

Increased Frequency of ICOS⁺ CD4 T Cells as a Pharmacodynamic Biomarker for Anti-CTLA-4 Therapy

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Abstract

Pharmacodynamic biomarkers can play an important role in understanding whether a therapeutic agent has "hit its target" to impact biologic function. A pharmacodynamic biomarker for anti-CTLA-4 therapy remains to be elucidated. We previously reported that anti-CTLA-4 therapy increases the frequency of CD4 T cells expressing the inducible costimulator (ICOS) molecule. To determine whether the frequency of ICOS⁺ CD4 T cells could be used as a pharmacodynamic biomarker for anti-CTLA-4 therapy, we carried out flow cytometric studies and statistical analyses on data from 56 individuals, which included 10 healthy donors, 36 patients who received anti-CTLA-4 monoclonal antibody (mAb), and 10 patients who received treatment with a different immunomodulatory agent (gp100 DNA vaccine). After treatment with anti-CTLA-4 mAb (ipilimumab; Bristol-Myers Squibb), we detected a statistically significant increase in the frequency of ICOS⁺ CD4 T-cells. After two doses of anti-CTLA-4 therapy, the assay was found to have an estimated specificity of 96% [95% confidence interval (CI), 88–100] and sensitivity of 71% (95% CI, 54–85), with positive expression defined as a frequency that is more than the upper bound of 95% CI among baseline samples from all subjects. Our data suggest that an increased frequency of ICOS⁺ CD4 T cells measured by flow cytometry can be used as a reproducible pharmacodynamic biomarker to indicate biologic activity in the setting of anti-CTLA-4 therapy, which should enable appropriate immune monitoring to determine whether patients receiving anti-CTLA-4 monotherapy or combination treatment strategies are having an adequate biologic response. *Cancer Immunol Res*; 1(4); 229–34. ©2013 AACR.

Introduction

Cytotoxic lymphocyte antigen-4 (CTLA-4) is an inhibitory coreceptor expressed on T cells and acts to attenuate T-cell immune responses (1–3). Blockade of CTLA-4 with a monoclonal antibody (mAb) has been shown to enhance T-cell responses and induce tumor rejection in a number of animal models (4, 5). Ipilimumab (Bristol-Myers Squibb), a mAb to human CTLA-4, has been found to elicit objective responses in clinical trials (6–10) and was shown to improve median overall survival of patients with metastatic melanoma in two different phase III clinical trials (11, 12).

In a presurgical clinical trial in which anti-CTLA-4 (ipilimumab) was administered to patients with localized bladder

cancer, we previously reported a significant increase in the frequency of T cells expressing the inducible costimulator (ICOS) molecule in both tumor tissues and blood samples obtained from patients. (13, 14) We subsequently reported that a sustained increase in the frequency of ICOS⁺ CD4 T cells was correlated with clinical benefit in a small cohort of patients with melanoma who received anti-CTLA-4 therapy (15). We also found that loss of the ICOS/ICOS-ligand pathway in a murine model led to impaired antitumor immune responses and tumor rejection (16). Another study, with a different anti-CTLA-4 antibody (tremelimumab; Pfizer), also reported an increase in ICOS⁺ CD4 T cells in blood samples obtained from patients with breast cancer (17). Collectively, these data indicated a biologically relevant role for ICOS in antitumor responses elicited by anti-CTLA-4 therapy.

ICOS is a T-cell-specific molecule that belongs to the CD28/CTLA-4 family (18, 19) and is expressed only after T-cell activation. As anti-CTLA-4 enhances T-cell activation, we hypothesized that ICOS expression on CD4 T cells could be used as a pharmacodynamic biomarker for anti-CTLA-4 therapy. To determine whether ICOS expression on CD4 T cells measured by flow cytometry could be used as a pharmacodynamic biomarker for anti-CTLA-4 therapy (ipilimumab), we analyzed blood samples from 56 individuals, consisting of 10 healthy donors, 36 patients who received ipilimumab (12 patients with bladder cancer and 24 patients with melanoma), and 10 patients with melanoma who received treatment with a different immunomodulatory agent (gp100 DNA vaccine).

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Materials and Methods

Normal healthy donors and cancer patients

Blood samples were collected after normal healthy donors provided informed consent on The University of Texas MD Anderson Cancer Center (Houston, TX) Institutional Review Board (IRB)-approved laboratory protocol 2005-0027. Blood samples from patients with cancer were obtained after they provided consent on IRB-approved protocols such that patients with bladder cancer were consented as per protocols 2006-0080 and 2005-0027, as previously published (13, 15), and patients with melanoma were either consented on the Memorial Sloan-Kettering Cancer Center (New York, NY) IRB-approved biospecimen collection protocols 95-090 or 00-144 before treatment with ipilimumab (NCT00495066), as previously published (15), or protocol 06-113 for treatment with gp100 DNA vaccine (NCT00398073), as previously published (20).

