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.

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 (Teff) 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 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 (Treg) cells. It has been reported that naïve CD4 T cells can differentiate into IL-10-producing Treg cells as a result of ICOS/(ICOS and its ligand; ICOSL) interactions (26). It was also reported that melanoma...
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 ICOS-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). Mice, cell lines, and reagents were kind gifts from Dr. Jim Allison. Cells were main-

**Flow cytometry**

Single cell suspension (2 × 10^5 to 5 × 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 stained with corresponding mAbs. To obtain T cells from

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out on a BD FACS Cantor 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 TRP1222–229. In some experiments, B16/BL6 tumor cells (2.5 × 10^5/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 of wt tumor-bearing mice 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 Treg 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^+ Treg 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. ID) 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^+ Treg cells, we next assessed the contribution of Foxp3^+ Treg 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 Teff to Treg 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 Teff/Treg 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 Treg cells and suggest that ICOS^- T cells may represent a population of Teff 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 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 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.

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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 (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.
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-\(\gamma\) 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-\(\gamma\) 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
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 antitumor responses mediated by anti-CTLA-4 therapy.
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/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+ Teff 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.
Anti-CTLA-4 Requires ICOS/ICOSL for Optimal Antitumor Responses

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