# Cytotoxic T lymphocyte-associated antigen 4 inhibition increases the antitumor activity of adoptive T-cell therapy when carried out with naïve rather than differentiated T cells

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**Abstract.** Although treatment with an antibody against cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) combined with multiple therapeutic interventions has been explored, the effect of combination therapy with CTLA-4 inhibition and adoptive T-cell therapy has not been determined. In the present study, our aim was to determine whether CTLA-4 inhibition, combined with adoptive transfer of T cells at different stages of differentiation, exhibits synergistic antitumor effects in a murine colon cancer model. Mice bearing subcutaneous tumors were administered adoptive T-cell transfer of $CD62L^{high}$ or $CD62L^{low}$ cells combined with an anti-CTLA-4 antibody (α-CTLA-4) or control immunoglobulin G. Subcutaneous tumors were harvested, and the antitumor effects and helper T-cell polarization were analyzed. CTLA-4 inhibition combined with $CD62L^{high}$ cell administration showed the strongest antitumor effect. Combination therapy increased the number of $CD3^+$ cells within the tumor. Moreover, CTLA-4 inhibition induced polarization of T cells infiltrating the tumor toward the T helper 1 lineage, and suppressed the frequency of regulatory T cells within the tumor, particularly in combination with $CD62L^{high}$ T-cell transfer. This is the first report demonstrating that the efficacy of α-CTLA-4 and adoptive T-cell transfer combination therapy depends on the state of differentiation of the transferred T cells. Our data support the notion that a combination of α-CTLA-4 and adoptive T-cell transfer containing an abundance of naïve phenotype cells could potentially exert antitumor effects in a clinical setting.

## Introduction

Antigen-specific response and tolerance to tumors of the immune system are regulated by multiple networks of stimulatory and inhibitory signals. Delivery of inhibitory signals to T cells mediated by cytotoxic T-lymphocyte antigen 4 (CTLA-4) may mediate the development of tumor antigen-specific T-cell tolerance. CTLA-4 is expressed on the cell surface of activated T cells and is critical to restrict cell cycle progression and inhibit the production of interleukin (IL)-2 (1). CTLA-4 presents a degree of sequence homology with the T-cell costimulatory molecule CD28 and binds with higher avidity and affinity than CD28 to its ligands, B7-1 and B7-2 (2). Consequently, CTLA-4 promotes the termination of immune responses by preventing continuous T-cell costimulation and activation (2). $CD4^+$ and $CD8^+$ T cells not expressing CTLA-4 exhibit an activated phenotype and increased proliferation potential both *in vitro* and *in vivo* (3,4). CTLA-4-deficient mice develop a CD28-dependent expansion of autoreactive T cells in lymph nodes, spleen and several peripheral organs, which leads to death within 4 weeks after birth due to diffuse lymphoproliferative disease (5).

Due to the central role of CTLA-4 in the inhibition of T-cell activation, targeting of this molecule holds great promise for several clinical applications. Clinical trials conducted with various anti-CTLA-4 antibodies (α-CTLA-4) demonstrated that selective inhibition of CTLA-4 enhances the endogenous antitumor immune response. The fully human antibodies tremelimumab and ipilimumab have been studied extensively in melanoma and were found to act by blocking the interaction of CTLA-4 with B7 ligands to enhance T-cell proliferation and activation (6-8). In a phase III study, tremelimumab administration did not improve overall survival when compared with dacarbazine chemotherapy (6). Conversely, ipilimumab

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*Abbreviations:* CTLA, cytotoxic T lymphocyte-associated antigen 4; Treg, regulatory T cell; Th, helper T; Teff, effector T cell; ADCC, antibody-dependent cellular cytotoxicity

*Key words:* CTLA-4 antibody, adoptive T-cell therapy, naïve T-cell, T helper 1

administration improved survival in comparison with patients with melanoma previously treated with a peptide vaccine (7). Furthermore, a phase III study demonstrated that ipilimumab and dacarbazine combination therapy is more effective than dacarbazine treatment alone (8). Subsequently, ipilimumab was approved in 2011 for the treatment of unresectable or metastatic melanoma by regulatory agencies in the US and the European Union.

CTLA-4 inhibition combined with multiple therapeutic interventions in murine tumor models has been explored (9). Synergistic effects were demonstrated in combination with chemotherapy (10), radiation (11,12), cryoablation (13) and surgery (14). These studies indicate that CTLA-4 inhibition can be an effective therapeutic strategy to extend and elicit the immune response in cancer-bearing hosts. Preclinical studies have demonstrated that CTLA-4 suppression is effective against tumors in combination with other immunotherapies such as cancer vaccines (15-18), cytosine-phosphate-guanine oligodeoxynucleotide (CpG-ODN) adjuvants (18,19) and antibodies (20,21). However, it is not clear whether CTLA-4 inhibition and adoptive T-cell transfer combination therapy is effective against tumors.

