

Two Distinct Mechanisms of Augmented Antitumor Activity by Modulation of Immunostimulatory/Inhibitory Signals

Jun Mitsui^{1,5}, Hiroyoshi Nishikawa¹, Daisuke Muraoka¹, Linan Wang², Takuro Noguchi^{1,5}, Eiichi Sato⁴, Satoshi Kondo⁵, James P. Allison^{6,7}, Shimon Sakaguchi⁹, Lloyd J. Old⁸, Takuma Kato³, and Hiroshi Shiku^{1,2}

Abstract

Purpose: Blockade of CTL-associated antigen-4 (CTLA-4), an inhibitory immunomodulatory molecule on T cells, has been shown to enhance T-cell responses and induce tumor rejection, and a number of clinical trials with anti-CTLA-4 blocking monoclonal antibody (mAb) are under way. However, accumulating evidence indicates that anti-CTLA-4 mAb increases the number of CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Treg) and that anti-CTLA4 mAb alone is often insufficient to reject established tumors in mice and humans. Thus, finding maneuvers to control Tregs and other immunosuppressive mechanisms remains a critical challenge.

Experimental Design: The potential to enhance antitumor immune responses by combining anti-CTLA-4 mAb with anti-glucocorticoid-induced tumor necrosis factor receptor family related gene (GITR) mAb, a costimulatory molecule that abrogates directly/indirectly Treg-mediated immune suppression or anti-CD25 mAb that depletes Tregs was analyzed with two tumor models, CT26 (a murine colon carcinoma cell line) and CMS5a (a murine fibrosarcoma cell line).

Results: Anti-CTLA-4/anti-GITR mAb combination treatment exhibited far stronger antitumor effects compared with either antibody alone. This strong antitumor effect was attributed to (a) increased numbers of CD8⁺ T cells infiltrating tumor sites in anti-CTLA-4 mAb-treated mice and (b) increased cytokine secretion and Treg resistance of tumor-specific CD8⁺ T cells with strongly upregulated CD25 expression in anti-GITR mAb-treated mice, indicating distinct quantitative/qualitative changes induced by modulating CTLA-4 and GITR signaling.

Conclusions: This study shows that combined treatment with different immune modulators can augment antitumor immune responses and provides justification for exploring anti-CTLA-4/anti-GITR mAb combination treatment in the clinic. *Clin Cancer Res*; 16(10); 2781-91. ©2010 AACR.

The molecular identification of tumor antigens recognized by the human immune system has prompted a renewed interest in the development of cancer vaccines (1, 2). Although many of these vaccines have resulted in the development of measurable humoral/cellular immune responses, only a limited number of treated patients experienced clinical benefit, such as tumor regression (3). The fact that most tumor antigens identified to date are nonmutated self-antigens and therefore may not induce strong CD4⁺ and CD8⁺ T-cell responses

by themselves is an important issue to be considered in the vaccine protocol (1, 2, 4). An increasingly attractive way to overcome this problem is by modulating costimulatory/inhibitory signals on T cells. CTL-associated antigen-4 (CTLA-4) is a negative immunomodulator expressed on activated T cells and delivers an inhibitory signal during immune responses (5). Blockade of CTLA-4 action by monoclonal antibody (mAb) enhances effector T-cell responses and induces T cell-mediated tumor rejection in mouse models (5, 6). Human anti-CTLA-4 mAb

Authors' Affiliations: Departments of ¹Cancer Vaccine, ²Immuno-Gene Therapy, and ³Cellular and Molecular Immunology, Mie University Graduate School of Medicine, Mie, Japan; ⁴Department of Anatomic Pathology, Tokyo Medical University, Tokyo, Japan; ⁵Department of Surgical Oncology, Division of Surgery, Hokkaido University Graduate School of Medicine, Hokkaido, Japan; ⁶Ludwig Center for Cancer Immunotherapy, Immunology Program, ⁷Howard Hughes Medical Institute, and ⁸Ludwig Institute for Cancer Research, New York Branch, Memorial Sloan-Kettering Cancer Center, New York; and ⁹Department of Experimental Pathology, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan

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J. Mitsui and H. Nishikawa contributed equally to this work.

Corresponding Authors: Hiroyoshi Nishikawa, Experimental Immunology, Immunology Frontier Research Center, Osaka University, Suita, Osaka 565-0871 Japan. Phone: 81-6-6879-4462; Fax: 81-6-6879-4464; E-mail: nishihiro@ifrec.osaka-u.ac.jp, Takuma Kato, Department of Cellular and Molecular Immunology, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan. Phone: 81-59-231-5187; Fax: 81-59-231-5276; E-mail: katotaku@doc.medic.mie-u.ac.jp, or Hiroshi Shiku, Department of Cancer Vaccine and Immuno-Gene Therapy, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan. Phone: 81-59-231-5062; Fax: 81-59-231-5276; E-mail: shiku@clin.medic.mie-u.ac.jp.

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Translational Relevance

Human clinical trials revealed that anti-CTLA-4 (CTLA-4) monoclonal antibody (mAb) alone is frequently insufficient to reject established tumors and sometimes can be associated with the increase of CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Treg). Thus, finding maneuvers to further augment antitumor effects would be critical. Here, we describe that anti-CTLA-4 mAb treatment combined with blocking of Treg function with anti-glucocorticoid-induced tumor necrosis factor receptor family related gene (GITR) mAb augments antitumor immune responses and induces rejection of large established tumors through two distinct mechanisms, which are characterized by quantitative (T-cell proliferation/expansion) enhancement with anti-CTLA-4 mAb and qualitative (cytokine secretion and Treg resistance) enhancement by anti-GITR mAb treatment. This is of particular interest to direct clinical application as a combinational mAb treatment and to the recent concept of integrated immunotherapy by showing the importance of combining immunomodulators with different modes of action to maximize antitumor activity.

has been found to elicit objective and durable clinical responses in a subset of patients, particularly melanoma patients (7).

CD4⁺CD25⁺ regulatory T cells (Treg), originally recognized for their suppression of autoimmune responses, are also critical in controlling antitumor immune responses (8–10). It has been shown that CD4⁺CD25⁺ Tregs constitutively express cell surface CTLA-4 and blocking/depleting of signaling through CTLA-4 impairs *in vivo* and *in vitro* suppressive functions of Tregs (9–12). Despite the critical roles of CTLA-4 signals in Treg-suppressive function, it has been reported that CTLA-4 blockade induces an increase in the number of Tregs as well as the number of CD8⁺ T cells at tumor local sites (13, 14), raising the possibility that treatment of anti-CTLA-4 mAb primarily mediates its effects through the activation of effector T cells rather than inhibition of Treg function alone.

Given the critical roles of Tregs in the suppression of antitumor immunity and the lack of objective clinical responses in a significant population of anti-CTLA-4 mAb-treated patients (7, 10), adding other approaches to anti-CTLA-4 mAb therapy for controlling Tregs and other immunosuppressive mechanisms represents promising strategies to improve clinical responses. Glucocorticoid-induced tumor necrosis factor receptor family related gene (GITR), a type I transmembrane protein with homology to tumor necrosis factor receptor family members, was originally reported as a molecule that inhibits T-cell receptor-induced apoptosis (15). GITR is a costimulatory molecule expressed at different levels in resting CD4⁺ and CD8⁺ T

cells and is upregulated after T-cell activation (15). GITR is also constitutively expressed on CD4⁺CD25⁺ Tregs at high levels (15, 16), and it has been shown that activation of GITR signaling can inhibit the suppressive activity of Tregs attributable to both the costimulatory activity of GITR on responder CD4⁺CD25⁻ T cells and a direct effect on CD4⁺CD25⁺ Tregs (15–17). GITR ligand expression is mainly detected on activated antigen presenting cells after stimulatory signals, such as TLR signals (15). Its expression in steady-state is limited, and combination of CD8⁺ T-cell epitope vaccination with augmented GITR-L expression by delivery of plasmids encoding GITR-L resulted in strong tumor inhibition in a CD8⁺ T cell-dependent manner (17).

