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# The ICOS/ICOSL Pathway Is Required for Optimal Antitumor Responses Mediated by Anti–CTLA-4 Therapy

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## Abstract

The anti–CTL-associated antigen 4 (anti–CTLA-4) antibody ipilimumab is the first agent to show improved survival in a randomized phase III trial that enrolled patients with metastatic melanoma. Studies are ongoing to identify mechanisms that elicit clinical benefit in the setting of anti–CTLA-4 therapy. We previously reported that treated patients had an increase in the frequency of T cells expressing the inducible costimulator (ICOS) molecule, a T-cell–specific molecule that belongs to the CD28/CTLA-4/B7 immunoglobulin superfamily. ICOS and its ligand (ICOSL) have been shown to play diverse roles in T-cell responses such as mediating autoimmunity as well as enhancing the development/activity of regulatory T cells. These seemingly opposing roles have made it difficult to determine whether the ICOS/ICOSL pathway is necessary for antitumor responses. To determine whether the ICOS/ICOSL pathway might play a causal role in the antitumor effects mediated by anti–CTLA-4, we conducted studies in ICOS-sufficient and ICOS-deficient mice bearing B16/BL6 melanoma. We show that $ICOS^+$ T cells comprised a population of Th1 cytokine producing and tumor antigen-specific effector cells. Furthermore, in the absence of ICOS, antitumor T-cell responses elicited by anti–CTLA-4 are significantly diminished, thereby impairing tumor rejection. Our findings establish that the ICOS/ICOSL pathway is necessary for the optimal therapeutic effect of anti–CTLA-4, thus implicating this pathway as a target for future combinatorial strategies to improve the efficacy of anti–CTLA-4 therapy. *Cancer Res; 71(16); 5445–54. ©2011 AACR.*

## Introduction

Although T-cell responses are initiated by T-cell receptor signaling, additional costimulatory and coinhibitory signals regulate the outcome of T-cell activation. The prototypic costimulatory molecule is CD28, a molecule on T cells that provides critical signals necessary for activation of naïve T cells (1, 2). CTL-associated antigen 4 (CTLA-4) is the prototypical coinhibitory molecule that opposes CD28-mediated costimulation (3–5). Blockade of CTLA-4 with a monoclonal antibody (mAb) has been shown to enhance effector T cell ($T_{eff}$) activity with subsequent antitumor responses in a variety of murine models (6–9).

An mAb to human CTLA-4 (ipilimumab, Bristol-Myers Squibb, BMS) has been found in clinical trials to elicit objective responses in cancer patients (10–13). Recently, a phase III clinical trial in patients with advanced melanoma showed that blockade of CTLA-4 with the mAb ipilimumab improved survival of treated patients (14). We previously conducted a presurgical clinical trial to obtain tumor tissues and peripheral blood for immunological studies after patients were treated with anti–CTLA-4. We observed a marked increase in the frequency of T cells expressing inducible costimulator (ICOS) in both tumor tissues and blood of treated patients (15, 16). In a retrospective analysis of a small cohort of patients with metastatic melanoma treated with anti–CTLA-4, we found that a sustained increase in $ICOS^+$ T cells correlated with increased survival (17). These data suggested that $ICOS^+$ T cells might play an important role in antitumor immune responses.

ICOS is a T-cell specific molecule that is a member of the extended CD28/B7/CTLA-4 immunoglobulin superfamily (18). Unlike CD28, which is constitutively expressed on T cells and provides costimulatory signals necessary for full activation of resting T cells, ICOS is expressed only after activation. ICOS has been implicated in diverse aspects of T-cell responses. It plays a critical role in the function of follicular T helper cells, formation of germinal centers, regulation of Th2 cytokine production, T/B cell collaboration, and immunoglobulin class switching (19, 20). ICOS-deficient mice show impaired germinal center formation and have decreased production of the Th2 cytokine interleukin (IL)-10 (21). It has also been shown that $ICOS^+$ T cells are involved in transplant rejection (22) as well as autoimmune responses (23–25). ICOS has also been linked to the function of regulatory T ($T_{reg}$) cells. It has been reported that naïve CD4 T cells can differentiate into IL-10–producing $T_{reg}$ cells as a result of ICOS/(ICOS and its ligand; ICOSL) interactions (26). It was also reported that melanoma

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cells expressing ICOS ligand promote the activation and expansion of $T_{reg}$ cells (27). These data would suggest that ICOS might play a role in suppressing antitumor responses. On the contrary, our finding that CTLA-4 blockade in cancer patients results in an increase in the frequency of $ICOS^+$ T cells that seem to correlate with clinical benefit is more consistent with a role for the ICOS/ICOSL pathway in enhancing antitumor responses.

To directly examine the role of $ICOS^+$ T cells in tumor rejection mediated by anti–CTLA-4, we conducted studies in wild-type (wt), ICOS-deficient ($ICOS^{-/-}$), and ICOSL-deficient ($ICOSL^{-/-}$) mice bearing B16/BL6 melanoma. We show for the first time that the ICOS/ICOSL pathway plays an important role in the therapeutic effect of anti–CTLA-4. Our data suggest that the ICOS/ICOSL pathway can be targeted to enhance the efficacy of CTLA-4 blockade in cancer patients.

## Materials and Methods

### Mice, cell lines, and reagents

C57BL/6 (B6) mice were purchased from The Jackson Laboratory and the National Cancer Institute. $ICOS^{-/-}$, and $ICOSL^{-/-}$ mice, all on the B6 background, were obtained from The Jackson Laboratory. All animal experiments were carried out under pathogen-free conditions according to approved protocols from UT MD Anderson Cancer Center IACUC.

The B16/BL6 murine melanoma cell line, the TRAMP-C2 murine prostate cancer line, and B16/BL6 expressing GM-CSF (Gvax) were kind gifts from Dr. Jim Allison. Cells were maintained, used, and tested for tumorigenicity and unique tumor antigen expression as previously published (7–9, 28–30). Cells were grown in minnimum essential medium supplemented with 10% FBS, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 1% nonessential amino acids, and vitamin (all from Invitrgen). Cell lines were tested for mycoplasma by MycoAlert Mycoplasma detection Kit from Lonza and were found to be mycoplasma free.

