, but that main antitumor effect is T cell-dependent.[31] Interestingly, a report suggests that anti-CD137 mAb can activate Tregs.[40] In the current study, we hypothesized that CP and GEM could inhibit Tregs, which might otherwise be activated after anti-CD137 mAb therapy, and MDSC, which were increased dramatically at tumor sites during the late tumor-bearing stage.

The combination treatment with intermittent chemotherapy and anti-CD137 mAb induced two therapeutic results: tumor regression and tumor stabilization. Such different responses might depend on whether the combination therapy induced tumor-reactive CTL. Therefore, we assessed whether tumor-reactive or tumor antigen peptide-reactive CTL were induced in the mice that responded differently. CT26-reactive CTL were induced from the spleen cells of either CT26-cured or CT26-stable mice (Fig. 4a). CTL activity from the spleen cells of CT26-progressing mice was low, and no activity was induced from the spleen cells of naïve mice. This suggests that tumor-reactive CTL were induced successfully even in tumor-bearing hosts with "stable disease," and suggests the clinical implication of anti-cancer immunotherapy. We also examined CTL activity against P815 cells that had been pulsed with either control or AH1 peptide. The P815 cells used in the current study were clearly positive for *gp70* mRNA (Fig. 3f), despite a previous report that they expressed small amounts of *gp70* mRNA.[35] P815 cells showed a similar sensitivity pattern with CT26 cells (Fig. 4b). Interestingly, CTL that were induced from the spleen cells of CT26-cured or CT26-stable mice showed higher cytotoxicity against AH1 peptide-pulsed P815 cells than against control peptide-pulsed P815 cells. This suggests that AH1 peptide-specific CTL and systemic antitumor immunity were induced in the mice that responded to the combination therapy. Accordingly, the combination therapy cured tumors to which anti-CD137 mAb was not delivered (Fig. 5).

In conclusion, this study demonstrated that intermittent chemotherapy using low-dose CP and GEM could act in synergy with local anti-CD137 mAb therapy. Pre-treatment with intermittent immunochemotherapy maintained a suitable microenvironment for the subsequent local anti-CD137 mAb therapy. Because tumor-reactive T cells are the main effectors of tumor regression after immunomodulating mAb therapy, intermittent immunochemotherapy might be a promising modality for supporting mAb-based anti-cancer treatment.

Acknowledgments

We thank Ms Tamami Moritani for her technical assistance.

Disclosure Statement

The authors have no conflict of interest to declare.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Gating strategy for examining tumor-infiltrating immune cells.