Whereas there are a few studies examining combination treatment with anti-CTLA-4 mAb and blocking/depleting of Tregs, the underlying mechanisms involved in the enhanced antitumor immunity have not been analyzed in detail (18, 19). In the present study, we examined whether anti-CTLA-4 mAb treatment combined with anti-GITR mAb or depletion of Tregs with anti-CD25 mAb could augment antitumor immune responses and induce rejection of well-established tumors. To address the influence of these strategies on the generation and activity of tumor-specific T-cell responses, we also used a new model in BALB/c mice involving tumors expressing NY-ESO-1, an extensively studied human cancer/testis antigen (20). Using these approaches, we show that anti-CTLA-4/anti-GITR mAb combination treatment exhibited stronger antitumor responses compared with either mAb alone and caused rejection of well-established tumors as large as 150 mm². The augmented antitumor effect of this combination treatment was due to increased tumor-infiltrating CD8⁺ T cells by anti-CTLA-4 mAb treatment and increased cytokine secretion and increased resistance of tumor-specific CD8⁺ T cells to CD4⁺CD25⁺ Treg suppression by anti-GITR mAb treatment. Finally, the Treg resistance of tumor-specific CD8⁺ T cells from mice receiving anti-GITR mAb was primarily detected in the CD8⁺ T-cell population with enhanced expression of CD25. Our results show that appropriate combinations of immune modulators further augment antitumor immune responses and could be a promising approach for antitumor immunotherapy.

Materials and Methods

Mice. Female BALB/c mice were purchased from CLEA Japan and used at 7 to 10 weeks of age. Mice were maintained at Animal Center of Mie University Graduate School of Medicine. The experimental protocol was approved by Ethics Review Committee for Animal Experimentation of Mie University Graduate School of Medicine.

Tumors. CMS5 is a 3-methylcholanthrene-induced sarcoma cell line of BALB/c origin (17, 21). CT26 is a colon epithelial tumor derived by intrarectal injections of *N*-nitroso-*N*-methylurethane in BALB/c mice (22). CT26 expressing NY-ESO-1, a human cancer/testis antigen,

was established.¹⁰ CT26 and CMS5a do not express GTR ligands (Supplementary Fig. S1A).

Tumor challenge. Groups of five mice were inoculated s.c. in the right hind flank with 1×10^6 CT26 or CMS5a and monitored thrice a week. In the indicated experiments, 2×10^6 CT26 expressing NY-ESO-1 were used.

Antibodies and reagents. Anti-CD4 (GK1.5, rat IgG2b), anti-CD8 (19/178, mouse IgG2a), anti-CD25 (PC61, rat IgG1), anti-GTR agonistic (DTA-1, rat IgG2a), and anti-CTLA-4 antagonistic (9D9, mouse IgG2b) mAbs were produced from each hybridoma and were purified by protein G columns. The endotoxin levels (final injection concentration) of anti-CD25, anti-GTR, and anti-CTLA-4 mAbs were 0.0202673, 0.0278168, and 0.0280718 EU/mL, respectively. Each mAb was i.v. injected as described (17, 21). Anti-CD3 mAb (145-2C11, hamster IgG1), phycoerythrin (PE)-anti-Foxp3 mAb (FJK-16s, rat IgG2a), and APC-conjugated anti-CD25 mAb (3C7, rat IgG2b) were purchased from eBioscience. FITC-conjugated anti-CD4 mAb (GK1.5, rat IgG2b), anti-CD8 mAb (53-6.7, rat IgG2a), and PE-conjugated anti-CD25 mAb (3C7, rat IgG2b) were purchased from BD Biosciences. Ki-67 staining was done by Ki-67 staining set (BD Biosciences) according to the instructions provided by the manufacturer. Synthetic mERK2₁₃₆₋₁₄₄-9m peptide QYIHSANVL (21) and NY-ESO-1₈₁₋₈₈ peptide RGPESRL¹⁰ were obtained from Sigma.

Flow cytometry and tetramer staining. Cells were stained for surface markers in PBS with 2% fetal bovine serum for 15 minutes at 4°C and analyzed on FACSCanto (BD Biosciences). Foxp3 staining was done by Treg staining kit (eBioscience) according to the instructions provided by the manufacturer. Tetramer staining was done as described (20). Briefly, CD8⁺ T cells were stained PE-NY-ESO-1₈₁₋₈₈/D^d tetramers (prepared at the Ludwig Institute Core Facility by Drs. P. Guillaume and I. Luescher, Lausanne, Switzerland) for 15 minutes at 37°C before additional staining of surface marker for 15 minutes at 4°C. After washing, cells were analyzed on FACSCanto (BD Biosciences) and FlowJo software (Tree Star).

Cell isolation. Spleen cell suspensions were mixed with anti-CD8 microbeads (Miltenyi Biotec) and separated into CD8⁺ T cells by positive selection on a MACS column. CD8⁺ T-cell populations were confirmed to contain >95% CD8⁺ T cells. In some experiments, these CD8⁺ T cells were further purified into CD8⁺ tetramer⁺ T cells on a FACSaria (BD Biosciences) after staining with FITC-anti-CD8 and PE-NY-ESO-1₈₁₋₈₈/D^d tetramer. The purity of these CD8⁺ NY-ESO-1₈₁₋₈₈/D^d tetramer⁺ T cells was >98%.

CD4⁺CD25⁺ T cells were purified using CD4⁺CD25⁺ T-cell isolation kit (Miltenyi Biotec) according to the instructions provided by the manufacturer, and the purity of these CD4⁺CD25⁺ T cells was >95%.

To collect tumor-infiltrating T cells, tumors were minced and treated with 1 mg/mL of collagenase IA (Sigma) in HBSS for 90 minutes at room temperature, followed by separation with 40% Percoll (GE Healthcare).

Immunohistochemistry. Immunofluorescence labeling of CD8 α was done on freshly frozen tumor specimens. Frozen sections of 3 μ m were mounted on glass slides and fixed with ice-chilled acetone for 15 minutes. Sections were then washed with PBS (0.01 mol/L, pH 7.4). Anti-CD8 α (53-6.7, BD Bioscience), diluted at the concentration of 3.0 μ g/mL, was applied and incubated in room temperature for 2 hours. The sections were then washed with PBS and incubated in room temperature for 1 hour with Alexa-488-conjugated anti-rat IgG secondary antibody (Invitrogen) diluted at the concentration of 20 μ g/mL. The sections were then washed with PBS and counterstained with 4',6-diamidino-2-phenylindole (DAPI). Specimens were aqueously mounted, and high power field was digitally photographed. Intratumoral infiltrated positive cells were counted on the photograph.

Proliferation assay. CD8⁺ effector T cells were cultured with irradiated splenic Thy-1⁻ APCs prepared from wild-type BALB/c mice in the presence of 1 μ g/mL anti-CD3 mAb in 96-well plates. To these cultures, CD4⁺CD25⁺ T cells were added. Proliferation was evaluated by pulsing with 0.5 μ Ci/well [³H]thymidine for the last 6 hours of the 72-hour culture. [³H]thymidine incorporation was measured by a scintillation counter.