Anti–CTLA-4 mAb (clone 9H10) was obtained from BioXcell. Mouse and human melanoma differential antigen-derived peptides, H-$2D^b$–restricted $hgp100_{25–33}$ (KVPRNQDWL), H-$2K^b$–restricted $TRP2_{180–188}$ (SVYDFFVWL), and $TRP1_{222–229}$ (TWHRYHLL; refs. 31, 32) were synthesized by Invitrogen with high-performance liquid chromatography to a purity of more than 95%.

### *In vivo* tumor growth and treatment

The tumor inoculation and treatment protocol has been previously described (7–9, 28) and was used here with minor modifications. Briefly, the anesthetized mice were injected in the right flank intradermally (i.d.) with $1 \times 10^4$ B16/BL6 melanoma cells at day 0, and then left untreated or treated with anti–CTLA-4 mAb plus Gvax started on day 3 after tumor inoculation. One million irradiated (150 Gy) Gvax cells were injected i.d. in 100 μL PBS in the left flank on days 3, 6, 9, while at the same time points 200, 100, and 100 μg of anti–CTLA-4 mAb were injected i.p. in 200 μL of PBS. Tumor growth was monitored against time, and tumor size was measured with an electronic caliper 2 to 3 times every week. Mice were euthanized when tumor reached 1.5 cm in diameter or ulceration or moribund occurred.

### *Ex vivo* study and tumor infiltrating lymphocytes isolation

Mice were inoculated i.d. with $5 \times 10^4$ of B16/BL6 cells and treated as described above for *in vivo* treatment. Six to 7 days after the last treatment, mice were euthanized with $CO_2$, spleen and tumor draining lymph nodes (DLN) as well as tumor tissues were removed for single cell suspension preparation. For analyses of intracellular cytokines, tumor DLN cells were stimulated with 1 μmol/L ionomycin and 50 ng/mL phorbol 12-myristate 13-acetate in the presence of monensin (3 μmol/L) for 5 hours. Cells were then washed and blocked with Fc receptor mAb (2.4G2) before cell surface and intracellular staining with corresponding mAbs. To obtain T cells from tumor infiltrating lymphocytes (TIL), we followed previously published methods (7–9, 28). Briefly, tumors were cut into small pieces in the presence of 1 to 2 mL of a collagenase-DNAse mix (Roche), incubated at 37°C for 30 minutes, passed through 40 micron filter, loaded on Histopaque-1077 (Sigma), and spun at 2,000 × *g* for 30 minutes. Cells at the interphase were collected and washed in 5% RPMI 1640 containing 1 mmol/L EDTA before resuspending in complete culture medium (CM, 10% FBS RPMI-1640 medium supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 50 μmol/L 2-mercaptoethanol, 12 mmol/L HEPES, and 2 mmol/L L-glutamine; all from Invitrogen). In some experiments, CD8 TILs were further purified by using Dynabeads FlowComp (Invitrogen). The purified $CD8^+$ TILs (>85% purity) were stimulated with irradiated (150 Gy) B16/BL6 and antigen–irrelevant TRAMP-C2 prostate cancer cells pulsed on splenic dendritic cells (DC) and Brefeldin A (10 μg/mL). $1 \times 10^5$ CD8 T cells were cocultured with $5 \times 10^4$ splenic DCs plus irradiated tumor cells for 18 hours. Cells were harvested and washed before being blocked with anti-FcR II/III (clone 2.4G2). After cell surface staining for CD8 and ICOS, cells were further stained for intracellular IFN-γ and analyzed by flow cytometry.

### Flow cytometry

Single cell suspension ($2 \times 10^5$ to $5 \times 10^5$) were first blocked with 10 μg/mL of anti-FcR II/III (clone 2.4G2) on ice for 10 minutes followed by staining with the following fluorescence-conjugated Abs against CD4(GK1.5), CD8 (53-6.7), and ICOS (C398.4A or 7E.17G9; all from eBioscience) on ice for 30 minutes. After being washed twice with fluorescent activated cell sorting (FACS) buffer (2% FBS–PBS/0.05% sodium azide), cells were fixed with 0.5 mL of 1% paraformaldehyde-PBS. For intracellular cytokine staining, cells were treated with BD Cytofix/Cytoperm solution and stained for IL-2 (JES6-1A12), -4 (BVD4-1D11), -10 (JES5-16E3), -13 (eBio13A), -17A (TC11-18H10), and IFN-γ (XMG1.2) on ice for 30 minutes, then washed with the washing buffer and fixed with 1% paraformaldehyde-PBS. Foxp3 expression was detected by allophycocyanin-conjugated mAb (FJK16s; eBioscience) according to manufacturer's staining protocol. Pentamer staining for $TRP2_{180–188}$H-$2k^b$ (Proimmune) was carried out according to manufacturer's guidelines. Flow cytometry was carried

out on a BD FACS Canton II. Data were analyzed by FACS Diva (BD Biosciences) and FlowJo (Tree Star).

### Enzyme-linked immunosorbent spot

IFN-γ enzyme-linked immunosorbent spot (ELISPOT) was conducted by using a kit from MabTech. Briefly, 100 μL of the cell suspension ($2.5 \times 10^4$ cells/well) in CM were seeded into polyvinylidene difluoride plates precoated with anti–mouse IFN-γ antibody (clone AN18), followed by another 100 μL of the CM either alone or containing melanoma-related MHC class-I peptides, $hgp100_{25–33}$, $TRP2_{180–188}$, and $TRP1_{222–229}$. In some experiments, B16/BL6 tumor cells ($2.5 \times 10^4$/w, irradiated by 150 Gy) were used. After incubation for 48 hours at 37°C, plates were extensively washed with PBS plus 0.05% Tween-20 and incubated for 2 hours at 37°C with 100 μL/well of biotinylated antibody against mouse IFN-γ (clone R4-6A2). Spots were developed and counted by an automated ELISPOT reader system with ImmunoSpot 3.2 software (CTL Analyzers LLC).

### Statistical analysis

Results from *in vitro* experiments were expressed as means or means ± SEM. Data were analyzed by using a 2-sided Student's *t* test. The log-rank test was used to determine the significance in the survival experiments. All analyses were conducted by using Prism 5.0 (GraphPad Software, Inc.), and $P < 0.05$ was considered statistically significant.

