CD4 T Cells Require ICOS-Mediated PI3K Signaling to Increase T-Bet Expression in the Setting of Anti-CTLA-4 Therapy

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Abstract

The transcription factor T-box expressed in T cells (T-bet) controls the Th1 genetic program in T cells for effective antitumor responses. Anti-CTLA-4 immunotherapy elicits dramatic antitumor responses in mice and in human patients; however, factors that regulate T-bet expression during an antitumor response mediated by anti-CTLA-4 remain to be elucidated. We were the first to report that treatment with anti-CTLA-4 led to an increase in the frequency of T cells expressing inducible costimulator (ICOS). In both treated patients and mice, our data revealed that CD4+ICOS+ T cells can act as effector T cells, which produce the Th1 cytokine IFN-γ. We also showed in a small retrospective analysis that an increased frequency of CD4+ICOS+ T cells correlated with better clinical outcome and the absence of ICOS or its ligand (ICOSL) in mouse models led to impaired tumor rejection. Here, we show that CD4+ICOS+ T cells from anti-CTLA-4–treated patients had an increase in signaling via the phosphoinositide-3-kinase (PI3K) pathway and an increase in expression of T-bet. An ICOS-specific siRNA transfected into human T cells led to diminished PI3K signaling and T-bet expression. Therefore, we hypothesized that ICOS, and specifically ICOS-mediated PI3K signaling, was required for T-bet expression. We conducted studies in ICOS-deficient and ICOS-YF mice, which have a single amino acid change that abrogates PI3K signaling by ICOS. We found that ICOS-mediated PI3K signaling is required for T-bet expression during an antitumor response elicited by anti-CTLA-4 therapy. Our data provide new insight into the regulation of T-bet expression and suggest that ICOS can be targeted to improve Th1 antitumor responses. Cancer Immunol Res; 2(2); 167–76. ©2013 AACR.

Introduction

Anti-CTLA-4 blocks the cytotoxic lymphocyte antigen-4 (CTLA-4) inhibitory pathway on T cells thereby leading to enhanced T-cell responses (1–3). We previously reported that patients treated with anti-CTLA-4 had an increase in the frequency of CD4 T cells expressing the inducible costimulator (ICOS), which correlated with clinical benefit (4–6). ICOS is expressed solely by T cells and the expression of ICOS increases upon T-cell activation (7). ICOS interacts with ICOS-ligand, which is expressed by antigen-presenting cells (7). Although ICOS can be expressed on multiple T-cell subsets, including Th2 cells, regulatory T cells, and follicular helper T cells (8), we have previously shown that ICOS is expressed predominantly on effector T cells that produce the Th1 cytokine IFN-γ after patients and mice received anti-CTLA-4 treatment (4, 9).

The transcription factor T-box expressed in T cells (T-bet) controls the Th1 genetic program in CD4 T cells for effective antitumor responses (10–13). Although T-cell receptor signaling and CD28 costimulation regulate T-bet expression during the early stages of a T-cell response (14–17, 7), other factors that regulate T-bet expression, especially during prolonged antitumor T-cell responses in vivo, remain to be elucidated.

Here, we show that increased ICOS expression correlated with increased phosphoinositide-3-kinase (PI3K) signaling and increased T-bet expression in CD4 T cells from patients treated with anti-CTLA-4. Therefore, we hypothesized that ICOS, which is a member of the CD28 family of proteins that can mediate signaling via the PI3K pathway, was an important factor in controlling T-bet expression. To test our hypothesis, we studied CD4 T cells ex vivo from ICOS−/− tumor-bearing mice treated with anti-CTLA-4. We found that CD4 T cells from ICOS−/− mice, which had intact CD28 expression, had diminished PI3K signaling, and T-bet expression, as compared with CD4 T cells from wild-type (WT) mice. To determine whether
ICOS-mediated PI3K signaling controlled T-bet expression, we conducted studies in ICOS-YF mice, which have a single amino acid change that abrogates PE3K signaling by ICOS (18). These studies revealed that ICOS-mediated PE3K signaling was critical for T-bet expression in the setting of anti-CTLA-4 therapy.

Our data provide new insight into the regulation of T-bet expression, which can be exploited for novel immunotherapy strategies to treat cancer and possibly infectious agents, by targeting ICOS to enhance PE3K signaling and T-bet expression in Th1 effector CD4 T cells.