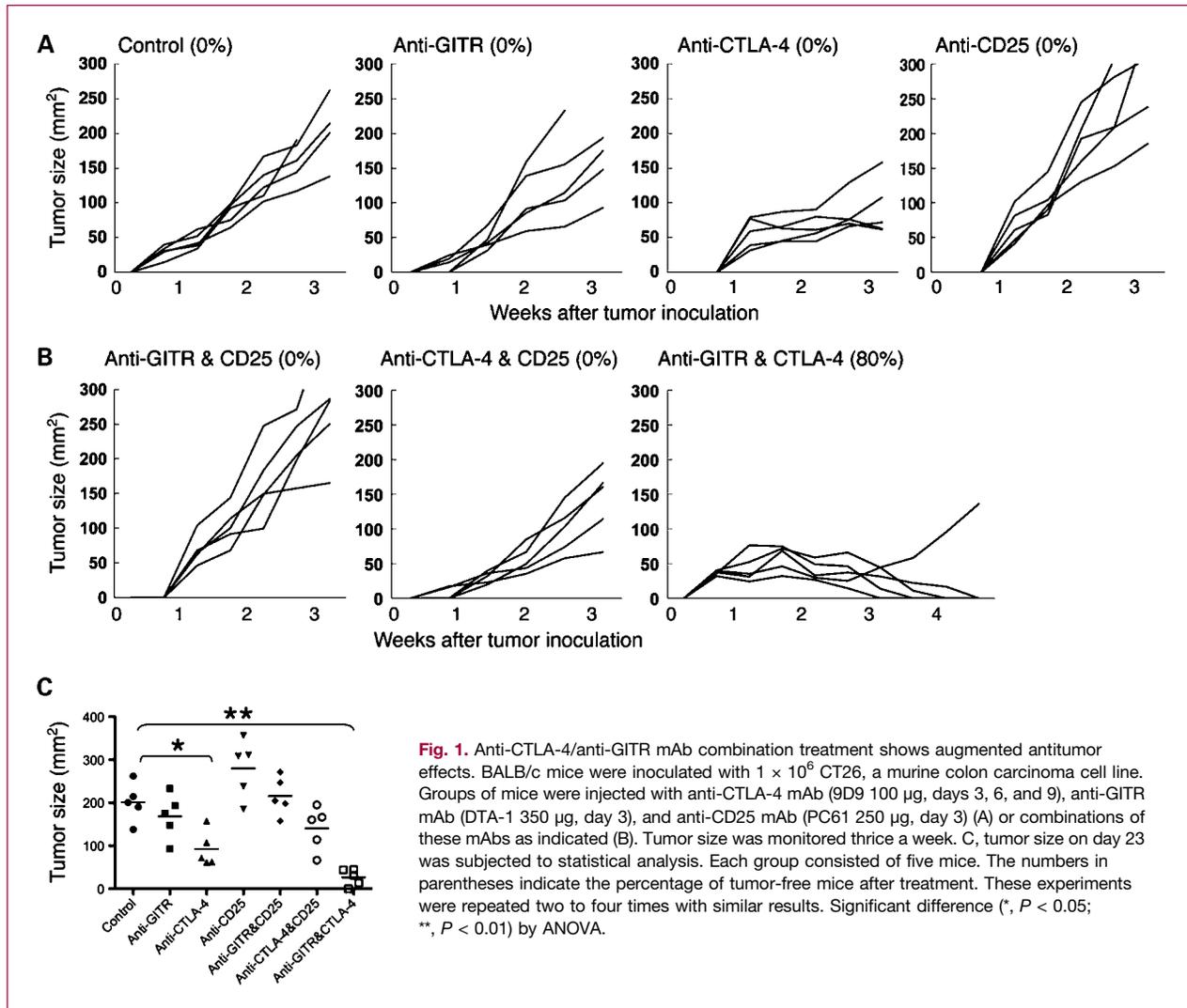
ELISA. The concentration of IFN- γ in supernatants from cultures was determined by IFN- γ ELISA kit (BD Biosciences) according to the instructions provided by the manufacturer.

Statistical analysis. Tumor curves were assessed by one-way ANOVA with a Bonferroni multiple comparison post-test. Single measurement comparison between two groups was evaluated by two-sided Student's *t* test. *P* values of < 0.05 were considered statistically significant.

Results

Anti-CTLA-4/anti-GTR mAb combination treatment shows augmented antitumor activity. To address the antitumor activity by anti-CTLA-4 mAb treatment in our mouse tumor models, BALB/c mice were inoculated with CT26 (a murine colon carcinoma cell line) or CMS5a (a murine fibrosarcoma cell line) and injected with anti-CTLA-4 mAb on days 3, 6, and 9 after tumor inoculation. Despite a clear antitumor effect of anti-CTLA-4 mAb, complete tumor rejection was not observed (Fig. 1A; Supplementary Fig. S2A). We next asked whether anti-CTLA-4 mAb treatment combined with anti-GTR mAb (direct/indirect blocking of Treg function) or anti-CD25 mAb (depletion of Tregs) augmented the antitumor immune response. BALB/c mice were inoculated with CT26 or CMS5a and injected with anti-CTLA-4 (days 3, 6, 9), anti-GTR (day 3), or anti-CD25 (day 3) mAb, either singly or in combination. The treatment protocol was based on antitumor effects

¹⁰ Manuscript in preparation.



observed in preliminary assays (Supplementary Fig. S1B and C). Because our aim is to find a new treatment strategy, anti-CD25 mAb was injected after tumor inoculation, although it is known that anti-CD25 mAb injection before tumor inoculation shows antitumor activity (23, 24). Anti-GITR mAb slightly inhibited tumor growth, but not significantly; anti-CD25 mAb did not exhibit any antitumor effects (Fig. 1A; Supplementary Fig. S2A). The combination of anti-CTLA-4/anti-GITR mAb showed significant antitumor responses and resulted in complete tumor rejection in 80% of mice with CT26 or CMS5a tumors (Fig. 1B and C; Supplementary Fig. S2B and C). We could not find any additive antitumor effects by combining either anti-CTLA-4 and anti-CD25 mAb or anti-GITR and anti-CD25 mAb (Fig. 1B and C; Supplementary Fig. S2B and C). Based on the strong antitumor effects of the anti-CTLA-4/anti-GITR mAb combination treatment, we further analyzed the detailed mechanism(s) of antitumor responses mediated by this mAb combination.

Anti-CTLA-4/anti-GITR mAb combination treatment controls large established tumors and is dependent on both $CD4^+$ and $CD8^+$ T cells. We assessed whether anti-CTLA-4/anti-GITR mAb combination treatment could reject large established tumors. BALB/c mice were inoculated with CT26, and injection of anti-CTLA-4 and anti-GITR mAb was started on 3, 5, 7, or 9 days after tumor inoculation. A high percentage of tumor regression was observed when treatment was started on days 3, 5, and 7 (when tumors reached 150 mm^2) but not on day 9, whereas anti-CTLA-4/anti-GITR mAb combination treatment started on day 9 markedly slowed tumor growth (Fig. 2A and B). A similar strong antitumor effect was observed in mice with large CMS5a tumors (Supplementary Fig. S3A and B). Mice receiving anti-CTLA-4/anti-GITR mAb combination treatment showed no manifestation of autoimmunity, such as colitis and thyroiditis, by detailed histologic analyses (data not shown).