## Results

### Anti–CTLA-4 therapy elicits an increase in CD4 and CD8 T cells with a concomitant increase in the frequency of $CD4^+ICOS^+$ and $CD8^+ICOS^+$ T cells

We previously reported that treatment of cancer patients with anti–CTLA-4 therapy led to an increase in the frequency of $ICOS^+$ T cells in both tumor tissue and systemic circulation (15–17). Here, to identify changes in T-cell subsets in mice after treatment with anti–CTLA-4 therapy, we conducted our studies in the setting of wt C57BL/6 (B6) and ICOS-deficient ($ICOS^{-/-}$) mice bearing B16/BL6 melanoma. We treated tumor-bearing mice with anti–CTLA-4 therapy as previously published (7–9) and assessed the impact of therapy on the major T-cell compartments in spleen, tumor DLN, and TILs. Wild-type and $ICOS^{-/-}$ mice had comparable numbers of CD4 and CD8 T cells prior to treatment (data not shown). TILs showed significant increases in CD4 (Fig. 1A) and CD8 (Fig. 1B) T cells after anti–CTLA-4 therapy. Interestingly, $ICOS^{-/-}$ mice had a greater increase in CD4 T cells as compared with wt mice (Fig. 1A). We then examined the frequency of $T_{reg}$ cells, as defined by expression of the Foxp3 transcription factor (33), in treated mice. The frequency of $CD4^+Foxp3^+$ T cells in splenocytes (Supplementary Fig. S1) and TILs (Fig. 1C) did not change significantly in wt tumor-bearing mice after treatment with anti–CTLA-4 but was shown to be increased in DLN (Supplementary Fig. S1), which was consistent with the previously published data (9). However, comparison between wt and $ICOS^{-/-}$ mice revealed that the $ICOS^{-/-}$ mice had a significantly lower frequency of $CD4^+Foxp3^+$ T cells prior to treatment (Supplementary Fig. S2) and after treatment with anti–CTLA-4 (Fig. 1C). This is consistent with reports that ICOS plays a role in the development/expansion of $Foxp3^+$ $T_{reg}$ cells (34). Next, we analyzed the frequency of $ICOS^+$ T cells in CD4 and CD8 populations from spleen, tumor DLN, and TILs of untreated and treated wt mice. As was the case in patients (17), the frequency of $ICOS^+$ T cells increased significantly in both the CD4 (Fig. 1D) and CD8 (Fig. 1E) T-cell populations in wt tumor-bearing mice treated with anti–CTLA-4 therapy.

### Anti–CTLA-4 therapy increases $ICOS^+$ T cells that consist of a population of $Foxp3^-$ cells to increase the ratio of $T_{eff}$ to $T_{reg}$ cells

Because $ICOS^+$ T cells have been previously reported to comprise a population of $Foxp3^+$ $T_{reg}$ cells, we next assessed the contribution of $Foxp3^+$ $T_{reg}$ cells to the increase in $ICOS^+$ CD4 T cells in TILs. As shown in Figure 2A, CD4 T cells from untreated mice consisted of approximately 7% $ICOS^+$ and approximately 93% $ICOS^-$ T cells. The $ICOS^+$ population consisted of approximately 36% $Foxp3^-$ and approximately 64% $Foxp3^+$ T cells (Fig. 2A, top). However, after anti–CTLA-4 therapy, the CD4 population contained approximately 25% $ICOS^+$ and approximately 75% $ICOS^-$ cells, and the $ICOS^+$ T cells comprised approximately 54% $Foxp3^-$ and approximately 46% $Foxp3^+$ T (Fig. 2A, bottom). Data summarized from 3 independent experiments indicate that the frequency of $CD4^+ICOS^+$ and $CD4^+ICOS^+Foxp3^-$ T cells increased significantly, whereas the frequency of $CD4^+ICOS^+FOXP3^+$ and total $CD4^+Foxp3^+$ T cells remained relatively unchanged after treatment of wt tumor-bearing mice with anti–CTLA-4 (Fig. 2B). The ratio of $T_{eff}$ cells to $T_{reg}$ cells was calculated based on frequency of total $CD4^+ICOS^+$ to total $CD4^+FoxP3^+$ T cells or $CD8^+ICOS^+$ to $CD4^+FoxP3^+$ T cells as shown in Figure 2C. The $T_{eff}/T_{reg}$ ratio was significantly increased in the spleen, tumor DLN, and TILs after treatment of wt tumor-bearing mice with anti–CTLA-4 (Fig. 2C). These data show that anti–CTLA-4 therapy favors expansion of $ICOS^+$ $T_{eff}$ over $T_{reg}$ cells and suggest that $ICOS^+$ T cells may represent a population of $T_{eff}$ cells that play an important role in antitumor immune responses evoked by anti–CTLA-4 therapy.

### $ICOS^+$ T cells comprise a population of effector cells that produce the Th1 cytokine IFN-γ and the cytokine IL-2

To determine whether $ICOS^+$ T cells might play a functional role in mediating antitumor immune responses, we assessed the nature of cytokine production by $ICOS^+$ T cells. After wt tumor-bearing mice were treated with anti–CTLA-4 therapy, we analyzed tumor DLN cells for intracellular cytokines present in both $ICOS^+$ and $ICOS^-$ CD4 T cells, which were all $Foxp3^-$ cells. A representative experiment is shown in Figure 3A showing that $ICOS^+$ CD4 cells consist of a population that produce IL-2 and IFN-γ whereas a lower frequency of cells are also capable of producing IL-4, -10, -17, and -13. Supplementary Figure S3 shows that both $ICOS^+$ and $ICOS^-$ CD4 T cells from untreated wt mice produce minimal amounts of cytokines. Figure 3B provides a summary of IL-2 (left) and IFN-γ