The state of differentiation of T cells is crucial to the success of adoptive T-cell therapy, and less-differentiated T cells are ideal due to their *in vivo* persistence, high proliferative potential, receptiveness to homeostatic and costimulatory signals, and their ability to target secondary lymphoid tissues and secrete IL-2 (22,23). CTLA-4 suppression has the potential to enhance the activation of less-differentiated transferred T cells *in vivo*. In the present study, we evaluated whether a combination of CTLA-4 inhibition and transfer of adoptive T cells at different stages of differentiation exhibit synergistic antitumor effects in a murine colon cancer model.

## Materials and methods

*Mice and cell line*. All experiments were performed according to the protocols approved by the Animal Care Committee of Kyoto Prefectural University of Medicine. BALB/c male mice were purchased from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan), fed a standard laboratory diet, and were provided with water *ad libitum* under standard laboratory conditions. Mice between 7 and 8 weeks of age were used for the subsequent experiments.

The colon-26 murine colon adenocarcinoma cell line was used. Cells were cultured in monolayer with RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), l-glutamine and penicillin/streptomycin at 37˚C in a humidified atmosphere containing 5% $CO_2$.

*Reagents*. For *in vivo* CTLA-4 inhibition, purified hamster anti-mouse CD152 (CTLA-4; clone UC10-4F10, #BE0032) immunoglobulin (Ig)G or hamster IgG control antibodies (#BE0091) were purchased from Bio X Cell (West Lebanon, NH, USA).

*Preparation of therapeutic CD62L$^{high}$ and CD62L$^{low}$ T cells*. The effects of CTLA-4 inhibition combined with transfer of adoptive T cells at different stages of differentiation were determined. To this end, CD62L$^{high}$ and CD62L$^{low}$ T cells were prepared for less-differentiated (naïve phenotype) and differentiated (effector phenotype) adoptive transfer therapy, respectively. T cells were obtained from spleens harvested from 7-week-old male BALB/c mice sacrificed by cervical dislocation. Splenocytes were mechanically dissociated and strained through a 40-$\mu$m nylon mesh to produce a single-cell suspension. CD3$^+$ T cells were separated by AutoMACS Pro using the Pan T isolation kit (both from Miltenyi Biotec, Tokyo, Japan) and seeded on 12-well plates (Thermo Fisher Scientific K.K., Yokohama, Japan) previously coated with 5 $\mu$g/ml of mCD3 antibody (R&D Systems, Rockville, MD, USA), and 5 $\mu$g/ml RetroNectin®. Cells were cultured in GT-T503 medium (Takara Bio, Inc., Otsu, Japan) containing 10% FBS, penicillin/streptomycin, nonessential amino acids, sodium pyruvate and 2-ME for 3 days ($1.5x10^6$ cells/2.5 ml/well). Subsequently, cells were transferred into a T225 flask (BD Falcon, Franklin Lakes, NJ, USA) and cultured with GT-503 containing 100 U/ml recombinant mouse IL-2 and 10 ng/ml recombinant mouse IL-7 (both from R&D Systems). Seven days after harvesting, CD62L$^{high}$ and CD62L$^{low}$ populations were sorted with a MACS CD62L$^+$ selection column (Miltenyi Biotec).

*Adoptive cell transfer and α-CTLA-4 administration*. Male BALB/c mice at 7-8 weeks of age were injected s.c. with $1x10^6$ colon-26 cells. Mice (n=9 for all groups) were treated with i.v. adoptive T-cell transfer ($5x10^7$ cells) 6 and 13 days after tumor challenge. α-CTLA-4 or control IgG [100 $\mu$g in phosphate-buffered saline (PBS)] was delivered intraperitoneally 5, 8, 10, 12 and 15 days after tumor challenge. The percentage of CD62L$^+$ cells in the transferred population was confirmed by flow cytometry. Tumor growth was monitored twice a week, and tumor volume was expressed as $(a \times b^2)/2$, where a is the largest and b is the smallest diameter of the tumor. Mice were sacrificed by cervical dislocation 17 days after tumor inoculation.

*Flow cytometry*. The phenotype of the lymphocytes in the transferred cells and in the draining lymph nodes was analyzed by flow cytometry. For lymph node analysis, the tissue was mechanically dissociated and strained through a 40-$\mu$m nylon mesh to produce a single-cell suspension.

Cells were stained with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, phycoerythrin-Texas Red (ECD)-, or phycoerythrin-cyanin (PC5)-conjugated monoclonal antibodies specific for CD3, CD4, CD8, CD62L (Beckman Coulter, Marseille, France), forkhead box P (Foxp)-3, and interferon (IFN)-γ (both from eBioscience, San Diego, CA, USA). Five hours before cell harvesting, brefeldin A (BD Biosciences, San Jose, CA, USA) was added for intracellular blocking of IFN-γ. A single aliquot was thawed, and mononuclear cells were stained with fluorescence-conjugated antibodies and analyzed with a FACSCalibur flow cytometer (BD Biosciences). Data acquisition and analysis were conducted with the CellQuest software version 6.0 for Mac OS 10 (BD Biosciences).