To gain insight into the cellular target(s) of the strong antitumor effects of anti-CTLA-4/anti-GITR mAb combination

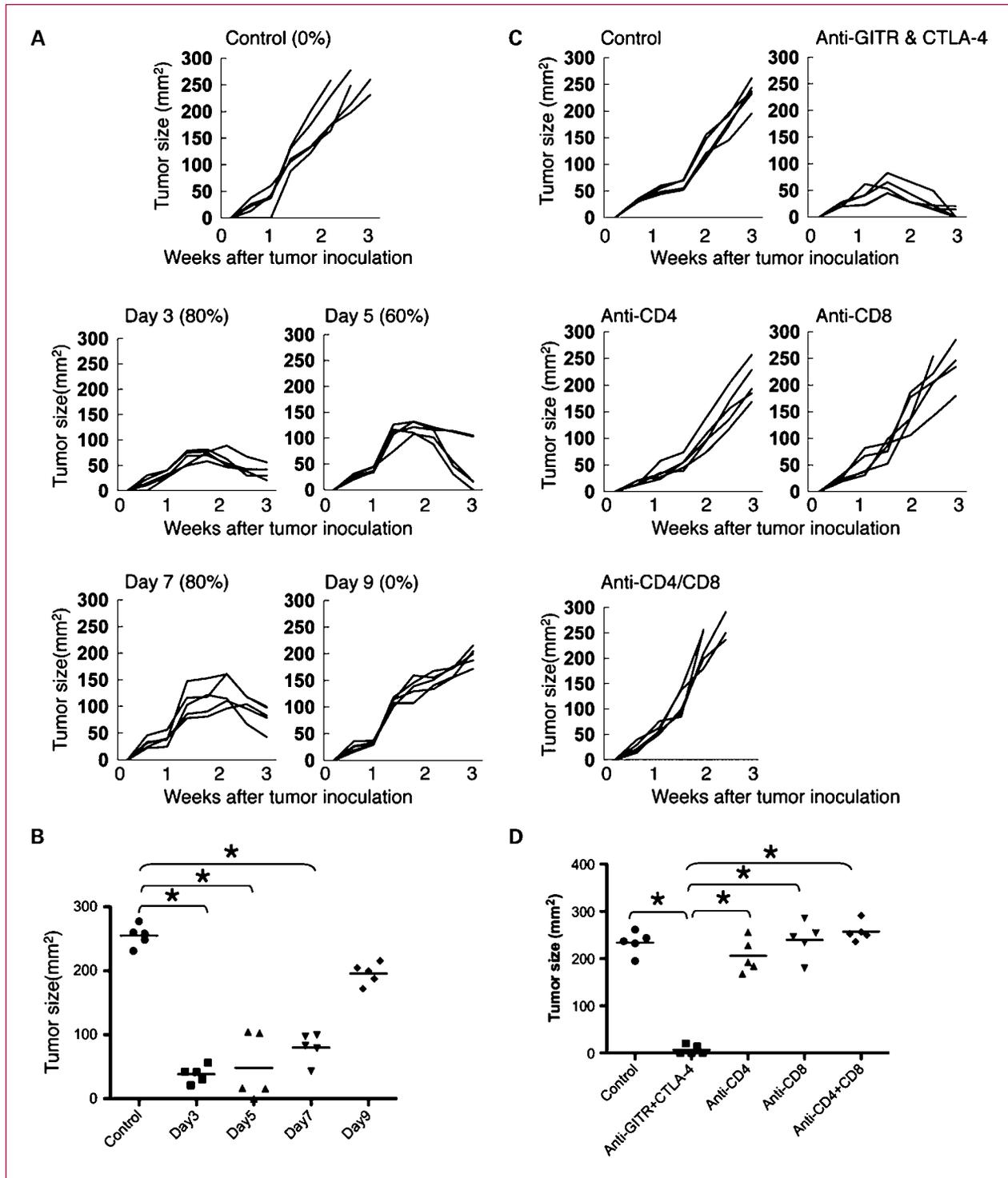


Fig. 2. Anti-CTLA-4/anti-GITR mAb combination treatment controls large established tumors and is dependent on both CD4⁺ and CD8⁺ T cells. A and B, BALB/c mice were inoculated with 1×10^6 CT26, and injection of anti-CTLA-4 mAb (9D9 100 μ g, three injections) and anti-GITR mAb (DTA-1 350 μ g, single injection) was started on the indicated day. Tumor size was monitored thrice a week. C and D, BALB/c mice were inoculated with 1×10^6 CT26 and injected with anti-CTLA-4 mAb (9D9 100 μ g, days 3, 6, and 9) and anti-GITR mAb (DTA-1 350 μ g, day 3). In addition, groups of mice received the administration with anti-CD4 (GK1.5, 200 μ g) and/or anti-CD8 mAb (19/178, 200 μ g) at the time of tumor inoculation (resulting in >90% of CD4⁺/CD8⁺ T-cell depletion). Mice were monitored thrice a week. B and D, tumor size on day 23 was subjected to statistical analysis. Each group consisted of five mice. The numbers in parentheses indicate the percentage of tumor-free mice after treatment. These experiments were repeated twice with similar results. Significant difference (*, $P < 0.05$) by ANOVA.

treatment, we examined the outcome of CD4⁺/CD8⁺ T-cell depletion. BALB/c mice bearing CT26 were injected with anti-CTLA-4 (days 3, 6, and 9) and anti-GITR (day 3) mAb and received anti-CD4 and/or anti-CD8 mAb (day 0). The depletion of CD4⁺ and CD8⁺ T cells totally abolished tumor regression induced by the anti-CTLA-4/anti-GITR mAb combination (Fig. 2C and D).

Modulation of two different immunomodulatory molecules provides distinct activation signals to CD8⁺ T cells. Whereas CD4⁺ T cells provide essential "help" to activate CD8⁺ T cells, CD8⁺ CTLs have the capacity to directly kill tumor cells (25). Thus, we focused on alterations in the properties of CD8⁺ T cells following anti-CTLA-4/anti-GITR mAb combination treatment. Because tumor progression/tumor

regression could first be distinguished around day 13, we chose day 13 tumors for immunohistochemical analyses of intratumoral CD8⁺ T-cell infiltration. The number of infiltrating CD8⁺ cells was augmented in tumors of mice treated with anti-CTLA-4 mAb alone or anti-CTLA-4/anti-GITR mAb combination compared with control animals (Fig. 3A). Furthermore, the number of tumor-infiltrating CD8⁺ cells in mice treated with anti-CTLA-4/anti-GITR mAb was higher than mice treated with anti-CTLA-4 mAb alone (Fig. 3A).

It is possible that the augmented CD8⁺ T-cell infiltration in tumors by anti-CTLA-4/anti-GITR mAb may reflect enhanced proliferation of CD8⁺ T cells. Therefore, we examined the effect of these mAbs on CD8⁺ T-cell proliferation