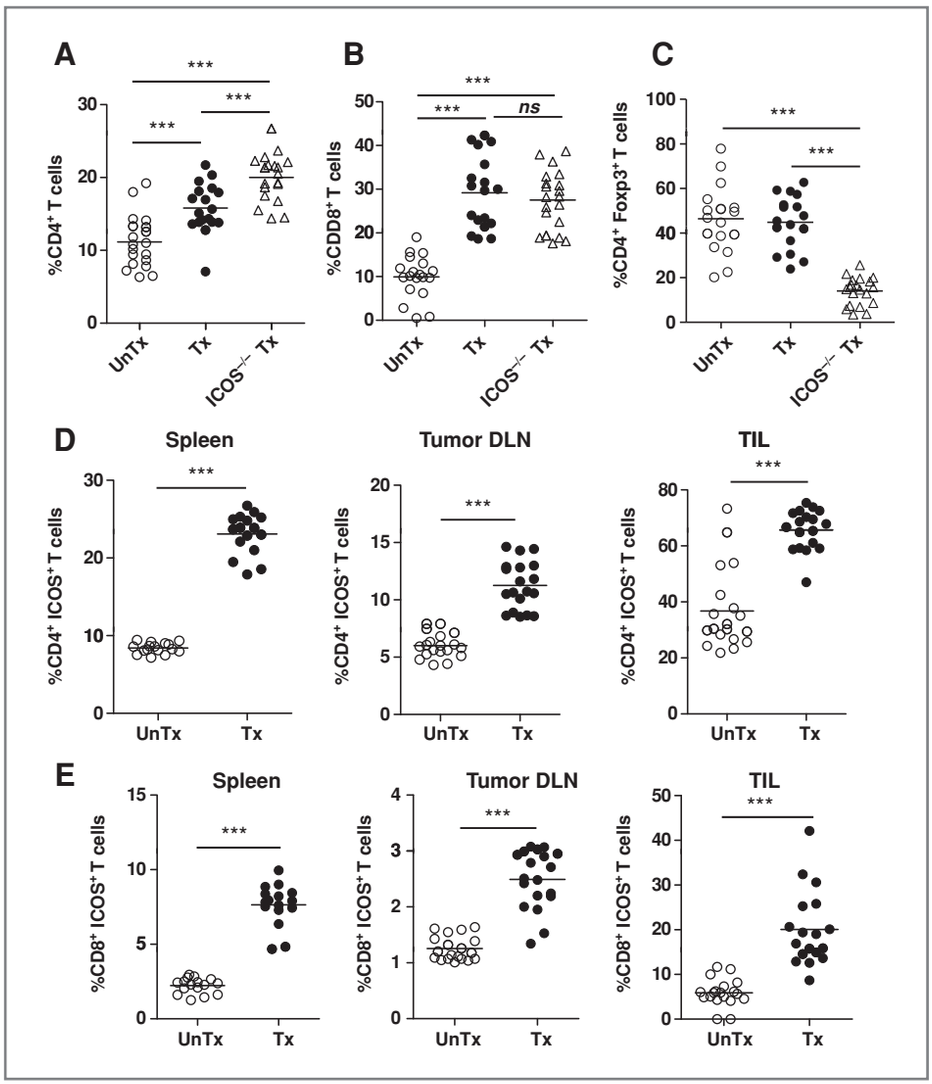


Figure 1. Anti–CTLA-4 therapy elicits an increase in CD4 and CD8 T cells with a concomitant increase in the frequency of $CD4^+ICOS^+$ and $CD8^+ICOS^+$ T cells. Mice inoculated with B16/BL6 tumor cells at day 0 were either untreated or treated with anti–CTLA-4 therapy at days 3, 6, and 9. Six to 7 days after the last treatment, they were sacrificed and spleen, tumor DLN cells, and TILs were analyzed by flow cytometry. A, CD4 and B, CD8 T cells were increased in wt and $ICOS^{-/-}$ mice in TILs after treatment with anti–CTLA-4 therapy as compared with untreated mice. C, the frequency of $CD4^+Foxp3^+$ T cells was not significantly different in TILs from untreated and treated wt mice but was significantly decreased in $ICOS^{-/-}$ mice. D, the frequency of $CD4^+ICOS^+$ and E, the frequency of $CD8^+ICOS^+$ T cells was increased in wt mice in spleen, tumor DLN, and TILs after treatment with anti–CTLA-4 therapy as compared with untreated mice. The results shown are from 3 independent experiments with 6 to 7 mice/group for each experiment. Horizontal solid lines intersecting circular data points represent means; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not statistically significant.

(right) production by $ICOS^+$ CD4 and $ICOS^-$ CD4 T cells obtained from 5 mice after treatment with anti–CTLA-4 (Fig. 3B, top). Similarly, $ICOS^+$ CD8 T cells, as opposed to $ICOS^-$ CD8 T cells, also consist of a population of cells that produce IL-2 and IFN-γ (Fig. 3B, bottom). Because IFN-γ and IL-2 are necessary for effective antitumor responses (35), our data implicate $ICOS^+$ T cells as containing the major population of cytokine-producing effector cells in the setting of anti–CTLA-4 therapy.

### $ICOS^+CD8T$ cells produce IFN-γ upon recognition of tumor antigen and the absence of ICOS results in diminished tumor antigen–specific T-cell responses

Because CD8 T cells have been shown to play a dominant role in tumor rejection in the B16/BL6 melanoma model treated with anti–CTLA-4 (7–9, 36), we investigated the impact of ICOS expression on the function of CD8 T cells. We first asked whether $ICOS^+$ CD8 T cells from TILs might be functionally associated with antitumor activity as judged by IFN-γ production after tumor antigen stimulation. To this end, CD8 TILs from B16/BL6 tumor-bearing wt mice treated with anti–CTLA-4 therapy were placed in coculture with splenic DC in the presence of B16/BL6 cells or TRAMP-C2 cells, as an irrelevant tumor control, and then intracytoplasmic cytokine production was analyzed by flow cytometry. Supplementary Figure S4 shows that in the absence of DCs, which limits antigen presentation to T cells, there is minimal cytokine production. As shown in Figure 4A, $ICOS^+$ CD8 T cells showed a 7-fold higher frequency of B16–specific IFN-γ production than $ICOS^-$ CD8 T cells ($P = 0.0001$; Fig. 4A). These data indicate that ICOS expression on CD8 T cells is functionally associated with T-cell responses consisting of IFN-γ production in the presence of tumor cells, which may indicate antitumor responses.