*Immunohistochemistry*. Seventeen days after subcutaneous transplantation of colon-26 cells, tumors were harvested, fixed in formalin and analyzed by immunohistochemistry. For immunostaining, 4-$\mu$m sections were cut, deparaffinized and subjected to heat-induced epitope retrieval before incubation

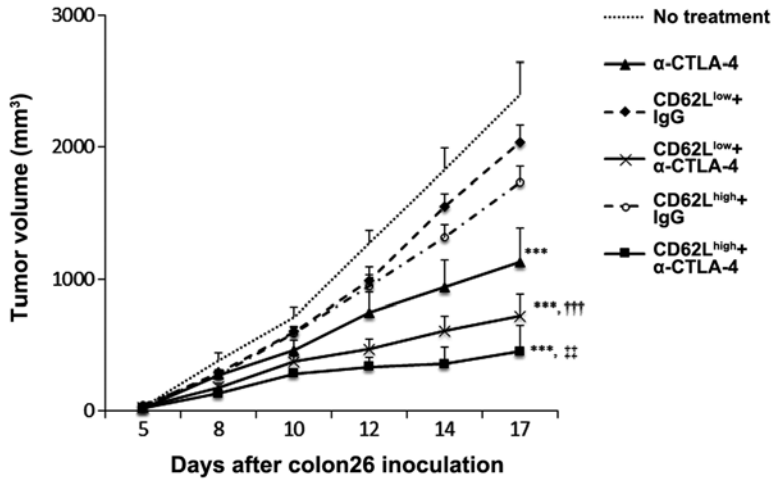


Figure 1. Effects of combination therapy with anti-CTLA-4 Ab and adoptive cell transfer on tumor growth. Tumor-bearing mice were treated with anti-CTLA-4 Ab or control IgG (day 5, 8, 10, 12 and 15) combined with adoptive cell transfer (day 6 and 13) of $CD62L^{low}$ or $CD62L^{high}$ cells. Results of tumor volume are the mean of measurements from 9 mice/group. Error bars represent means ± SEM. $^{***}P<0.001$ vs. no treatment. $^{†††}P<0.001$ vs. $CD62L^{low}$ + IgG. $^{‡‡}P<0.01$ vs. $CD62L^{high}$ + IgG. CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; IgG, immunoglobulin G.

with the antibodies. Sections were immersed in sodium citrate buffer at pH 7.0 and heated in a high-pressure cooker, treated with 3% $H_2O_2$ in methanol for 15 min, and blocked with Dako Protein Block Serum-Free solution for 30 min. Two consecutive sections were then incubated for 2-3 h at room temperature with a rabbit anti-CD3 antibody (ab5690; Abcam, Cambridge, MA, USA) and a rabbit anti-Foxp3 antibody (14-5773-82; eBioscience) at a dilution of 1:100 and 1:300, respectively. After incubation with anti-rabbit MAX-PO secondary antibody (Nichirei Bioscience, Tokyo, Japan), color development was performed using a DAB substrate kit (Nichirei Bioscience).

*Detection of T-bet, GATA-3 and Foxp3 expression by western blot analysis*. Subcutaneous tumors were harvested and frozen immediately. Total cell protein was extracted by thawing on ice and homogenizing at 4˚C in a solution of 50 mmol/l Tris-HCl, pH 7.6, 300 mmol/l NaCl, 0.5% Triton X-100, 10 $\mu$g/ml aprotinin, 10 $\mu$g/ml leupeptin, 1 mmol/l phenylmethylsulfonyl fluoride, 1.8 mg/ml iodoacetamide, 50 mmol/l NaF and 1 mM DTT. Equal quantities of protein (25 $\mu$g) were added to lysis buffer containing protease inhibitors and boiled at 70˚C for 10 min. The proteins were separated by 10% NuPAGE® Novex Bis-Tris Gel and electroblotted to nitrocellulose membranes (iBlot® Transfer Stack) (both from Thermo Fisher Scientific, Hampton, NH, USA). Membranes were incubated in blocking buffer (AE-1475; ATTO Corporation, Tokyo, Japan) for 20 min, followed by primary antibodies (20 h) raised against mouse T-bet (1:500 dilution, sc21003), GATA-3 (1:500 dilution, sc9009) (both from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), Foxp3 (1:500 dilution, 320002; BioLegend, Inc., San Diego, CA, USA), tubulin (1:500 dilution, T9026; Sigma-Aldrich, St. Louis, MO, USA) in Tris-buffered saline containing 0.1% Tween-20 (TBS-T). Subsequently, membranes were incubated with secondary anti-mouse or rabbit antibodies (GE Healthcare, Tokyo, Japan) in TBS-T (diluted 1:10,000) for 50 min at room temperature. Immunocomplexes were detected using a commercial kit (ECL Plus; GE Healthcare Bio-Sciences K.K., Tokyo, Japan) according to the manufacturer's recommendations.

*Statistics*. The results are presented as mean ± SEM. Statistical significance of differences between means was analyzed by one-way ANOVA, followed by Tukey's multiple comparison test, and $P<0.05$ indicates a statistically significant difference. All analyses were performed using the GraphPad Prism 4 program (GraphPad Software, Inc., La Jolla, CA, USA).