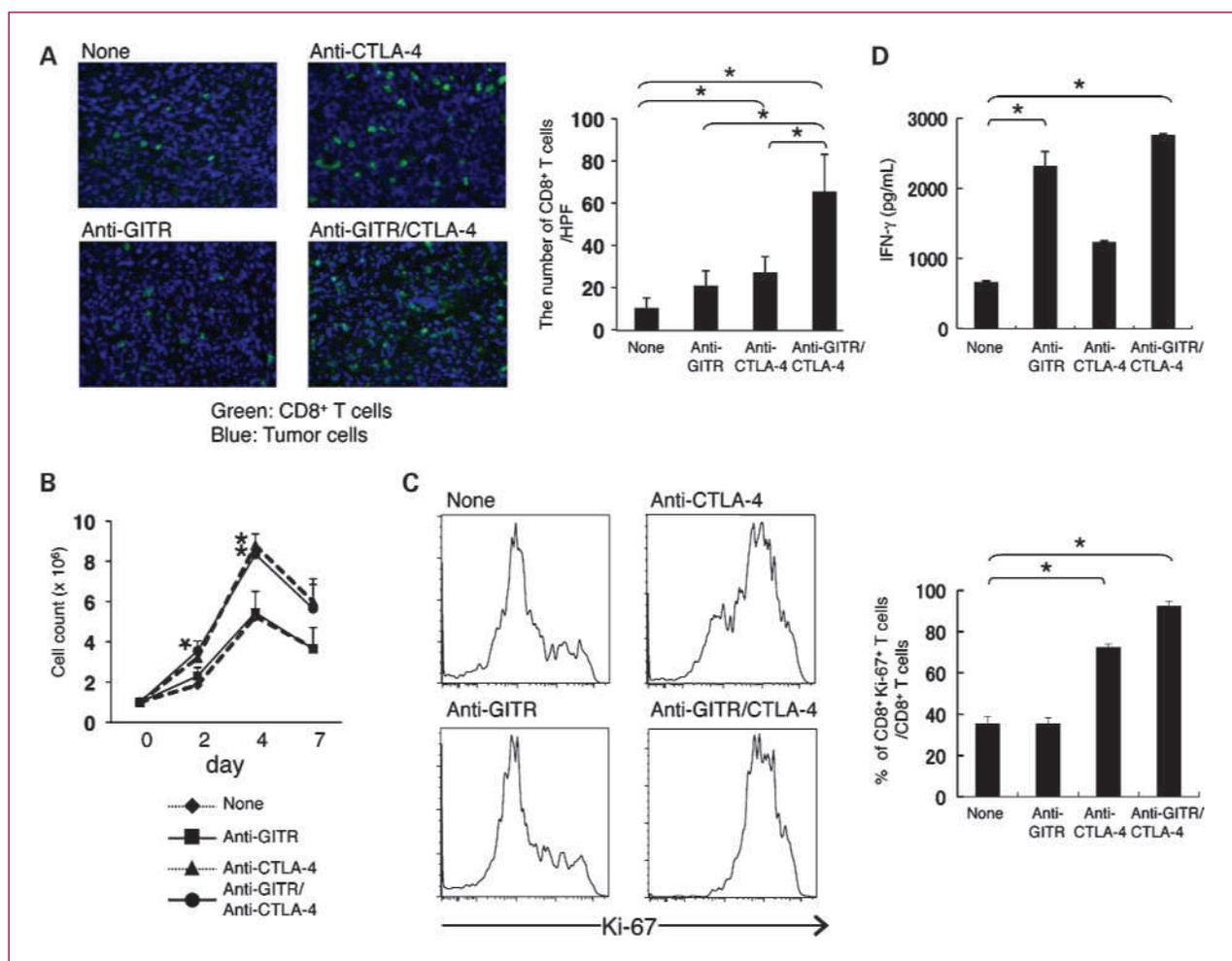


Fig. 3. The modulation of two different immunomodulatory molecules provides distinct activation signals to CD8⁺ T cells. A, BALB/c mice were inoculated with 1×10^6 CT26 and injected with anti-CTLA-4 mAb (9D9 100 μ g, days 3, 6, and 9) and/or anti-GITR mAb (DTA-1 350 μ g, day 3). Thirteen days later, tumors were removed and analyzed immunohistochemically as described in Materials and Methods. Tumor-infiltrating T cells labeled with anti-CD8 mAb (green) were counted in 40 HPF. B and D, splenic CD8⁺ T cells were obtained from naive BALB/c mice, and 1×10^6 CD8⁺ T cells were cultured with 2×10^6 irradiated splenic Thy-1⁻ APCs with anti-CD3 mAb with or without anti-CTLA-4 mAb and/or anti-GITR mAb. The number of CD8⁺ T cells (B) and IFN- γ secretion (D) were analyzed. C, BALB/c mice were inoculated with 1×10^6 CT26 and injected with anti-CTLA-4 mAb (9D9 100 μ g, days 3, 6, and 9) and/or anti-GITR mAb (DTA-1 350 μ g, day 3). Thirteen days later, tumor-infiltrating T cells were prepared as described in Materials and Methods and were stained with PE-Ki-67 mAb and APC-CD8 mAb. Ki-67 expression gated with CD8⁺ T cells was analyzed with FACSCanto. These experiments were repeated twice or thrice with similar results. Data are mean \pm SD. Significant difference (*, $P < 0.05$) by two-sided Student's t test.

using an *in vitro* CD8⁺ T-cell culture system. Splenic CD8⁺ T cells (1×10^6) purified from naive BALB/c mice were cultured with 2×10^6 irradiated BALB/c splenic Thy-1⁻ APCs with anti-CD3 mAb with or without anti-CTLA-4 mAb and/or anti-GITR mAb, and CD8⁺ T-cell proliferation was analyzed. An augmented *in vitro* CD8⁺ T-cell proliferation was observed in cultures containing anti-CTLA-4 mAb or anti-CTLA-4/anti-GITR mAb, but not in cultures containing anti-GITR mAb alone compared with control culture (Fig. 3B). In accordance with *in vitro* data, we observed a higher frequency of tumor-infiltrating CD8⁺ Ki-67⁺ T cells in mice treated with anti-CTLA-4 mAb alone or anti-CTLA-4/anti-GITR mAb combination compared with control animals (Fig. 3C). We also analyzed a functional feature by cytokine secretion in the supernatants of these cultures. IFN- γ secretion was significantly enhanced in cultures containing anti-GITR mAb or anti-CTLA-4/anti-GITR mAb, but not anti-CTLA-4 mAb alone (Fig. 3D).

Tumor antigen-specific CD8⁺ T cells from mice injected with anti-GITR mAb show enhanced resistance to CD4⁺CD25⁺ Tregs. We and others have recently reported that GITR signaling delivered with antigen stimulation renders effector cells resistant to CD4⁺CD25⁺ Treg suppression (15, 17). It has also been shown that IFN- γ inhibits proliferation/activation of CD4⁺CD25⁺ Tregs (26, 27). Given the finding that anti-GITR mAb augments IFN- γ secretion of CD8⁺ T cells, we asked whether CD8⁺ T cells in mice injected with anti-GITR mAb were resistant to suppression by CD4⁺CD25⁺ Tregs. To determine *in vivo* effect of anti-CTLA-4 mAb and/or anti-GITR mAb on Treg susceptibility of tumor antigen-specific CD8⁺ T cells, we attempted to collect CD8⁺ T cells specific for the envelope protein (gp70) of an endogenous ecotropic murine leukemia provirus expressed by CT26, which is the target of CD8⁺ T cells (28). However, the frequency of these antigen-specific CD8⁺ T cells was too low (<0.01%) to be subjected to functional analyses. Thus, we used a new tumor model (CT26-NY-ESO-1) with stable expression of NY-ESO-1, a human cancer/testis antigen that has been the focus of much attention. Anti-CTLA-4 mAb and/or anti-GITR mAb treatment showed similar antitumor effects in the CT26-NY-ESO-1 tumor model compared with CT26 parental tumors (Supplementary Fig. S4). NY-ESO-1-specific T cells were identified as CD8⁺NY-ESO-1/D^d tetramer⁺ T cells and were purified on a FACSAria; purity was >98% as reported (17). Splenic CD4⁺CD25⁺ Tregs (5×10^3) prepared from naive BALB/c mice were added to cultures of 5×10^3 CD8⁺NY-ESO-1/D^d tetramer⁺ T cells with 1×10^5 irradiated BALB/c splenic Thy-1⁻ APCs with anti-CD3 mAb. Ratio of Tregs to effector cells for *in vitro* analysis was determined based on the ratio at CT26-NY-ESO-1 tumor local site that was close to 1:1 (Fig. 4C; data not shown). CD8⁺NY-ESO-1/D^d tetramer⁺ T cells derived from mice injected with anti-GITR mAb or anti-CTLA-4/anti-GITR mAb showed strong proliferation in the presence of Tregs, and the suppression by Treg was not significant (Fig. 4A). In contrast, proliferation of CD8⁺NY-ESO-1/D^d tetramer⁺ T cells derived from mice without treatment or

with anti-CTLA-4 mAb treatment was completely suppressed by CD4⁺CD25⁺ Tregs (Fig. 4A). Taken together, these results indicate that anti-GITR mAb treatment renders tumor antigen-specific CD8⁺ T cells more resistant to suppression by CD4⁺CD25⁺ Tregs.

