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

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Abstract

The anti–CTLA-4-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.
cells expressing ICOS ligand promote the activation and expansion of T<sub>reg</sub> 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<sup>+</sup> 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<sup>+</sup> T cells in tumor rejection mediated by anti–CTLA-4, we conducted studies in wild-type (wt), ICOS-deficient (ICOS<sup>−/−</sup>), and ICOSL-deficient (ICOSL<sup>−/−</sup>) 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<sup>−/−</sup> and ICOSL<sup>−/−</sup> 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 minimum essential medium supplemented with 10% FBS, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 1% nonessential amino acids, and vitamin (all from Invitrogen). 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<sup>−</sup>–restricted hgp100<sub>25–33</sub> (KYPQRQDWL), H-2K<sup>−</sup>–restricted TRP2<sub>140–148</sub> (SVYDFDVWL), and TRP1<sub>222–239</sub> (TWHRHYLL; 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 × 10<sup>6</sup> 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 × 10<sup>6</sup> 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<sub>2</sub>, 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 lonomycin 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<sup>+</sup> 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 × 10<sup>5</sup> CD8 T cells were cocultured with 5 × 10<sup>4</sup> 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 × 10<sup>6</sup> to 5 × 10<sup>6</sup>) 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 (C98.4A 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 (JES5-16E3), -10 (JESS-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<sub>140–148</sub>H-2K<sup>β</sup> (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 × 10^6 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, hgp10025–33, TRP2180–186 and TRP2222–229. In some experiments, B16/BL6 tumor cells (2.5 × 10^6/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^+Foxp3^+ 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 DNL, 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 DNL (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 DNL, 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 Teff to Treg 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 cells (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 to T_reg cells was calculated based on frequency of total CD4^+ICOS^+ to total CD4^+Foxp3^+ T cells or CD8^+ICOS^+ to CD8^+Foxp3^+ T cells as shown in Figure 2C. The T_eff/T_reg ratio was significantly increased in the spleen, tumor DNL, 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 anti-tumor 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 T 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
We first asked whether ICOS expression on the function of CD8 T cells. Similarly, ICOS expression on the frequency of 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

**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
were made between untreated and treated mice. Because TRP2180 mice are ICOS \textsuperscript{+} T cells that consist of a population of Foxp3\textsuperscript{+} cells in the presence of melanoma-specific peptides (gp100, TRP1, and TRP2) and B16/BL6 tumor cells. We found that CD8 T cells from ICOS \textsuperscript{−} mice produced significantly less IFN-\gamma in response to the tumor antigens and B16/BL6 cells as compared with T cells from wt mice (Fig. 4B). 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\textsubscript{reg} cells (26, 34, 38). Indeed, we observed a lower frequency of CD4\textsuperscript{+}Foxp3\textsuperscript{+} T cells in ICOS \textsuperscript{−/−} mice (Fig. 1C). These data suggest a strong role for ICOS \textsuperscript{+} T cells in immune suppression. However, our previous data in melanoma patients indicate that an increase in the frequency of ICOS \textsuperscript{+} 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 \textsuperscript{−/−}, and ICOSL \textsuperscript{−/−} 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
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
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 importantly, 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...
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 Treg cells also express ICOS, as previously published (42, 43). Indeed, we detected a population of CD4+ ICOS+Foxp3+ Treg cells. ICOS has previously been shown to play an important role in the function of Foxp3+ Treg 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 Teff and Treg 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+ T effector 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 effector and Treg 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.

In summary, our findings establish that anti–CTLA-4 therapy permits T-cell activation, such as in 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
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