## Results

*Phenotype of transferred cells*. $CD62L^{high}$ and $CD62L^{low}$ cells from mouse spleen were cultured, and before cell separation, double $CD62L^+$ and $CD3^+$ T cells contributed to 55% of the total cell population. After separation, the fractions of double $CD62L^+$ and $CD3^+$ T cells among total $CD62L^{high}$ T cells from the first and second adoptive transfer were 92.16 and 96.78%, respectively. In contrast, the fraction of double-positive T cells among total $CD62L^{low}$ T cells was 26.63% in the first and 36.71% in the second adoptive transfer. Subsequently, we considered T-cell separation between $CD62L^{high}$ and $CD62L^{low}$ successful, and we used these cells for further analysis.

*CTLA-4 inhibition enhances the therapeutic potential of adoptive T-cell transfer*. To determine whether CTLA-4 inhibition enhances the antitumor effects of adoptive cell transfer, $1x10^6$ colon-26 cells were injected subcutaneously, followed by intravenous injection of $CD62L^{high}$ or $CD62L^{low}$ T cells with or without administration of α-CTLA-4. Tumor growth was monitored twice a week. The body weight of the mice was not affected by the procedure and did not change over time (data not shown). Administration of $CD62L^{high}$ T cells exhibited a tendency toward higher antitumor activity than administration of $CD62L^{low}$ T cells (Fig. 1). α-CTLA-4 monotherapy displayed significant antitumor activity. Administration of α-CTLA-4 combined with $CD62L^{low}$ or $CD62L^{high}$ cell administration enhanced the antitumor activity to a greater