Anti-GITR mAb treatment does not alter CD4⁺CD25⁺ Treg activity but reduces their tumor accumulation. Next, we investigated the effect of anti-CTLA-4 mAb and/or anti-GITR mAb treatment against CD4⁺CD25⁺ Tregs. BALB/c mice were injected with CT26-NY-ESO-1 tumors and received anti-CTLA-4 mAb and/or anti-GITR mAb. As Treg infiltration increases with tumor growth (19, 29), mice were sacrificed on day 13 when the size of tumors was not different among groups. CD4⁺CD25⁺ Tregs were isolated from draining lymph nodes of these animals. CD4⁺CD25⁺ Tregs (5×10^4) were added to cultures of splenic CD4⁺CD25⁻ T cells (5×10^4) prepared from naive BALB/c mice with 1×10^5 irradiated BALB/c splenic Thy-1⁻ APCs with anti-CD3 mAb. CD4⁺CD25⁺ Tregs from all groups exhibited similar suppressive capacity (Fig. 4B). To further analyze the influence of mAb treatment on CD4⁺ Tregs, we analyzed the percentage of Tregs relative to total CD4⁺ T cells at tumor sites by flow cytometry. Treg frequency in tumors was decreased by anti-GITR mAb or anti-CTLA-4/anti-GITR mAb combination treatment, but not following anti-CTLA-4 mAb treatment (Fig. 4C).

Tumor-specific CD8⁺ T cells resistant to Tregs show enhanced expression of CD25. To explore the mechanism (s) involved in Treg resistance, we examined activation markers in Treg-resistant and Treg-sensitive CD8⁺ T cells. Splenic CD8⁺ T cells (1×10^6) derived from naive BALB/c mice were cultured with 2×10^6 irradiated BALB/c splenic Thy-1⁻ APCs with anti-CD3 mAb with or without anti-CTLA-4 mAb and/or anti-GITR mAb, and phenotypic characterization of CD8⁺ T-cell activation markers was analyzed. Among the markers tested (CD25, CD69, CD62L, and CD45RB), the expression level of CD25 only exhibited a striking difference (Fig. 5A; data not shown). CD8⁺ T cells cultured with anti-GITR mAb or anti-CTLA-4/anti-GITR mAb showed enhanced levels of CD25 expression compared with CD8⁺ T cells in control culture (Fig. 5A). We next determined Treg sensitivity of CD8⁺ T cells cultured with anti-GITR mAb. In addition to *in vivo* data shown in Fig. 4A, CD8⁺ T cells cultured with anti-GITR mAb or anti-CTLA-4/anti-GITR mAb maintained proliferative capacity in the presence of CD4⁺CD25⁺ Tregs (Fig. 5B). In contrast, proliferation of CD8⁺ T cells in control culture or cultured with anti-CTLA-4 mAb was completely suppressed by CD4⁺CD25⁺ Tregs (Fig. 5B). These data indicate a potential association between Treg resistance and CD25 expression. To explore this possibility, we further separated CD8⁺ T cells stimulated with anti-GITR mAb based on CD25 expression, namely CD25^{high} and CD25^{low} population (Fig. 5C). Surprisingly, CD25^{high}CD8⁺ T cells completely maintained proliferative capacity in the presence of CD4⁺CD25⁺ Tregs. In sharp contrast, the proliferation of CD25^{low}CD8⁺ T cells was significantly suppressed, indicating a clear relationship