Materials and Methods

Mice, cell lines, and reagents

C57BL/6 WT and ICOS−/− mice were obtained from the Jackson Laboratory. ICOS-YF mice were generated as previously described (18) and have been backcrossed more than ten generations into C57BL/6 background. B16/BL6 murine melanoma cell line was maintained, used, and grown as previously published (9). No additional authentication was performed on the murine tumor cell lines used. Anti-CTLA4 mAb (clone 9H10) was obtained from Bio X cell. Animals were cared for in accordance with NIH and American Association for the Accreditation of Laboratory Animal Care International regulations. Experiments were performed according to an Institutional Animal Care and Use Committee-approved protocol.

Tumor growth and treatment

B16/BL6 tumor cells were injected into mice and treatment with anti-CTLA plus Gvax was administered as previously described (9). Briefly, the anesthetized mice were injected with 1 × 10^6 B16/BL6 melanoma cells at day 0 and then untreated or treated with anti-CTLA4 monoclonal antibody (mAb) plus Gvax on day 3, 6, and 9. The spleen, tumor-draining lymph nodes (DLN), and T cells from tumor-infiltrating lymphocytes (TIL) were obtained as previously published (9). Total CD4 T cells were isolated using microbeads (Miltenyi Biotech). CD4+ ICOS^hi and CD4+ ICOS^low cells were further sorted by using FACS Aria II sorter. Protein lysates from these cells were obtained for Western blot analyses.

Patients and blood processing

Patients with diagnoses of localized urothelial carcinoma received 2 doses of anti-CTLA-4 antibody ipilimumab at 3 mg/kg or 10 mg/kg, with a 3-week interval between doses on an Institutional review board-approved clinical trial (2006-0080) as previously published (4–6). Ipilimumab is a fully human monoclonal immunoglobulin (IgG1) specific for human CTLA-4 (CD152). Blood was collected before the first dose, 3 weeks after dose #1, and 3 to 4 weeks after dose #2.

Peripheral blood mononuclear cells (PBMC) were isolated from patients' whole blood by density gradient centrifugation using lymphocyte separation Medium (Mediatech, Inc.) and Leucosep tubes (Greiner Bio-one). Cells recovered from the gradient interface were washed twice in RPMI-1640 medium.

Reverse phase protein array assays and analysis

CD4+ T cells were obtained pre- and posttherapy from patients who were treated with anti-CTLA-4 mAb ipilimumab. CD4+ T cells from the patient's PBMCs were obtained by negative immunoselection using CD4+ T cell Isolation Kit II (Miltenyi Biotech Inc.). Cells were lysed in reverse phase protein array (RPPA) lysis buffer [1% Triton X-100, 50 mmol/L Hepes (pH 7.4), 150 mmol/L NaCl, 1.5 mmol/L MgCl2, 1 mmol/L EGTA, 100 mmol/L NaF, 10 mmol/L NaPPI, 10% glycerol, 1 mmol/L phenylmethysulfonyl fluoride, 1 mmol/L Na3VO4, and whole proteinase and phosphatase inhibitor tablets (Boehringer/Roche)], then added to 4 × SDS/2-ME sample buffer (35% glycerol, 8% SDS, 0.25M Tris-HCL pH 6.8; with 10% β-mercaptoethanol added before use) for 20 to 30 minutes with frequent vortexing on ice, centrifuged for 15 minutes at 14,000 rpm, and the supernatant collected. Lysates were transferred into a PCR 96-well plate. The plates were covered and incubated for 5 minutes at 95°C and then centrifuged for 1 minute at 2,000 rpm.