We then asked whether there were measurable differences in tumor antigen–specific CD8 T-cell responses between cells from wt and $ICOS^{-/-}$ mice. Untreated wt and $ICOS^{-/-}$ mice had similar data (Supplementary Fig. S5), and comparisons

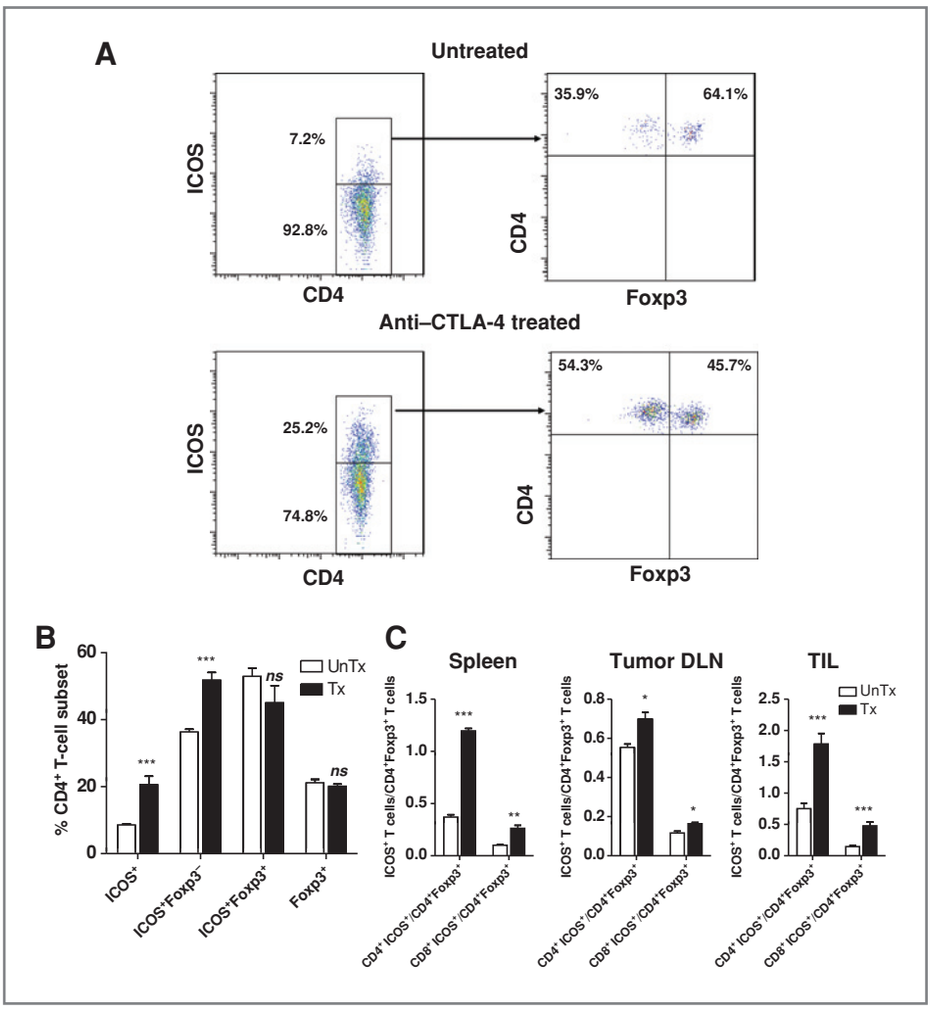


**Figure 2.** Anti–CTLA-4 therapy increases $ICOS^+$ T cells that consist of a population of $Foxp3^-$ cells to increase the ratio of $T_{eff}$ to $T_{reg}$ cells. A, as shown in a representative experiment, in untreated mice, 7.2% of CD4 T cells are $ICOS^+$ and the $ICOS^+$ T cells comprised 35.9% $Foxp3^-$ and 64.1% $Foxp3^+$ T cells (top); however, after treatment with anti–CTLA-4, 25.2% of CD4 T cells are $ICOS^+$ and the $ICOS^+$ T cells comprised 54.3% $Foxp3^-$ and 45.7% $Foxp3^+$ T cells (bottom). B, data from 3 independent experiments are summarized and indicate that the frequency of $CD4^+ICOS^+$ and $CD4^+ICOS^+Foxp3^-$ T cells increase significantly whereas the frequency of total $CD4^+Foxp3^+$ T cells remain relatively unchanged after treatment with anti–CTLA-4. C, the ratio of $T_{eff}/T_{reg}$ cells as determined by the frequency of total $CD4^+ICOS^+$ or $CD8^+ICOS^+$ T cells to total $CD4^+Foxp3^+$ T cells is significantly increased in spleen, tumor DLN, and TILs after treatment with anti–CTLA-4 therapy. The results shown are from 3 independent experiments with 6 to 7 mice/group for each experiment. Vertical bars represent means; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not statistically significant.

were made between untreated and treated mice. Because $TRP2_{180–188}$ is the predominant antigen in B16/BL6 tumors that is recognized by CD8 T cells from mice treated with anti–CTLA-4 therapy (37), we compared endogenous antigen-specific CD8 TILs from wt and $ICOS^{-/-}$ tumor-bearing mice treated with anti–CTLA-4 therapy. As shown in a representative experiment (Fig. 4B), anti–CTLA-4 therapy leads to an increase in T cells specific for $TRP2_{180–188}$ from 1% to 13.7% as detected by pentamer staining. However, this increase is significantly diminished to 3.3% in $ICOS^{-/-}$ mice, suggesting that the ICOS pathway may play a role in the expansion or survival of antigen-specific T cells. Data summarized from 3 independent experiments are also shown (Fig. 4C).

To determine whether there were functional differences in CD8 T cells from wt and $ICOS^{-/-}$ mice, we carried out ELISPOT assays for IFN-γ production in the presence of melanoma-specific peptides (gp100, TRP1, and TRP2) and B16/BL6 tumor cells. We found that CD8 T cells from $ICOS^{-/-}$ mice produced significantly less IFN-γ in response to the tumor antigens and B16/BL6 cells as compared with T cells from wt mice (Fig. 4D). Taken together, these data indicate that tumor antigen–specific T-cell responses induced by anti–CTLA-4 therapy are diminished in the absence of ICOS.

### Impaired tumor rejection in ICOS- and ICOSL-deficient mice treated with anti–CTLA-4 therapy

It has been shown that the ICOS/ICOSL pathway plays a significant role in the development, expansion/survival, and function of $T_{reg}$ cells (26, 34, 38). Indeed, we observed a lower frequency of $CD4^+Foxp3^+$ T cells in $ICOS^{-/-}$ mice (Fig. 1C). These data suggest a strong role for $ICOS^+$ T cells in immune suppression. However, our previous data in melanoma patients indicate that an increase in the frequency of $ICOS^+$ T cells induced by anti–CTLA-4 therapy may be associated with clinical benefit (17). To determine whether the ICOS/ICOSL pathway plays a role in immune suppression or tumor immunity in the setting of anti–CTLA-4, we compared the efficacy of anti–CTLA-4 therapy in wt, $ICOS^{-/-}$, and $ICOSL^{-/-}$ mice. As shown in Figure 5A, 10/10 untreated wt mice developed tumors and without intervention all mice died from tumor within 43 days after tumor inoculation, as previously published (7, 8, 28). Treatment of tumor-bearing wt mice with anti–CTLA-4 therapy resulted in tumor rejection in