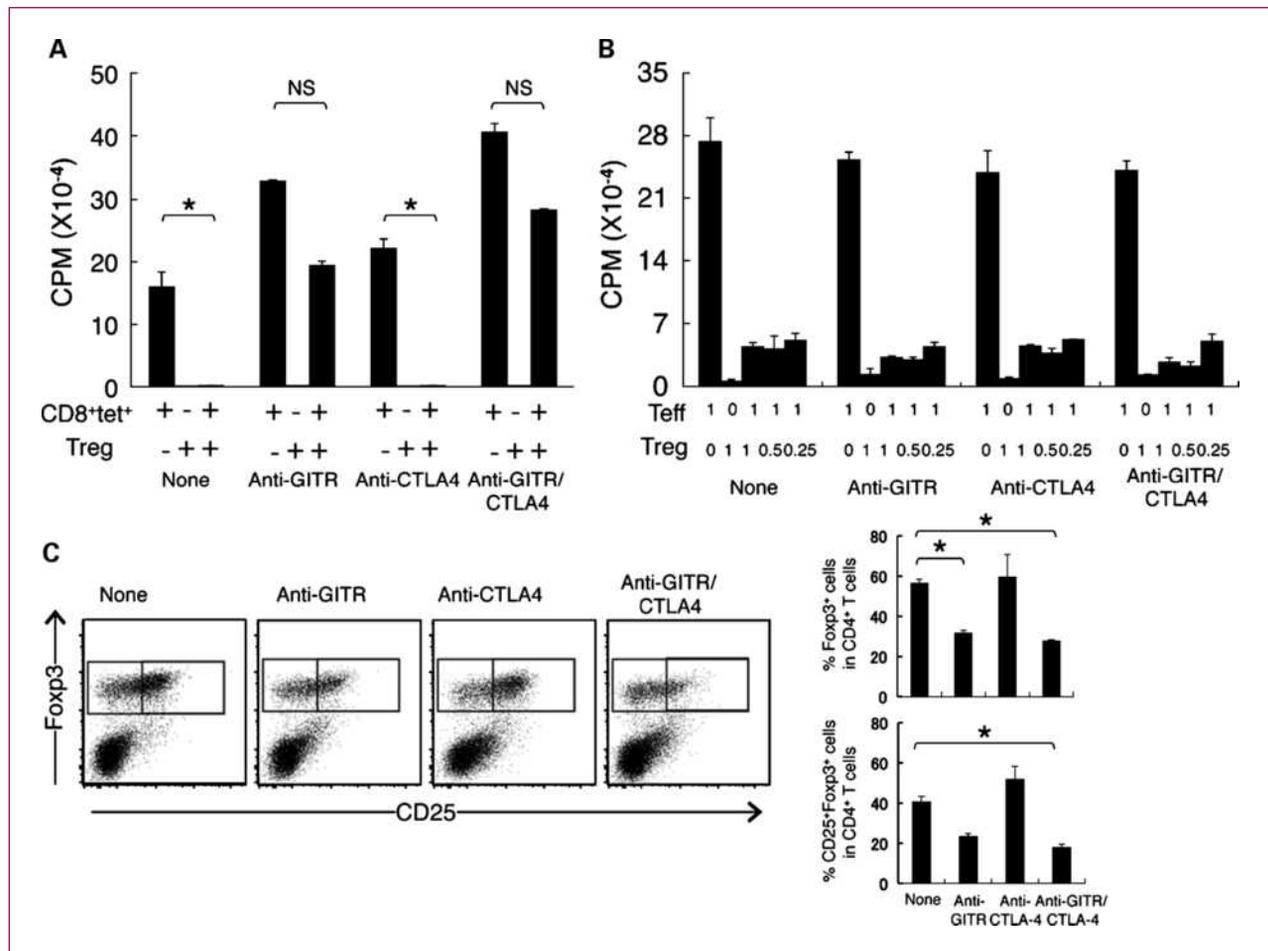


Fig. 4. Tumor antigen-specific CD8⁺ T cells from mice injected with anti-GITR mAb show enhanced resistance to CD4⁺CD25⁺ Tregs, and anti-GITR mAb treatment does not alter CD4⁺CD25⁺ Treg activity but reduces their tumor accumulation. BALB/c mice were inoculated with CT26-NY-ESO-1 and injected with anti-CTLA-4 mAb and/or anti-GITR mAb. Mice were sacrificed on day 13 when tumors were similar size among groups. A, CD8⁺ T cells were prepared from draining lymph nodes and were subjected to staining with FITC-CD8 mAb and PE-NY-ESO-1/D^d tetramer. CD8⁺NY-ESO-1/D^d tetramer⁺ T cells were sorted using FACSARIA. Purity of sorted populations was >98%. Splenic CD4⁺CD25⁺ Tregs (5×10^3) prepared from naive BALB/c mice were added to cultures of 5×10^3 CD8⁺NY-ESO-1/D^d tetramer⁺ T cells with 1×10^5 irradiated BALB/c splenic Thy-1⁻ APCs with anti-CD3 mAb. Proliferation was assessed as described in Materials and Methods. To obtain sufficient number of cells, a pool of lymph nodes from 10 mice were used for each group. B, CD4⁺CD25⁺ T cells were obtained from draining lymph nodes of mice treated with anti-CTLA-4 mAb and/or anti-GITR mAb. CD4⁺CD25⁺ Tregs (5×10^4) were added to cultures of splenic 5×10^4 CD4⁺CD25⁻ T cells prepared from spleens of naive BALB/c mice with 1×10^5 irradiated BALB/c splenic Thy-1⁻ APCs with anti-CD3 mAb. Proliferation was assessed as described in Materials and Methods. C, tumor-infiltrating T cells were prepared as described in Materials and Methods and were stained with FITC-CD4 mAb, PE-Foxp3 mAb, and APC-CD25 mAb. Foxp3 and CD25 expression gated with CD4⁺ T cells was analyzed with FACSCanto. Data are expressed as mean \pm SD. Significant difference (*, $P < 0.05$) by two-sided Student's *t* test.

between Treg resistance and CD25 expression. To extend these findings to the *in vivo* effects of anti-CTLA-4 mAb and/or anti-GITR mAb, we analyzed CD25 expression of NY-ESO-1-specific CD8⁺ T cells in mice treated with anti-CTLA-4 mAb and/or anti-GITR mAb. CD8⁺NY-ESO-1/D^d tetramer⁺ T cells obtained from draining lymph nodes of mice treated with anti-GITR mAb or anti-CTLA-4/anti-GITR mAb also exhibited higher CD25 expression compared with T cells from untreated mice (Fig. 5D). The other activation markers tested were comparable among groups (Supplementary Fig. S5). Taken together, NY-ESO-1-specific CD8⁺ T-cell resistance to Tregs is associated with high CD25 expression.

Discussion

Although significant progress has been made in understanding immune responses elicited by cancer, it is becoming increasingly clear that the compensatory down-regulation of immunity that occurs during the course of immune response plays a major role in limiting the effectiveness of cancer immunity (2, 3, 10, 30). A plethora of cell types, cell surface molecules, and soluble factors mediate this suppressive activity, and this homeostatic immunosuppressing circuitry must be understood and controlled if we are to maximize the promise of cancer vaccines, adoptive immunotherapy, and other immunotherapeutic

approaches to cancer (2, 30). Antibodies that activate or neutralize immunostimulatory and immunoinhibitory factors have proved valuable in dissecting their individual roles, and their use in animal models of cancer is showing the exciting therapeutic potential of this approach (5, 30). In fact, anti-CTLA-4 antibody is now under intense clinical evaluation, and the antitumor activity of anti-CTLA-4 mAb evident in animal models is clearly being seen in melanoma patients (5, 7, 30). Clinical

trials with anti-programmed death-1, anti-programmed death-L1, indole-2,3 dioxygenase inhibitors, and other modulators of cancer immunosuppression (MOI) are now under way, and a number of other MOIs are being prepared for human testing.

With the diverse activities mediated by these different molecules or pathways, there are many opportunities to explore their combined therapeutic efficacy. In the present study, we found that anti-CTLA-4 mAb and anti-GITR