Protein extracts were serially diluted and probed for the expression of validated antibodies by RPPA (19, 20). Specifically, five serial 2-fold dilutions of the protein extracts were performed using RPPA lysis buffer containing 1% SDS. The diluted lysates were spotted on nitrocellulose-coated FAST slides (Whatman, Schleicher & Schuell BioScience, Inc.) by an Aushon 2470 arrayer (Aushon Biosystems) as per manufacturer's protocol. Each spotted slide was incubated with a primary antibody in the appropriate dilution. The specific protein-antibody interaction was recognized by biotin-conjugated secondary antibody and amplified by tyramide deposition. The analyte was detected by avidin-conjugated peroxidase reactive to its substrate diaminobenzidine (DAKO catalyzed signal amplification system, DAKO). The stained RPPA slides were scanned by the Hewlett-Packard (HP) scanner and its accompanying scanning software, and the slide images were quantified for raw signal intensities by the software MicroVigene (VigeneTech Inc.) as per manufacturer's protocol. The raw signal intensities were then processed by the R package SuperCurve (19) developed by the Department of Bioinformatics and Computational Biology at M.D. Anderson Cancer Center (http://bioinformatics.mdanderson.org/Software/OOMPA/). The log2-scaled protein concentrations were normalized by global sample median normalization. The median of all protein marker intensities for a single sample is subtracted from each of the data points (21, 22). To generate heat maps, Treeview and Cluster software were used (23). Student t test was used to evaluate statistical significance of differences in protein expression levels between different samples groups. Box plot analysis of RPPA assay was generated by “mixed-effects model”. Mixed-effects models were fitted to incorporate multiple observations from an individual patient. All P values were two-sided, at a significance level of 0.05.

Flow cytometry analyses

PBMCs from patients were assessed using anti-CD4 PerCP-Cy5.5 (BD Biosciences) and anti-ICOS PE-Cy7 (eBioscience). Flow cytometry was carried out on a BD FACs Canto II. Data were analyzed by FACS Diva software. Gates were set according to appropriate isotype controls. In addition, CD4+ ICOS^hi and CD4+ ICOS^low cells were isolated by FACS Aria II cell sorter (BD Bioscience). For murine studies, antibodies used were CD4 (GK1.5), CD28 (37.51), ICOS (7E.17G9), and T-bet (eBio4B10)
were all from eBioscience. Data were analyzed by FACS Diva and Flowjo software (TreeStar).

**Western blot analysis and antibodies**

Human CD4 T cells were harvested and lysed in RPPA lysis buffer. The concentration of each sample was determined by a standard Bradford assay. Equal amounts of protein (20 μg) from cells were subjected to Western blot analysis. Anti-phospho-Akt (Ser473), anti-Bcl-XL, anti-phospho-S6 ribosomal protein (Ser235/236), and β-actin were probed using appropriate murine or human antibodies available from Cell Signal- ing Technology. Anti-Cyclin B1 (Epitomics), anti-TBX21/T-bet, and anti-GATA3 were obtained for assessment of human samples. Anti-cyclin B1 (Cell Signaling Technology), anti-TBX21/T-bet, and anti-GATA3 were obtained for assessment of murine samples (ProteinTech group). Semi-quantification of protein was performed by densitometric digital analysis of protein bands (TIF image) using Adobe Photoshop CS3 software. Band values (mean intensities) were measured on each area of interest, subtracting the background value from the result and multiplying the remainder with the size of the area of interest. The relative amount of protein (normalized intensity) was calculated using the expression ratio of a target protein versus β-actin as a reference protein.

**RNA interference**

On-TARGET plus SMARTpool targeting human ICOS (siRNA-ICOS) and non-targeting (Negative control, siRNA-NC) unrelated siRNAs were purchased from Dharmacon RNAi Technologies. Approximately 1 million posttherapy (week 7) PBMCs from patients were transfected with either human ICOS siRNAs (50 nmol/L) or control siRNAs (50 nmol/L) or mock transfected. Electroporation transfection method was performed according to manufacturer’s instructions of 4D Nucleofector Device and Amass P3 primary cell 4D-Nucleofector X kit from Lonza. Some PBMCs were also transfected with pmax-GFPVector (1 μg/sample, Lonza) to interrogate the transfection efficiency. Transfection efficiency was monitored by fluorescence microscopy and measured by flow cytometry at various time points. The post-transfected PBMCs were incubated in 12-well culture plates and CD4 T cells were sorted using a FACS Aria II sorter. Sorted CD4 T cells were stained with propidium iodide (BD) and assessed for viability by flow cytometry before performing Western blot analyses and real-time PCR.