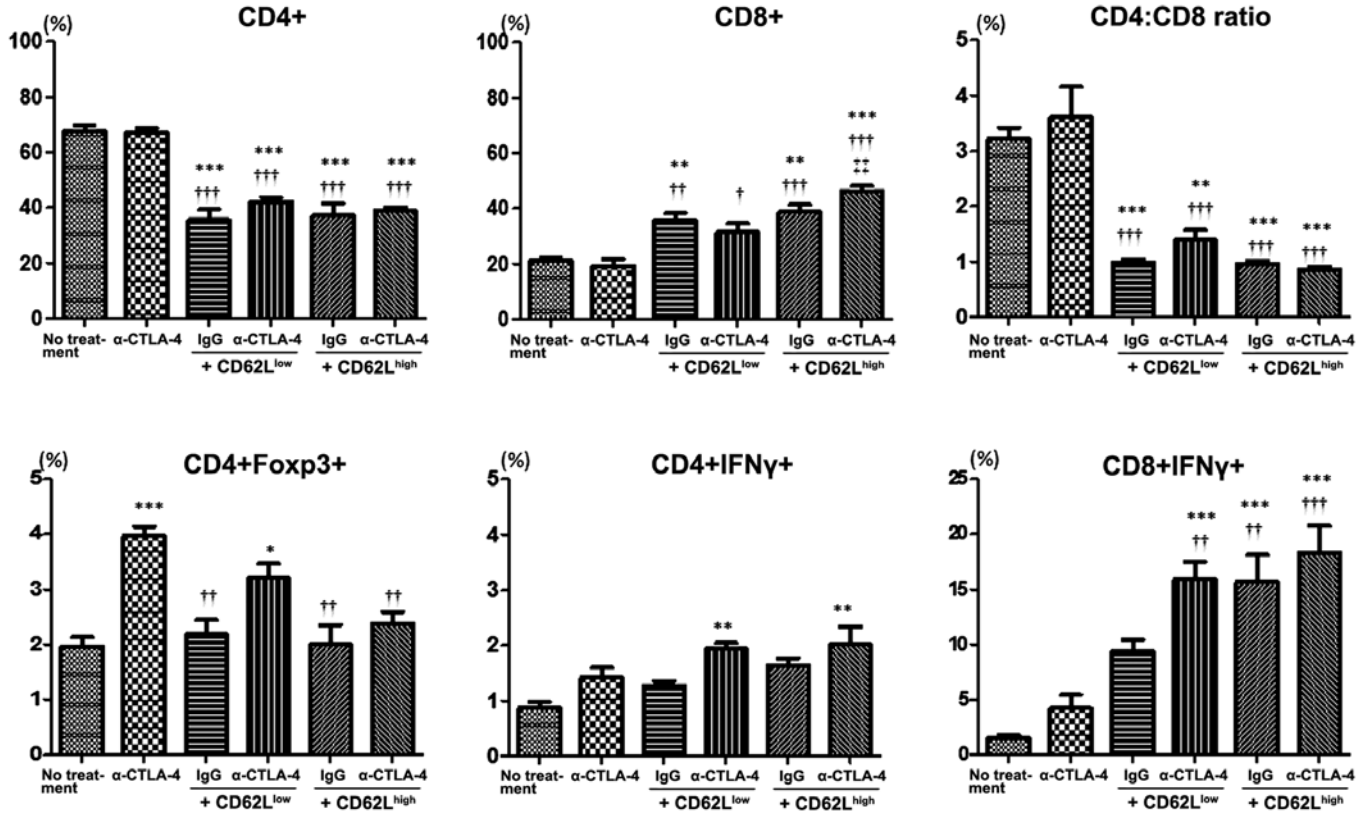


Figure 2. Flow cytometric analysis of lymphocytes from the spleen of control mice (no treatment), mice treated with anti-CTLA-4 Ab and mice treated with adoptive cell transfer (CD62L$^{low}$ or CD62L$^{high}$ subsets) combined with control IgG or anti-CTLA-4 Ab at day 17 after tumor challenge. The percentage of CD4$^+$, CD8$^+$, CD4$^+$Foxp3$^+$, CD4$^+$IFNγ$^+$ and CD8$^+$IFNγ$^+$ T cells was analyzed using flow cytometry (n=3 mice in each group). Error bars represent means ± SEM. $^*$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001 vs. no treatment. $^†$P<0.05, $^{††}$P<0.01, $^{†††}$P<0.001 vs. anti-CTLA-4 Ab. $^{‡‡}$P<0.01 vs. CD62L$^{low}$ + anti-CTLA-4 Ab. CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; IgG, immunoglobulin G.

extent than did administration of CD62L$^{low}$ or CD62L$^{high}$ in combination with IgG injection (P<0.001). CTLA-4 suppression combined with administration of CD62L$^{high}$ T cells exhibited a tendency toward a higher efficacy against tumors than injection of CD62L$^{low}$ T cells, although the effect was not statistically significant.

*Adoptive T-cell transfer and CTLA-4 inhibition modifies the population of lymphocytes within the spleen and draining lymph nodes*. We assessed the phenotype of lymphocytes in the spleen and draining lymph nodes of tumor-bearing mice. In the spleen of mice subjected to adoptive T-cell transfer, the frequency of CD4$^+$ lymphocytes was decreased whereas that of CD8-positive cells was increased (Fig. 2). The CD4/CD8 ratio was significantly lower in mice subjected to adoptive cell transfer than this ratio in the controls (no treatment) or mice injected with α-CTLA-4. Monotherapy with α-CTLA-4 did not affect the frequency of CD4- and CD8-positive cells or the CD4/CD8 ratio in the spleen. The frequency of Tregs (CD4$^+$ and Foxp3$^+$) in the spleen was higher in mice treated with α-CTLA-4 than the frequency in the controls. The frequency of IFN-γ-producing cells in CD4$^+$ lymphocytes was higher in mice subjected to α-CTLA-4 and adoptive cell transfer combination therapy when compared with the frequency in the control mice. The frequency of IFN-γ-producing cells among CD8$^+$ lymphocytes was higher in the context of α-CDLA-4 and CD62L$^{low}$ adoptive transfer combination therapy, and in mice subjected to CD62L$^{high}$ cell transfer irrespective of CTLA-4 suppression, than under control conditions. The frequency in the context of α-CTLA-4 monotherapy or CD62L$^{low}$ adoptive transfer was higher than under basal conditions, yet the effect reached statistical significance.

Similar trends were observed in draining lymph nodes (Fig. 3). The frequency of CD4$^+$ T cells was decreased in mice administered CD62L$^{high}$ T cells combined with control IgG or α-CTLA-4 in comparison to control mice or mice subjected to α-CTLA-4 monotherapy. The frequency of CD8$^+$ T cells was significantly higher in the context of adoptive CD62L$^{low}$ or CD62L$^{high}$ transfer than under control conditions, and this effect was more pronounced in the mice subjected to CD62L$^{high}$ T-cell transfer than in those administered CD62L$^{low}$ T cells. The CD4/CD8 ratio was lower in mice treated with α-CTLA-4 or subjected to adoptive T-cell transfer, either alone or in combination, than this ratio in the control mice. The lowest CD4/CD8 ratio was observed in the mice subjected to CD62L$^{high}$ T-cell transfer and α-CTLA-4 combination therapy. The frequency of Tregs increased in all mice treated with α-CTLA-4 or subjected to adoptive T-cell transfer. The frequency of IFN-γ-producing cells among CD4-positive lymphocytes was not affected by the treatments. Mice injected with α-CTLA-4 or T cells, either alone or in combination, exhibited a tendency toward a higher frequency of IFN-γ-producing cells among CD8$^+$ lymphocytes than control mice, yet the effect did not reach statistical significance.