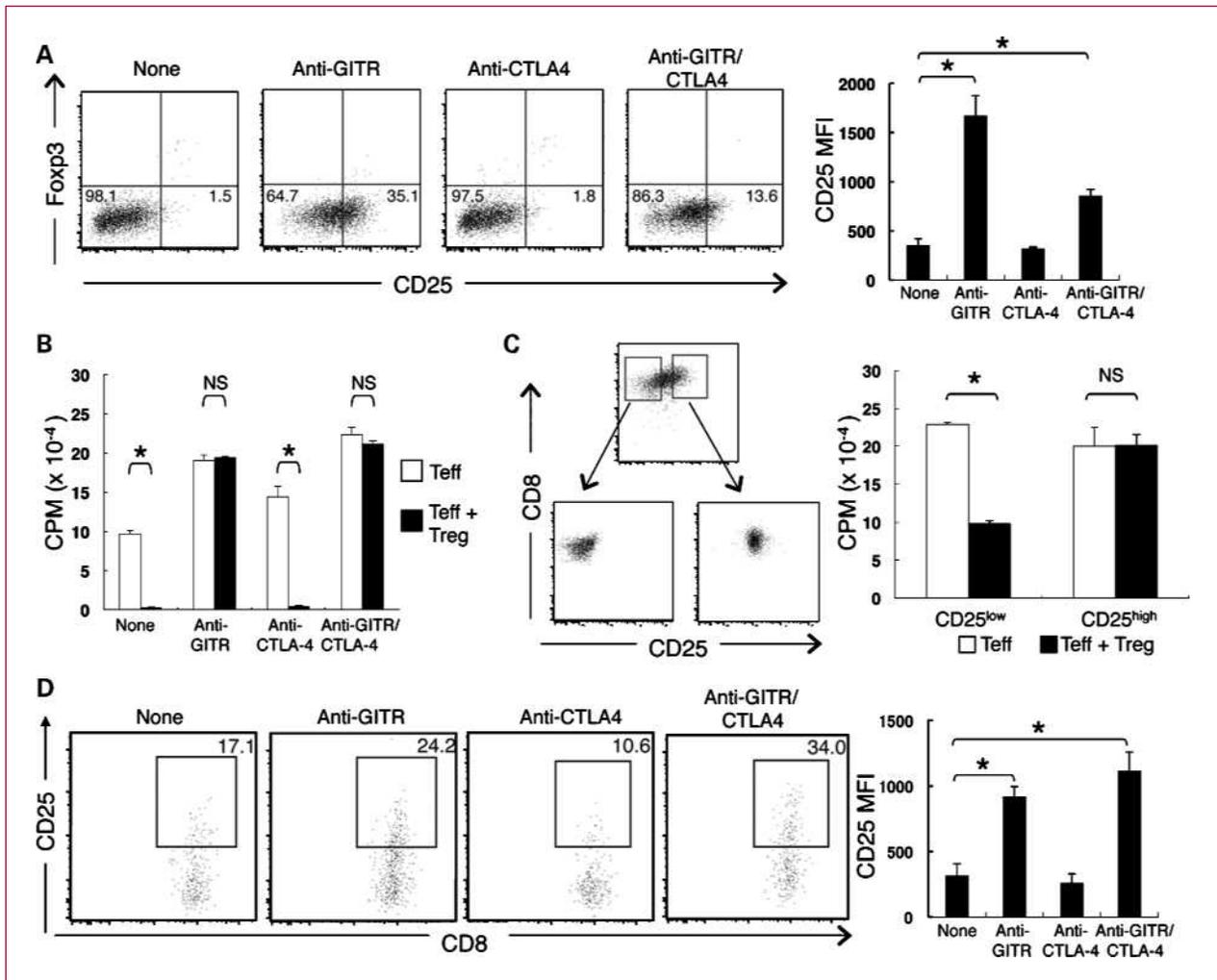


Fig. 5. CD8⁺ T cells with enhanced expression of CD25 show resistance to Tregs. Splenic CD8⁺ T cells (1×10^6) were cultured with 2×10^6 irradiated BALB/c splenic Thy-1⁻ APCs with anti-CD3 mAb with or without the indicated mAb. A, 3 d later, cells were subjected to staining with FITC-CD8, PE-Fcpx3, and APC-CD25. Fcpx3 and CD25 expression and mean fluorescence intensity (MFI) of CD25 staining gated with CD8⁺ T cells were analyzed with FACSCanto. B, the cultured CD8⁺ T cells were resorted using FACSaria. Splenic CD4⁺CD25⁺ Tregs (5×10^4) prepared from naive BALB/c mice were added to cultures of 5×10^4 CD8⁺ T cells with 1×10^5 irradiated BALB/c splenic Thy-1⁻ APCs with anti-CD3 mAb. Proliferation was assessed as described in Materials and Methods. C, CD8⁺ T cells stimulated with anti-GITR mAb were separated using FACSaria based on CD25 expression, namely CD25^{high} and CD25^{low} population. These 5×10^4 CD25^{high/low} CD8⁺ T cells were added to cultures of 5×10^4 CD4⁺CD25⁺ Tregs with 1×10^5 irradiated BALB/c splenic Thy-1⁻ APCs with anti-CD3 mAb. Proliferation was assessed as described in Materials and Methods. D, BALB/c mice ($n = 10$) inoculated with CT26-NY-ESO-1 were injected with anti-CTLA-4 mAb and/or anti-GITR mAb and sacrificed on day 13. CD8⁺ T cells were isolated from draining lymph nodes and subjected to staining with FITC-CD8 mAb, PE-NY-ESO-1/D^d tetramer, and APC-CD25 mAb. CD8 and CD25 expressions gated with NY-ESO-1/D^d tetramer⁺ T cells and mean fluorescence intensity (MFI) of CD25 staining gated with CD8⁺ NY-ESO-1/D^d tetramer⁺ T cells were analyzed with FACSCanto. These experiments were repeated two to four times with similar results. Data are expressed as mean \pm SD. Significant difference (*, $P < 0.05$) by two-sided Student's *t* test.

mAb, both with antitumor activity by themselves, have augmented antitumor activity when combined. We found that both CD4⁺ and CD8⁺ T cells were critical in mediating this antitumor effect and defined several *in vitro* and *in vivo* features that distinguished anti-CTLA-4 mAb and anti-GITR mAb responses. For instance, anti-CTLA-4 mAb treatment induced augmented proliferation of CD8⁺ T cells, whereas anti-GITR mAb treatment did not. Anti-GITR mAb, on the other hand, enhanced T cells to produce IFN- γ , whereas anti-CTLA-4 mAb had limited activity in this regard. An increased number of CD8⁺ T cells at tumor sites was found in anti-CTLA-4 mAb-treated but not in anti-GITR mAb-treated mice, and upregulation of CD25 on CD8⁺ T cells was a characteristic of anti-GITR mAb but not anti-CTLA-4 mAb treatment. Finally, anti-GITR mAb treatment, both *in vitro* and *in vivo*, led to CD8⁺ T-cell resistance to Treg-mediated suppression, whereas anti-CTLA-4 mAb did not alter Treg sensitivity.

We found that anti-CTLA-4/anti-GITR mAb combination treatment induced very strong antitumor effects and could regress tumors that reached 150 mm². However, complete tumor regression was not observed when anti-CTLA-4/anti-GITR mAb combination treatment was started on day 9, although the treatment markedly slowed tumor growth. To investigate whether higher doses of either mAb in combined treatment had improved antitumor activity, we focused day 9 tumors that are more resistant to anti-CTLA-4/anti-GITR mAb treatment. However, we did not observe any additive antitumor effects by increasing the doses of mAbs (data not shown). To optimize the effect of anti-CTLA-4/anti-GITR mAb treatment, studies assessing the combination of adoptive T-cell therapy (14) or immunization with CD8⁺ T-cell epitopes (17) with these mAbs are being investigated. Another issue under study is why 20% of treated mice seem to be resistant to anti-CTLA-4/anti-GITR mAb treatment. Understanding the basis for differences within groups would suggest other ways to overcome resistance.

Although it has been reported that anti-CTLA-4 mAb and anti-GITR mAb enhance T-cell proliferation, cytokine secretion, and CD25 expression (5, 15, 31), they have not been evaluated and compared in the same system. In our analysis, we found qualitative/quantitative differences in their influence on CD8⁺ T-cell proliferation, cytokine release, CD25 upregulation, and Treg susceptibility. While we focused on CD8⁺ T cells, similar distinctions were observed with CD4⁺ T-cell responses (Supplementary Fig. S6). In addition, unconventional T cells, such as NKT or $\gamma\delta$ T cells, may be involved in antitumor effects of anti-CTLA-4/anti-GITR mAb combination treatment, because it has been shown that the GITR signal also stimulates these T cells (32–34).

To address the *in vivo* kinetics and activation status of tumor antigen-specific T cells, we used the CT26-NY-ESO-1 model. Using this NY-ESO-1 mouse model, we found that anti-GITR mAb treatment induced Treg-resistant NY-ESO-1-specific CD8⁺ T cells and that these

Treg-resistant T cells were mainly present in the CD25^{high} population. Given the fact that CD4⁺CD25⁺ Tregs express CD25 and that exogenous interleukin-2 (IL-2) inhibits Treg suppression (9, 10, 35), it is plausible that CD8⁺CD25^{high} T cells induced by stimulation with anti-GITR mAb having their augmented high-affinity IL-2 receptor compete with CD4⁺CD25⁺ Tregs for IL-2. Alternatively, high CD25 expression may simply reflect a status of activation, and other unknown signals may be important for Treg resistance of CD25^{high} population. To resolve this issue, it will be critical to understand the intrinsic cell signaling in these cells, and comprehensive gene expression analyses are now planned.

Another intriguing point is that anti-GITR mAb decreased the number of CD4⁺CD25⁺ Tregs at tumor sites, as previously reported (19). It is unlikely that anti-GITR mAb deletes CD4⁺CD25⁺ Tregs (CD4⁺CD25⁺ Tregs express high levels of GITR) because activated CD8⁺ T cells also express GITR as well. It has been reported that costimulatory signals through GITR inhibit conversion of CD4⁺CD25⁻ effector T cells to Tregs (36). Another possibility similar to the one raised above is that CD8⁺CD25^{high} T cells infiltrating into tumors compete with CD4⁺CD25⁺ Tregs for available IL-2. Because of the greater dependence of Tregs on IL-2 compared with effector T cells (37, 38), CD4⁺CD25⁺ Treg survival at tumor sites and/or local infiltration may be inhibited due to IL-2 competition with CD8⁺CD25^{high} T cells. It has also been reported that neutralization of IL-2 selectively reduces the number of CD4⁺CD25⁺ Treg but not CD4⁺CD25⁻ effector T cells in autoimmune models (38). The fact that the frequency of CD4⁺CD25⁺ Tregs is markedly downregulated in the anti-CTLA-4/anti-GITR mAb treatment group and is associated with higher frequency of Treg-resistant CD8⁺CD25^{high} T-cell population compared with anti-CTLA-4 mAb alone might also be explained on the basis of IL-2 consumption by CD8⁺CD25^{high} T cells.

In summary, anti-CTLA-4 mAb treatment increases the number of effector T-cell infiltration in tumors, but these effector T cells remain sensitive to CD4⁺CD25⁺ Tregs and would be suppressed by the CD4⁺CD25⁺ Tregs. In contrast, anti-GITR mAb increases the resistance of effector T cells to Treg suppression but does not increase the number of effector T cells in tumors. These differences in Treg sensitivity/resistance may be a critical element in the success of anti-CTLA-4/anti-GITR mAb combination treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Two Distinct Mechanisms of Augmented Antitumor Activity by Modulation of Immunostimulatory/Inhibitory Signals

Jun Mitsui, Hiroyoshi Nishikawa, Daisuke Muraoka, et al.

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