**Quantitative RT-PCR**

Total RNA was isolated from CD4 T cells, as well as CD4+ ICOShi and CD4+ ICOSlow T cells using RNAeasy Kit (Qiagen) according to manufacturer’s instructions. cDNAs were generated using the SuperScript III Reverse Transcriptase Kit (Invitrogen). Real-time quantitative PCR was performed by a 7500 Fast Real-Time PCR system (Applied Biosystems) according to the manufacturer’s instructions. Samples were used as templates in reactions to obtain the threshold cycle (Ct) that were normalized with the Ct of CD3-ε from the same sample (ΔCt) in T cells. To compare the relative levels of gene expression in different T-cell types, ΔΔCt values were calculated with the ΔCt values associated with the lowest expression levels as 1. Fold-induction was calculated using 2ΔΔCt. TaqMan Gene Expression Assays of ICOS, CD3-ε, IFN-γ, IL-2, IL-4, IL-10, IL12A, IL17A, IL-21, TNF-α, T-bet, and GATA3 were purchased from Applied Biosystems. To determine the relative levels of gene expression in ICOS knockdown studies, ΔΔCt values were calculated with the ΔCt values associated with the control expression levels (transfected with siRNA-NC) as 1.

**Statistical analysis**

All statistical analyses except for RPPA, which were described above, were conducted by using Prism 5.0 (GraphPad software, Inc). Results were represented as means ± SEM with a 2-sided student t test. Significant values were those with P ≤ 0.05.

**Results**

**ICOS expression correlates with increased PI3K signaling, T-bet expression, and Th1 cytokines**

Ipilimumab, a fully human monoclonal immunoglobulin IgG1 that blocks a T-cell inhibitory pathway mediated by CTLA-4 (1, 2) was U.S. Food and Drug Administration-approved for treatment of patients with melanoma based on data from a phase III clinical trial demonstrating survival benefits in treated patients (3). In some patients, anti-CTLA-4 therapy may take several months before tumor regression can be detected (24), suggesting the need for a prolonged T-cell response that eventually facilitates tumor cell death. We evaluated the anti-CTLA-4 immune responses against bladder cancer in a clinical trial (Supplementary Fig. S1A) in which 12 patients received two doses of anti-CTLA-4, and peripheral blood was collected pretherapy at week 0, posttherapy at week 3, and posttherapy at week 7 (4–6). We obtained matched pre- and posttherapy (week 7) purified CD4 T cells from 7 patients and conducted ex vivo studies to evaluate changes in different signaling pathways. Protein lysates from these cells were analyzed by RPPA, which includes 84 validated antibodies related to PI3K signaling, T-bet expression, and Th1 cytokines.

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Figure 1. ICOS expression correlates with increased PI3K signaling, T-bet expression, and Th1 cytokines. A, RPPA data with unsupervised hierarchical clustering of 27 proteins that were significantly changed (P < 0.05) in posttherapy CD4 T cells as compared with matched pretherapy CD4 T cells. B, box plots to indicate changes in the expression levels of 7 proteins selected to represent PI3K signaling in posttherapy (after) CD4 T cells as compared with pretherapy (before) CD4 T cells. C, greater expression of proteins indicative of PI3K signaling was detected in posttherapy CD4 T cells at weeks 3 and 7 as compared with pretherapy CD4 T cells. D, greater expression of proteins indicative of PI3K signaling was detected in posttherapy CD4 T cells as compared with matched pretherapy CD4 T cells and posttherapy CD4 "ICOS\textsuperscript{hi}" T cells and pretherapy CD4 T cells. E, greater expression of T-bet protein was detected in posttherapy CD4 "ICOS\textsuperscript{hi}" T cells as compared with matched posttherapy CD4 T cells and pretherapy CD4 T cells. F, higher mRNA levels were observed for IFN-γ and TNF-α in posttherapy CD4 "ICOS\textsuperscript{hi}" T cells as compared with matched posttherapy CD4 T cells and pretherapy CD4 T cells.