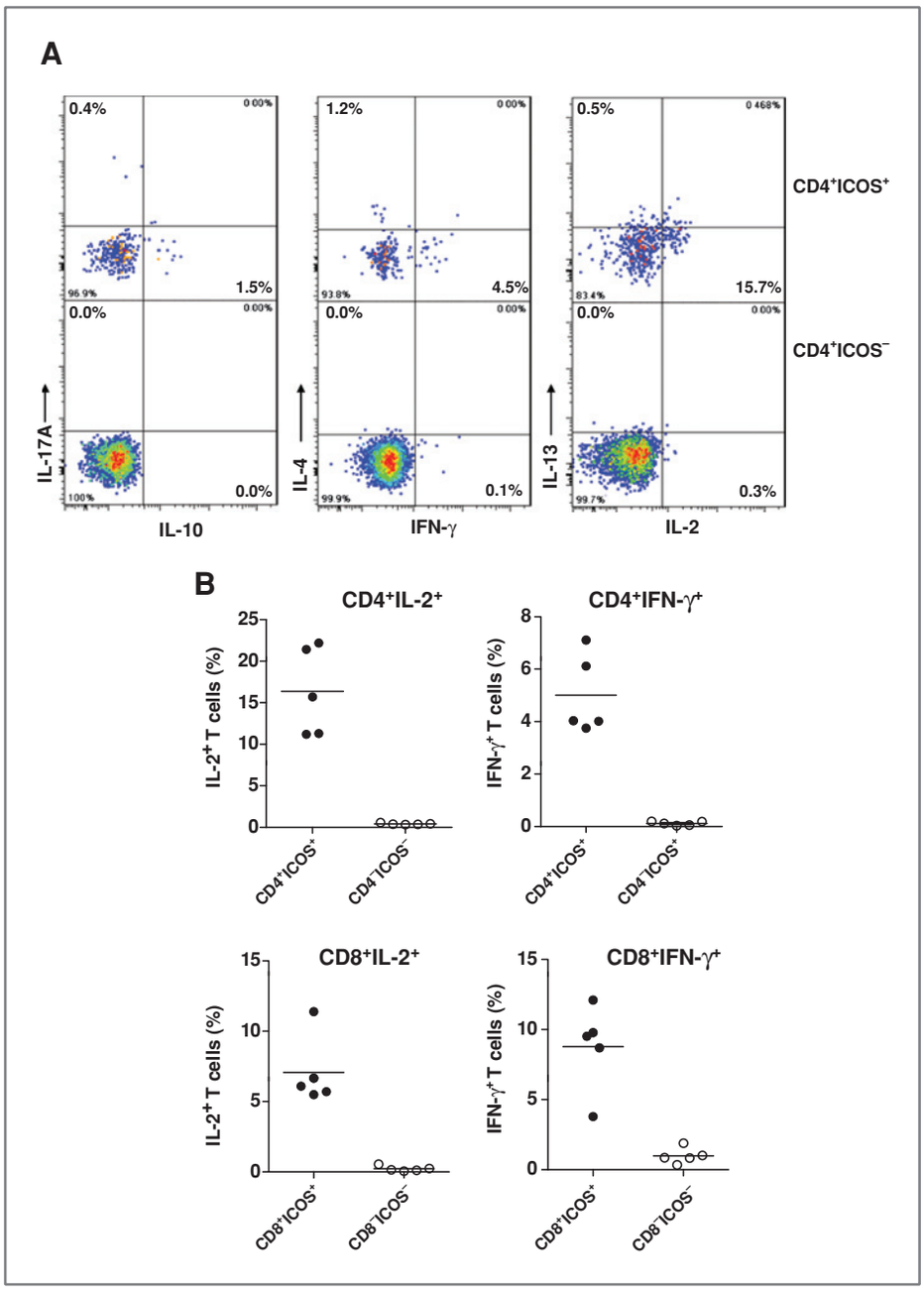


Figure 3. ICOS$^+$ T cells comprise a population of effector cells that produce the Th1 cytokines IL-2 and IFN-γ. A, tumor DLN Foxp3$^-$cells from anti–CTLA-4 treated wt mice ($n = 5$) were analyzed by flow cytometry. A representative experiment showing intracellular cytokine staining for ICOS$^+$ CD4 and ICOS$^-$ CD4 cells is shown. B, summary data for IL-2 and IFN-γ production are shown for CD4$^+$ICOS$^+$ and CD4$^+$ICOS$^-$ cells (top) as well as for CD8$^+$ICOS$^+$ and CD8$^+$ICOS$^-$ cells (bottom). The data are statistically significant with $P < 0.001$. The results shown are from 1 of 2 independent experiments with 5 mice/group for each experiment. Horizontal solid lines intersecting circular data points represent means.

the majority of mice, consistent with previous reports (7, 8, 28). However, the efficacy of treatment was greatly impaired in mice deficient in either ICOS or ICOSL (Fig. 5A). As shown in Figure 5A, tumors developed after anti–CTLA-4 therapy in 6/10 ICOS$^{-/-}$ and 7/10 ICOSL$^{-/-}$ mice, in contrast to wt mice that had only 2/10 mice with tumors. Of note, tumor development in ICOSL$^{-/-}$ mice was delayed at times as compared with wt and ICOS$^{-/-}$ mice. Survival data revealed that anti–CTLA-4 therapy cured approximately 80% of tumor-bearing wt mice with long-term survival of over 80 days, but only approximately 40% of ICOS$^{-/-}$ or ICOSL$^{-/-}$ mice were cured (Fig. 5B). These data clearly highlight the importance of the ICOS/ICOSL pathway in mediating optimal antitumor responses in the setting of anti–CTLA-4 therapy.