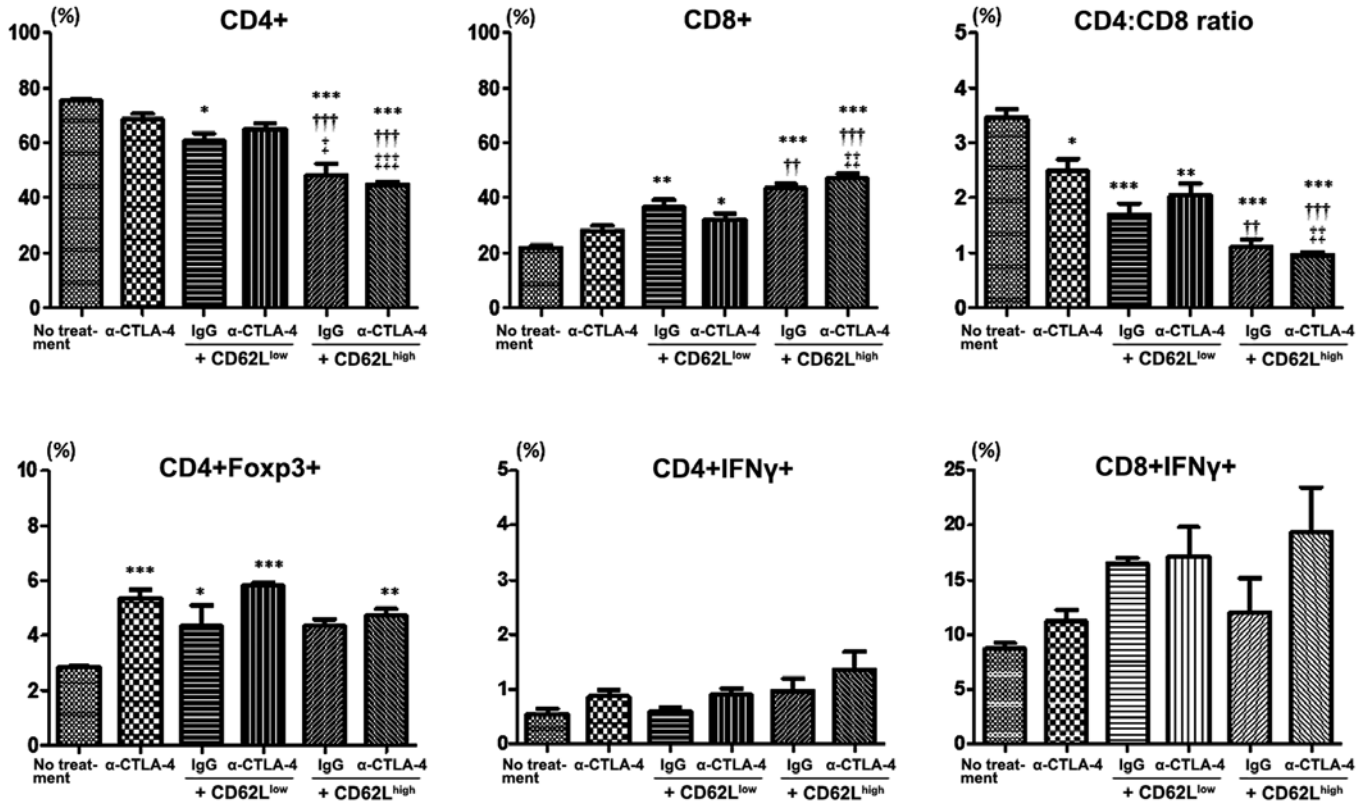


Figure 3. Flow cytometric analysis of lymphocytes from draining lymph nodes of control mice (no treatment), mice treated with anti-CTLA-4 Ab and mice treated with adoptive cell transfer ($CD62L^{low}$ or $CD62L^{high}$ subsets) combined with control IgG or anti-CTLA-4 Ab at day 17 after tumor challenge. The percentage of $CD4^+$, $CD8^+$, $CD4^+Foxp3^+$, $CD4^+IFN\gamma^+$ and $CD8^+IFN\gamma^+$ T cells was analyzed using flow cytometry (n=3 mice in each group). Error bars represent means ± SEM. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. no treatment. $^{††}P<0.01$, $^{†††}P<0.001$ vs. anti-CTLA-4 Ab. $^{‡‡}P<0.01$ vs. $CD62L^{low}$ + anti-CTLA-4 Ab. CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; IgG, immunoglobulin G.