Since the cytoplasmic tail of ICOS has an YIMPM P3K-binding motif (7, 8), we investigated whether ICOS expression correlated with the increase in PI3K signaling. We studied CD4 T cells from pre- and posttherapy (week 7) samples. CD4 T cells were sorted according to ICOS expression (hi vs. low) by flow cytometry using gating strategies based on isotype controls (Supplementary Fig. S1C) and as described (4–6). As previously published, ICOS expression can be denoted as "positive" versus "negative" expression (9, 25, 26) or "hi" versus "low" expression (4, 8, 27). Here, we refer to the different populations of cells as ICOS\textsuperscript{hi} versus ICOS\textsuperscript{low} (Supplementary Fig. S1C). Both ICOS\textsuperscript{hi} and ICOS\textsuperscript{low} CD4 T cells consisted of activated T cells as measured by the expression of the IL2-receptor alpha subunit (CD25; ref. 28; Supplementary Fig. S1D). We sorted CD4 "ICOS\textsuperscript{hi}" and CD4 "ICOS\textsuperscript{low}" T cells from pre- and posttherapy (week 7) samples from 3 patients and pooled one million cells from each subset to obtain sufficient protein lysates for Western blot studies. We found that posttherapy CD4 "ICOS\textsuperscript{hi}" T cells had significantly greater expression of proteins indicative of PI3K signaling compared with posttherapy CD4 "ICOS\textsuperscript{low}" T cells or pretherapy CD4 T cells, with greater expression of pAKT (S473), cyclin B1, pS6 (S235/236), and Bcl-X\textsubscript{L} detected in CD4 "ICOS\textsuperscript{hi}" T cells and pretherapy CD4 T cells. E, greater expression of T-bet protein was detected in posttherapy CD4 "ICOS\textsuperscript{hi}" T cells as compared with matched posttherapy CD4 T cells and pretherapy CD4 T cells. F, higher mRNA levels were observed for IFN-γ and TNF-α in posttherapy CD4 "ICOS\textsuperscript{hi}" T cells as compared with matched posttherapy CD4 T cells and pretherapy CD4 T cells.

\( P < 0.05 \).
To evaluate whether ICOS expression correlated with an increase in the expression of the transcription factor T-bet, which controls Th1 cytokine production by CD4 T cells, we studied ex vivo CD4 T cells from pre- and posttherapy patients’ samples. Posttherapy (week 7) CD4⁺ICOS⁺ T cells had significantly greater T-bet expression compared with posttherapy CD4⁺ICOS⁻ T cells (Fig. 1E). There was no significant difference in the expression of the transcription factor GATA-3, which controls Th2 cytokine production by CD4 T cells (29). Additional studies revealed that posttherapy CD4⁺ICOS⁺ T cells had higher expression of mRNA for the Th1 cytokines IFN-γ and TNF-α as compared with posttherapy CD4⁺ICOS⁻ T cells (Fig. 1F). There were no differences in the expression of Th2 cytokines. These data indicate that ICOS expression correlated with the increase in PI3K signaling, in T-bet expression and in Th1 cytokine production in CD4 T cells from anti-CTLA-4 treated patients.

**ICOS-specific siRNA diminishes PI3K signaling, T-bet expression, and Th1 cytokines**

To determine whether ICOS was directly driving PI3K-signaling and expression of T-bet and Th1 cytokines, we utilized an ICOS-specific siRNA to decrease ICOS expression in CD4 T cells obtained from anti-CTLA-4 treated patients (N = 3). Transfection of ICOS-specific siRNA decreased ICOS expression (Fig. 2A, representative patient data, right panel and summary data, left) and diminished expression of proteins indicative of PI3K-signaling, with decreased expression of pAKT, pS6, cyclin B1 and Bcl-XL protein (Fig. 2B, representative Western blot). The ICOS-specific siRNA also led to diminished protein expression for T-bet (Fig. 2C). Summary data from three independent experiments are shown in Fig. 2D. In addition, ICOS-specific siRNA significantly decreased expression of mRNA for T-bet and the Th1 cytokines IFN-γ and TNF-α (Fig. 2E). The ICOS-specific siRNA did not have any detectable impact on IL-4, a Th2 cytokine, or IL-17α (Fig. 2E).

![Figure 2. ICOS-specific siRNA diminishes PI3K signaling, T-bet expression and Th1 cytokines.](image-url)
These data indicate that ICOS expression directly impacted PI3K-signaling, T-bet expression and Th1 cytokine production in activated human T cells.