## Discussion

In this study we provide the first direct evidence that anti–CTLA-4 therapy elicits an increase in the frequency of ICOS$^+$ T cells to play an important role in mediating antitumor immune responses and tumor rejection. Anti–CTLA-4 therapy permits activation of T cells thus leading to an increased

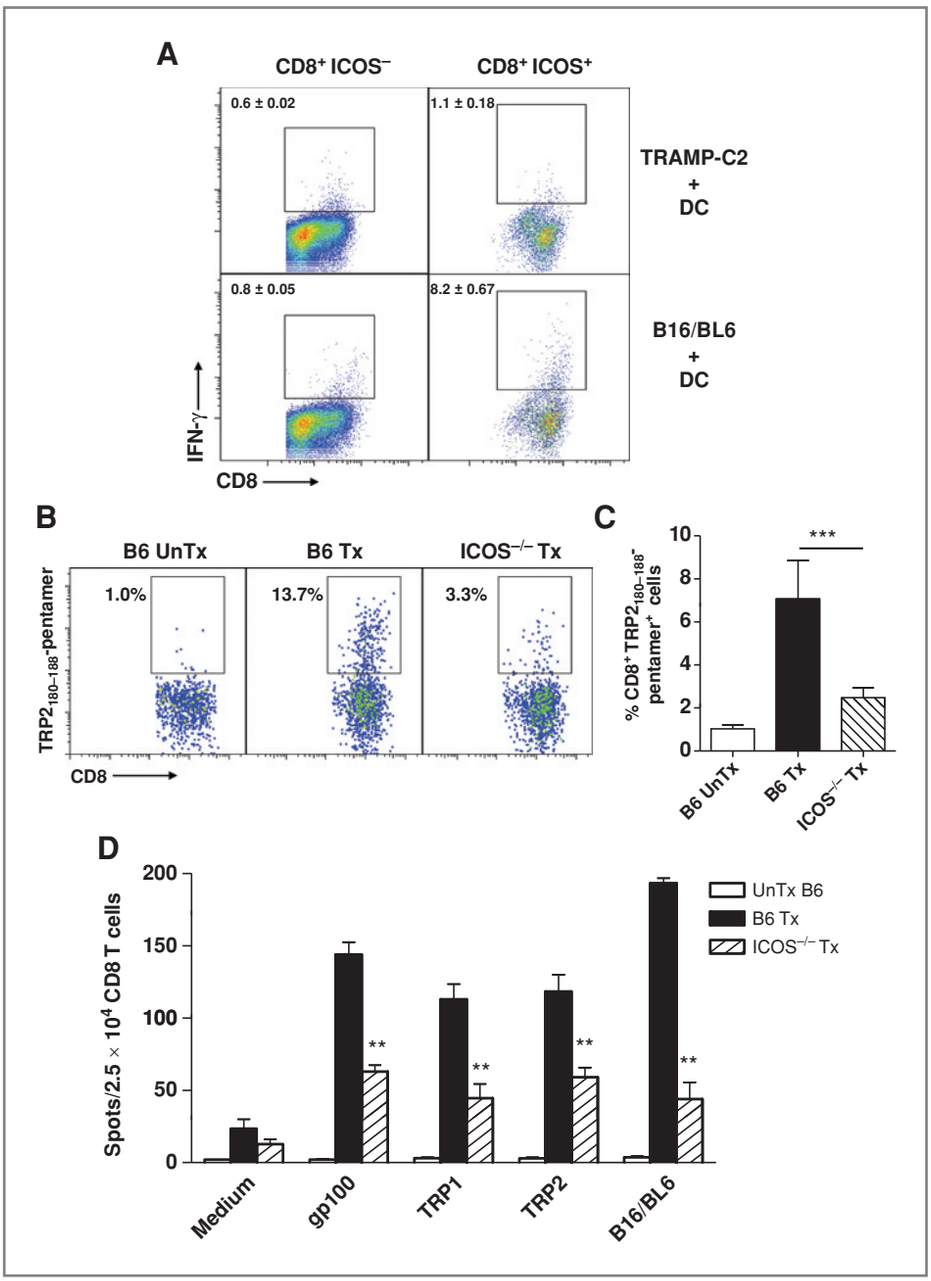


**Figure 4.** $CD8^+ICOS^+$ T cells produce IFN-γ upon antigen recognition, and absence of ICOS results in diminished antigen–specific T-cell responses. CD8 TILs were obtained from B16/BL6 melanoma tissues of treated wt tumor-bearing mice ($n = 5$) and stimulated with B16/BL6 melanoma tumor cells or antigen-irrelevant control TRAMP-C2 prostate tumor cells in the presence of DCs. A, a representative experiment showing IFN-γ production is shown for $ICOS^-$ CD8 and $ICOS^+$ CD8 T cells in the presence of DCs pulsed with control TRAMP-C2 or B16/BL6 cells. The average IFN-γ expression ± SEM from triplicate wells is indicated. The data are statistically significant with $P < 0.001$. The results shown are from 1 of 2 independent experiments with 5 mice/group for each experiment. B, TILs from the indicated mice ($n = 5$) were analyzed for recognition of $TRP2_{180–188}$-pentamer by gating on CD8 population in a representative experiment, and C, summary data expressed as mean ± SEM from 3 independent experiments are presented with 5 mice/group for each experiment. D, CD8 T cells from wt and $ICOS^{-/-}$ mice were cultured with melanoma peptide antigens (gp100, TRP1, and TRP2) or B16/BL6 melanoma tumor cells and analyzed by ELISPOT assays for IFN-γ production. Vertical bars represent means. The results shown are summary data expressed as mean ± SEM from 1 of 2 independent experiments with 5 mice/group for each experiment. **, $P < 0.01$; ***, $P < 0.001$.

frequency of $ICOS^+$ T cells in cancer patients (15–17, 39). Here, we provide data to show a functional role for $ICOS^+$ T cells and the ICOS/ICOSL pathway in mediating antitumor responses, which should be taken into consideration for further studies aimed at improving the efficacy of anti–CTLA-4 therapy in the treatment of cancer patients.

Anti–CTLA-4 antibodies (ipilimumab, BMS and Tremelimumab, Pfizer) are currently in clinical trials. The ipilimumab antibody was recently shown to lead to improved survival in a subset of patients with advanced melanoma (14). Mechanistic investigations as to why a subset of patients derives benefit or how to improve the numbers of patients who derive benefit are currently ongoing. Here, we show that anti–CTLA-4 therapy leads to an increase in the frequency of $CD4^+ICOS^+$ and $CD8^+ICOS^+$ T cells. Both $CD4^+ICOS^+$ and $CD8^+ICOS^+$ T cells from anti–CTLA-4 treated tumor-bearing mice produced Th1 cytokines, IL-2, and IFN-γ. In addition, the presence of tumor antigen-specific T cells is critical for successful immunotherapy and, as shown in Figure 3, ICOS plays an important role in the activation/development of functional antitumor CD8 T cells (Fig. 3). Most importantlyy, in the absence of the ICOS/ICOSL pathway, antitumor responses mediated by CTLA-4 blockade were greatly impaired. The data presented here support $ICOS^+Foxp3^-$ T cells as important effector cells in