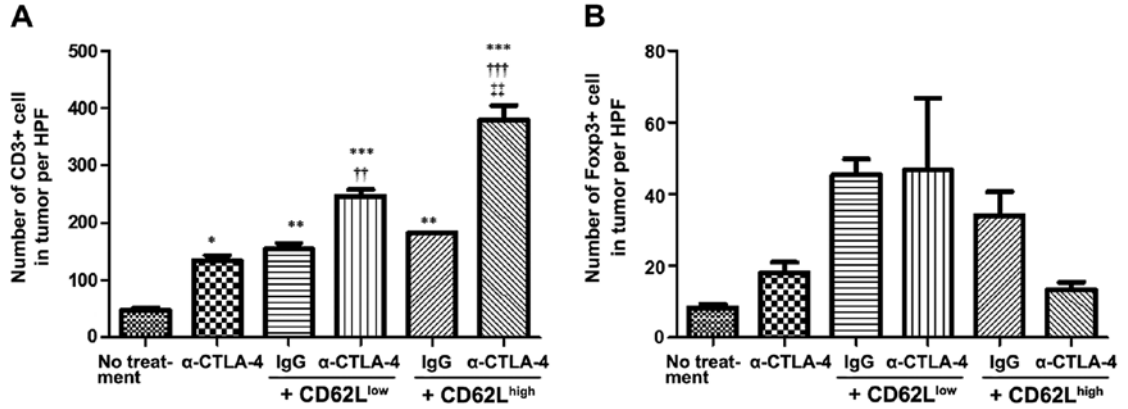


Figure 4. Immunohistological analysis of infiltrating T cells within the tumors of control mice (no treatment), mice treated with anti-CTLA-4 Ab and mice treated with adoptive cell transfer ($CD62L^{low}$ or $CD62L^{high}$ subsets) combined with control IgG or anti-CTLA-4 Ab at day 17 after tumor challenge. The graph shows the average number of (A) $CD3^+$ and (B) $CD4^+Foxp3^+$ T cells within tumors counted in 3 random microscopic views under x200 magnification for each tumor. Error bars represent means ± SEM. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. no treatment. $^{††}P<0.01$, $^{†††}P<0.001$ vs. anti-CTLA-4 Ab. $^{‡‡}P<0.01$ vs. $CD62L^{low}$ + anti-CTLA-4 Ab. HPF, high power field; CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; IgG, immunoglobulin G.

*CTLA-4 inactivation promotes T-cell migration and lowers the frequency of Foxp3-positive cells within the tumor.* We assessed the number of infiltrating T cells within the tumor by immunohistochemistry. Quantitative analysis represents the mean counts from three high-power fields. The number of intratumoral $CD3^+$ cells in mice subjected to α-CTLA-4 monotherapy, or to adoptive $CD62L^{low}$ or $CD62L^{high}$ T-cell transfer, either alone or in combination, was higher than in untreated controls (Fig. 4). α-CTLA-4 and $CD62L^{low}$ and $CD62L^{high}$ adoptive transfer promoted the migration of CD3 positive cells better than adoptive T-cell transfer combined with administration of control IgG. The number of $CD3^+$ cells migrating within the tumor was the highest in mice subjected to α-CTLA-4 and $CD62L^{high}$ adoptive transfer combination

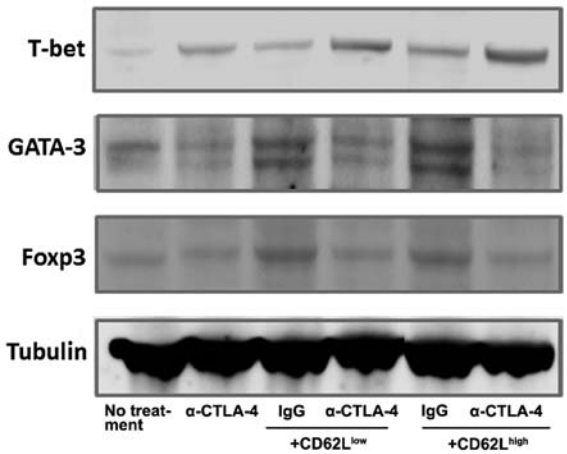


Figure 5. Western blot analysis of the expression of T-bet, GATA-3 and Foxp3 within tumors of the control mice (no treatment), mice treated with anti-CTLA-4 Ab and mice treated with adoptive cell transfer ($CD62L^{low}$ or $CD62L^{high}$ subsets) combined with control IgG or anti-CTLA-4 Ab at day 7 after tumor challenge. CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; IgG, immunoglobulin G.

therapy. The number of $Foxp3^+$ cells within the tumor was higher in mice administered either $CD62L^{low}$ or $CD62L^{high}$ T cells than this number in the untreated mice. Mice administered α-CTLA-4 presented a tendency toward an increased number of intratumoral $Foxp3^+$ cells, although the effect did not reach statistical significance. Administration of α-CTLA-4 reduced the number of Foxp3-positive cells in the mice administered $CD62L^{high}$ but not $CD62L^{low}$ T cells.

*CTLA-4 inhibition alters the expression of T-bet, GATA-3 and Foxp3 in tumors of mice subjected to adoptive T-cell transfer.* Western blot analysis was performed to investigate helper T (Th) differentiation in the tumors. The differentiation of Th1 lymphocytes is associated with a specific transcription factor, T-bet, which is a key regulator of cytokine expression by Th1. The expression of T-bet was increased under all experimental conditions in comparison to basal conditions (Fig. 5). CTLA-4 inhibition and adoptive $CD62L^{low}$ and $CD62L^{high}$ T-cell transfer combination therapy enhanced the T-bet expression levels. T-bet expression was the highest in the mice treated with α-CTLA-4 in combination with $CD62L^{high}$ cell transfer. Blocking CTLA-4 attenuated the expression of the Th2 lineage transcription factor GATA3, whereas adoptive T-cell transfer had the opposite effect. CTLA-4 inhibition and $CD62L^{low}$ or $CD62L^{high}$ adoptive transfer combination therapy attenuated GATA3 expression. Foxp3 expression was higher in the mice subjected to adoptive T-cell transfer than that in the controls. Although CTLA-4 inactivation alone did not affect Foxp3 expression, α-CTLA-4 and adoptive T-cell transfer combination therapy attenuated Foxp3 expression in comparison to mice administered $CD62L^{low}$ and $CD62L^{high}$ cells combined with control IgG.