**ICOS expression is required for increased PI3K signaling and T-bet expression during an in vivo antitumor immune response**

To investigate PI3K signaling and T-bet expression during prolonged antitumor T-cell responses, we obtained CD4 T cells 7 days after mice completed treatment with anti-CTLA-4 and performed ex vivo analyses. We found that CD4 T cells from spleen, tumor draining lymph nodes (DLN) and TILs of treated WT mice had greater expression of proteins indicative of PI3K-signaling as compared to CD4 T cells from treated ICOS−/− mice (Fig. 3A, representative experiment). Summary data from three independent experiments are shown in Fig. 3B. Proteins indicative of PI3K signaling were expressed to a much greater extent in CD4 ICOShi cells as compared to CD4 ICOSlow T cells from WT mice or CD4 T cells from ICOS−/− mice (Fig. 3C, representative experiment). Summary data from three independent experiments are shown in Fig. 3D. We also found that T-bet expression was markedly higher in T cells from spleen, DLN and TILs of WT mice as compared to ICOS−/− mice (Fig. 3E, representative experiment). Summary data from three independent experiments are shown in Figure 3F. CD4 ICOShi T cells from WT mice also had greater T-bet expression as compared to CD4 ICOSlow T cells from WT mice or CD4 T cells from ICOS−/− mice (Fig. 3G, representative experiment). Summary data from 3 independent experiments are shown in Fig. 3H. There was no difference in expression of GATA-3. These data indicated that anti-CTLA-4 therapy increases ICOS expression, which mediates an increase in T-bet expression through PI3K signaling.

**Discussion**

We have analyzed samples from patients treated with anti-CTLA-4 (ipilimumab, BMS) to investigate and identify the immunologic biomarkers and/or mechanisms that play important roles in mediating antitumor immune responses. We were the first to detect an increase in CD4 ICOShi T cells in both patients (4, 6) and mice (9) after treatment with anti-CTLA-4. Other investigators also reported a similar increase in the frequency of CD4 ICOShi T cells after patients received treatment with a different anti-CTLA-4 antibody known as tremelimumab (30). We were also the first to report that the increased frequency of ICOS+ CD4 T cells may serve as a biomarker of clinical benefit for patients treated with ipilimumab (6) and similar findings were recently reported by another group (31). We were also the first to report that the increased frequency of ICOS+ CD4 T cells may serve as a pharmacodynamic biomarker for anti-CTLA-4 therapy (32). We also evaluated ICOS expression on T cells in the context of another immunotherapy strategy (gp100 vaccine); however, we did not observe an increase in the frequency of ICOS+ CD4 T cells after that vaccination (32), which suggests that the increased frequency of ICOS+ CD4 T cells may serve as a specific biomarker for anti-CTLA-4 therapy. In addition to serving as a biomarker of therapy, we reported that ICOS and its ligand (ICOSL) played important functional roles in mediating optimal antitumor immune responses, including the production of the Th1 cytokine IFN-γ and tumor rejection in murine models (9).

It is well established that early T-cell immune responses rely on CD28-mediated PI3K signaling (14, 15, 7); however, it has remained unclear whether CD28-mediated PI3K signaling also plays a role in later T-cell responses. We did not detect a change in the frequency of CD28-expressing T cells either after patients received anti-CTLA-4 treatment or after tumor-bearing mice...
ICOS-Mediated PI3K Signaling Regulates T-Bet

received anti-CTLA-4 treatment. Our data demonstrated an important role for ICOS in antitumor T-cell responses, and since the cytoplasmic tail of ICOS has an YMPM PI3K-binding motif (7, 16), we hypothesized that ICOS may enable PI3K signaling to increase T-bet expression for later T-cell responses in the setting of anti-CTLA-4 therapy. Our results demonstrated the expression of T-bet in ICOS-deficient T cells after 48 hours of in vitro activation (Supplementary Fig. S2C) but the expression of T-bet was impaired in ICOS-deficient T cells 7 days after in vivo anti-CTLA-4 therapy (Fig. 3C). These results suggest that T-bet expression is regulated temporally such that the initial T-cell activation via the T-cell receptor and CD28 costimulation can induce PI3K signaling and T-bet expression but, this is a limited event, and sustained expression of T-bet, which may be required for Th1 antitumor responses and tumor rejection, requires ICOS-mediated PI3K signaling. Additional studies will be needed to further test this concept.

Our studies indicated impaired PI3K signaling and T-bet expression in CD4 T cells obtained ex vivo from ICOS−/− mice as compared with those from WT mice. To determine whether there was a link between ICOS-specific PI3K signaling and T-bet expression, we used ICOS-YF knock-in mice, which have a single amino acid change (Y to F) thus preventing PI3K signaling by ICOS. In studies with ICOS-YF mice, we found impaired T-bet expression, which was similar to the data observed in ICOS−/− mice. Collectively, our studies provide evidence for ICOS-mediated PI3K signaling as an important regulator of T-bet expression for Th1 antitumor immune responses that occur after treatment with anti-CTLA-4.