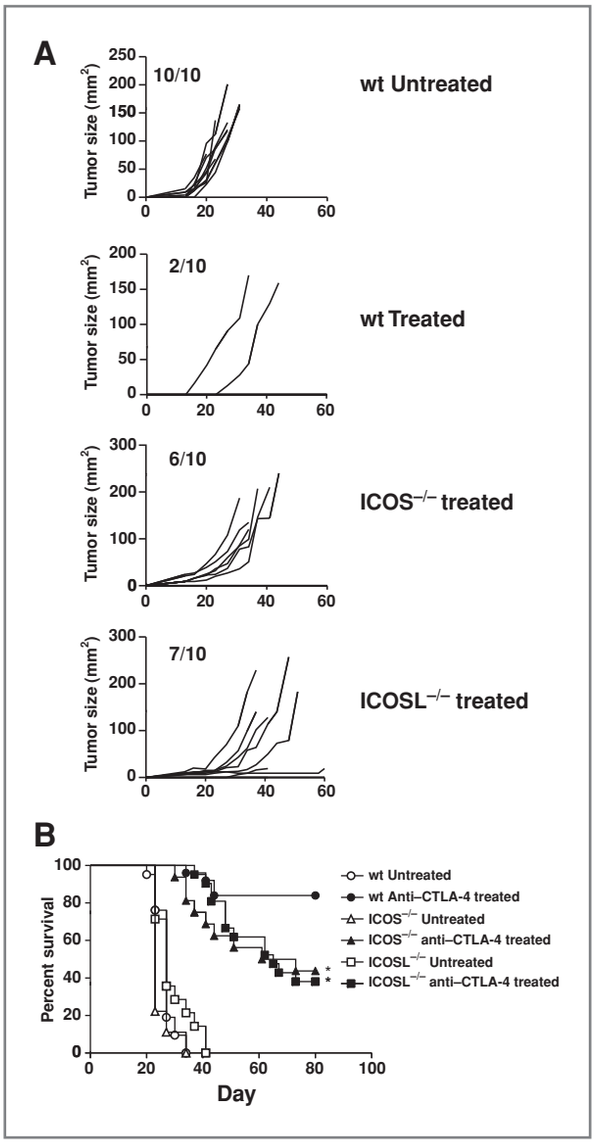


Figure 5. Impaired tumor rejection in ICOS$^{-/-}$ and ICOSL$^{-/-}$ mice treated with anti–CTLA-4 therapy. B16/BL6 tumor cells were injected into B6 wt, ICOS$^{-/-}$, and ICOSL$^{-/-}$ mice at day 0. Anti–CTLA-4 therapy was started 3 days later as described in Materials and Methods. Tumor size was calculated as longest diameter × shortest diameter. Each line represents 1 mouse with the tumor growth kinetics. The tumor incidence in each group is indicated. A, a representative of 3 independent experiments is shown. B, percentage of long-term survival is compared among wt, ICOS$^{-/-}$, and ICOSL$^{-/-}$ mice after anti–CTLA-4 therapy. Untreated wt, ICOS$^{-/-}$, and ICOSL$^{-/-}$ mice served as controls, respectively. Each curve represents 3 independent experiments with 5 mice/group for 1 experiment and 10 mice/group for the subsequent 2 experiments. *, $P = 0.003$ versus wt group treated with anti–CTLA-4 therapy, respectively.

the setting of CTLA-4 blockade and provide a rationale for considering these cells as potential predictive biomarkers of clinical outcome with anti–CTLA-4 therapy and as targets for the development of novel cancer immunotherapy strategies in combination with CTLA-4 blockade.

The mechanisms responsible for the upregulation of ICOS after anti–CTLA-4 therapy are not completely clear. Previous studies in humans (40) and mice (41) have suggested a relationship between IL-2 and ICOS expression, which can be tested in future studies in the setting of anti–CTLA-4 therapy. In addition, studies with *sanroque* mice and *ICOS* mRNA binding have also established an important role for *roquin* in the regulation of ICOS expression (25). Future studies will need to address whether CTLA-4 blockade would affect *roquin*-mediated ICOS repression.

Because ICOS is expressed on all T cells after activation, it is actually not surprising that $T_{reg}$ cells also express ICOS, as previously published (42, 43). Indeed, we detected a population of CD4$^+$ICOS$^+$Foxp3$^+$ $T_{reg}$ cells. ICOS has previously been shown to play an important role in the function of Foxp3$^+$ $T_{reg}$ cells (34), however, although ICOS$^{-/-}$ mice had fewer Foxp3$^+$ T cells (Fig. 1C), they did not show enhanced antitumor responses in the setting of CTLA-4 blockade. Therefore, it is likely that in the absence of ICOS, both $T_{eff}$ and $T_{reg}$ cells are impacted, either equally or selectively, as recently published (34). However, the net effect of the host immune response is dependent on other factors within a given setting, which can lead to seemingly controversial results when studying ICOS$^+$ T cells in different disease and treatment models.

It is possible that targeting the ICOS/ICOSL pathway may lead to the development of novel cancer immunotherapy strategies. Previous studies showed limited success with monotherapy strategies consisting of ICOSL-immunoglobulin fusion protein (44, 45) or ICOSL-expressing tumor cells (46, 47). It is important to note that the ICOS/ICOSL pathway is tightly regulated by feedback loops (48), and careful consideration will need to be given to the approaches that are undertaken to target this pathway for cancer immunotherapy. Careful consideration will also need to be given to additional pathways or possible compensatory mechanisms that play a role in antitumor responses because, as we show here, loss of the ICOS/ICOSL pathway leads to impaired antitumor responses but not complete loss of antitumor responses. Future studies will need to investigate possible combinatorial strategies that can successfully target the ICOS/ICOSL pathway for improved antitumor responses. Because ICOS expression on T cells is only increased after T-cell activation, combination strategies should be considered whereby initial therapy permits T-cell activation, such as in the setting of anti–CTLA-4, and subsequent therapy is then given to target the ICOS/ICOSL pathway.

In summary, our findings establish that anti–CTLA-4 therapy increases the frequency of ICOS$^+$ $T_{eff}$ cells to promote antitumor responses thus highlighting the ICOS/ICOSL pathway as another therapeutic target that may have implications in the development of novel combinatorial cancer immunotherapy strategies.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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