## Discussion

The present study provides evidence that blocking CTLA-4 enhances the antitumor efficacy of adoptive T-cell transfer therapy, particularly when $CD62L^{high}$ T cells, characterized by a high frequency of naïve T cells, were administered. Our results also indicate that α-CTLA-4 and adoptive T-cell transfer combination therapy increases the number of $CD3^+$ cells within the tumor, and that CTLA-4 inhibition leads to polarization of tumor-infiltrating T cells toward the Th1 lineage. Furthermore, α-CTLA-4 combined with $CD62L^{high}$ yet not $CD62L^{low}$ T cells decreased the frequency of Tregs within the tumor. Although CTLA-4 suppression combined with cancer vaccines (15-18) and therapeutic antibodies (20,21) is effective against tumors in preclinical models, there is limited evidence of a synergistic antitumor effect of CTLA-4 suppression and adoptive T-cell therapy (24,25). This is the first report on the effects of α-CTLA-4 on Th polarization of tumor-infiltrating T cells following adoptive T-cell transfer, and suggests that the effects of combination therapy depend on the state of T-cell differentiation. These data may have important implications in the clinical application of α-CTLA-4 combined with adoptive T-cell therapy.

The exact mechanism mediating the antitumor effects of CTLA-4 inhibition remains undefined. Although antitumor activity of CTLA-4 suppression may be mediated by interference with the negative regulation of effector T-cell (Teff) function, recent reports suggest a secondary mechanism, wherein CTLA-4 inhibition affects Teff suppressive activity or mediates Treg depletion (25-27). In agreement with our results pertaining to the expression of CD3 and Foxp3, prior reports have demonstrated that CTLA-4 suppression decreases the number of Tregs within tumors, yet not those occurring in the draining lymph node (25,26), and increases the Teff/Treg ratio, which suggests an imbalanced proliferation of Tregs over Teffs within the tumor microenvironment (28-30). Recently, Simpson *et al* demonstrated in a mouse model that α-CTLA-4 depletes tumor-infiltrating Tregs and that this effect is dependent on the presence of Fcγ receptor-expressing macrophages (25). These findings indicate that antibody-dependent cellular cytotoxicity (ADCC) is likely to be involved in Treg depletion in response to α-CTLA-4. However, a hamster α-CTLA-4 was used in the present study, so that under these experimental conditions, α-CTLA-4 may decrease the number of Foxp3-positive cells by ADCC-independent mechanisms. Previous studies have demonstrated that induced Tregs, a subset of Tregs, develop as a consequence of activation of mature T cells under specific conditions in the tumor periphery, at local tumor sites, or in lymphoid organs (31,32). Furthermore, $Foxp3^+CD25^+CD4^+$ Tregs can be present in a tumor as a result of conversion from the $CD25^-CD4^+$ population in the adoptive transfer system (33,34). Therefore, α-CTLA-4 may have blocked this conversion such that the number of Foxp3-positive cells within the tumor was decreased in the mice subjected to adoptive transfer with $CD62L^{high}$ T cells, represented mostly by naïve T cells.

In agreement with our results, CTLA-4 inhibition has been found to enhance the Th1 response (35-37). van Elsas *et al* reported that T cells from mice treated with α-CTLA-4 in combination with a GM-CSF-producing tumor cell vaccine exhibited enhanced IFN-γ secretion *in vivo*. In addition, the severity of experimental allergic encephalomyelitis, a classical Th1-mediated autoimmune disease model, is exacerbated by CTLA-4 suppression (36,37). Contrary to these findings, it

was demonstrated that engagement of CTLA-4 with B7 led to polarization of naïve $CD4^+$ cells toward the Th1 subset and that the Th1 polarization was inhibited by CTLA-4 suppression *in vitro* (38). However, our findings support the notion that blocking CTLA-4 caused polarization of transferred naïve $CD4^+$ T cells toward the Th1 subset. Differences in these studies may be explained by the use of different experimental models and by the complexity of the events that regulate Th cell subset polarization and interactions of the immune system with tumors.

It is not clear whether the effects of blocking CTLA-4 on Th-cell subset polarization are mediated by an effect on the transferred T cells or on endogenous T cells, as these cells cannot be distinguished within the tumor site. Although analysis of the behavior of transferred cells is important, this is a significant challenge, since in light of our findings, the efficacy of antitumor therapy and the effects on Th-cell subset polarization by α-CTLA-4 are determined by the state of T-cell differentiation. Recently, we reported that expansion of T cells in the presence of fibronectin CH296 (FN-CH296) leads to higher yields of naïve T cells, and that FN-CH296-stimulated T-cell adoptive transfer therapy was very well tolerated with a level of efficacy in a phase 1 clinical trial (39). Based on these results, we intend to conduct a clinical trial to clarify the efficacy of α-CTLA-4 and adoptive transfer with FN-CH296-stimulated T-cell combination therapy.

In conclusion, α-CTLA-4 enhances the antitumor activity of adoptive T-cell transfer therapy, and the effects are more pronounced in the context of naïve T-cell administration. CTLA-4 suppression may enhance Th1 polarization and attenuate Treg differentiation of T cells infiltrating the tumor. These findings suggest that α-CTLA-4 and FN-CH296-stimulated T-cell adoptive transfer combination therapy holds potential as an effective antitumor clinical intervention.

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