Figure 3. ICOS expression is required for increased PI3K signaling and T-bet expression during an in vivo antitumor immune response. A, greater expression of proteins indicative of PI3K-signaling in CD4 T cells from spleen, draining lymph nodes (DLN) and TILs of treated WT mice as compared to CD4 T cells from treated ICOS−/− mice (representative experiment). B, summary data from three independent experiments with mean and SEM. C, greater expression of proteins indicative of PI3K signaling in CD4+ T cells from treated WT mice as compared to CD4+ T cells from treated ICOS−/− mice (representative experiment). D, summary data from three independent experiments with mean and SEM. E, greater expression of T-bet protein in CD4 T cells from spleen, draining lymph nodes (DLN) and tumor-infiltrating lymphocytes (TIL) of treated WT mice as compared with treated ICOS−/− mice (representative experiment). F, summary data from three independent experiments with mean and SEM. G, greater expression of T-bet protein in CD4+ T cells from treated WT mice as compared with CD4+ T cells from treated ICOS−/− mice (representative experiment). H, summary data from three independent experiments with mean and SEM.
Additional studies will be required to understand the mechanisms that occur during later T-cell responses to facilitate ICOS-mediated PI3K signaling as opposed to CD28-mediated PI3K signaling. Previous studies highlighted the possibility that CD28-PI3K complexes may undergo preferential internalization thus limiting further CD28 signaling, or ICOS may have higher affinity for certain adaptor subunits of PI3K thus outcompeting CD28 for PI3K signaling (7). Moreover, T-bet expression can be driven by IL-12/STAT4 signaling (12) and future studies will be required to determine whether the IL-12/STAT4 signaling pathway is distinct from the ICOS-mediated PI3K signaling pathway. In addition, ICOS has been shown to play a role in Th2 immune responses (7) and it will be necessary to evaluate whether, under specific conditions, ICOS regulates the expression of GATA-3. Furthermore, PI3K signaling has been established as a critical pathway for tumorigenesis and tumor progression (33). Therefore, PI3K inhibitors are being tested in the clinic as therapeutic agents for patients with cancer (34). However, the impact of these PI3K inhibitors on human immune responses has not been well studied and will need to be addressed.

Figure 4. T-bet expression requires ICOS-mediated PI3K signaling during an in vivo antitumor immune response. A, tumor-bearing WT mice that received treatment with anti-CTLA-4 had an increased frequency of ICOS⁺ CD4 T cells, which was not found for CD4 T cells from ICOS⁻/⁻ mice but was observed for CD4 T cells from ICOS-YF mice (B). Treated WT mice had a significant increase in the frequency of T-bet⁺ CD4 T cells, which was not observed in treated ICOS⁻/⁻ and ICOS-YF mice (representative experiment). C, summary data from three independent experiments with mean and SEM. D, sorted CD4⁺ ICOS⁺ and CD4⁺ ICOS⁻/⁻ T cells from WT and ICOS-YF revealed greater protein expression of T-bet in CD4⁺ ICOS⁺ cells from WT mice as compared with CD4⁺ ICOS⁻/⁻ cells from WT mice or CD4⁺ ICOS⁻/⁻ and CD4⁺ ICOS-YF cells from ICOS-YF mice (representative experiment). E, summary data from three independent experiments with mean and SEM. *, P < 0.05; ns, no statistical significance. Tx, treated; UnTx, untreated.
In summary, we report here the regulation of T-bet expression through ICOS-mediated PI3K signaling, which will impact design of future cancer immunotherapy strategies. We propose that CTLA-4 blockade enhances CD28-mediated signaling during the early phases of a T-cell immune response thus facilitating increased expression of ICOS, which regulates PI3K signaling and T-bet expression for the late-phases of a T-cell response. Novel combination treatment with anti-CTLA-4 plus an agonistic agent to target ICOS should be developed and tested as a strategy to improve antitumor responses and tumor rejection. Importantly, our studies suggest that ICOS-mediated PI3K signaling can regulate T-bet expression for the late phases of in vivo Th1 responses that may be necessary for other immune responses such as the eradication of prolonged bacterial and viral infections, which should be tested in future studies and considered for the development of novel therapeutic strategies to target ICOS.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: P. Sharma

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