Blood processing

All blood samples were collected from the clinic and transported at room temperature within 3 hours after being drawn from each individual. Whole blood was collected in Vacutainer tubes containing sodium heparin (BD Vacutainer). Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by density gradient centrifugation using Lymphocyte Separation Medium (Mediatech) and Leucosep tubes (Greiner Bio-one). In brief, whole blood was centrifuged at $410 \times g$ for 10 minutes and the plasma layer was collected, centrifuged for 10 minutes at $885 \times g$, and the supernatant frozen at -80°C for subsequent experiments. The whole blood was diluted 1:1 with RPMI (Mediatech) and the PMBCs isolated per manufacturer's guidelines. Cells at the interface were harvested and washed once with RPMI, and red blood cells were lysed with ammonium chloride lysis buffer. Cells were washed, counted, and immediately used fresh for staining and flow cytometry analysis as previously described (13), or 10 million PBMCs were frozen per milliliter of freezing solution (10% DMSO 10% FBS-supplemented RPMI) per NUNC cryovial. PBMCs were frozen in a Nalgene Cryo 1°C freezing container per manufacturer's instructions and stored in liquid nitrogen thereafter.

In vitro activation

PBMCs were resuspended at 1.5×10^6 cells/mL in RPMI (Mediatech) supplemented with MEM nonessential amino acids, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 10% human AB serum (Life Technologies); 30×10^6 PBMCs were added to appropriate T25 tissue culture flask (Corning). PBMCs were activated for 48 hours with immobilized anti-CD3 5 $\mu\text{g}/\text{mL}$ clone UCHT1 and soluble anti-CD28 2 $\mu\text{g}/\text{mL}$ clone CD28.2 (BD Biosciences). PBMCs were collected after detachment from the flask with 4 mmol/L NaEDTA and washed twice with PBS + 2% FBS, followed immediately by labeling for flow cytometry or freezing as above for approximately 24 hours before labeling for flow cytometry.

Flow cytometry

Antibodies used for flow cytometry analysis were CD4 PerCP Cy5.5 (BD Biosciences), ICOS PE-Cy7 (eBioscience), and FoxP3-PE. Lineage was excluded using a cocktail of

FITC-conjugated antibodies, CD303 (Miltenyi Biotec), CD20, CD16, CD14, CD56, TCR $\gamma\delta$, and CD19 (BD Biosciences). Triplicate samples were analyzed using the FACSCanto II (Becton Dickinson). Data were analyzed using the BD FACSDiva software. Gates were set according to appropriate isotype controls. D.N. Tang and J. Sun conducted studies independently.

Statistical analyses

Summary statistics such as mean, median, SD, and 95% confidence interval (CI) were provided for the frequency of $\text{CD4}^+\text{ICOS}^+$ T cells. The histograms and bar plots were used to display ICOS expressions by different subject group. Wilcoxon signed-rank test for paired data was used to assess the difference on the frequency of ICOS^+ CD4 T cells between prior and posttreatment. Wilcoxon rank-sum test was also used to assess the difference on the frequency of ICOS^+ CD4 T cells at baseline between donor controls and patients with different tumor types. All *P* values presented are two-sided. *P* values less than 0.05 were considered to be statistically significant. Statistical analyses were carried out using SPLUS 7 (Insightful Corp).

Results

ICOS expression on CD4 T cells was consistently increased after patients received treatment with anti-CTLA-4

In a subset of 3 to 6 patients with bladder cancer who received two doses of anti-CTLA-4 and for whom PBMCs were available, we evaluated multiple potential pharmacodynamic biomarkers, including expression of ICOS, HLA-DR, and CD25 on CD4 T-cells; frequency of total CD4 and CD8 T-cell populations; and absolute lymphocyte count. As shown in Table 1, an increased frequency of ICOS^+ CD4 T cells was consistently found after patients received treatment with dose 1 and dose 2 of anti-CTLA-4. However, we did not observe a consistent change in the other 5 evaluated biomarkers after patients received treatment with two doses of anti-CTLA-4. These data suggested that ICOS expression on CD4 T cells was a reasonable biomarker to further pursue as a potential pharmacodynamic biomarker for anti-CTLA-4 therapy.

Flow cytometric studies can reproducibly measure frequency of ICOS^+ CD4 T cells

Because we found that anti-CTLA-4 (ipilimumab) led to an increased frequency of ICOS^+ CD4 T cells, which was due to enhanced T-cell activation, we conducted studies to determine whether *in vitro* activation with anti-CD3 and anti-CD28 would also lead to an increased frequency of ICOS^+ CD4 T cells. We conducted *in vitro* activation with anti-CD3 and anti-CD28 on human PBMCs obtained from normal healthy donors to determine whether our flow cytometric assay could reliably detect a change in ICOS expression on CD4 T cells. We found that *in vitro* activation of human PBMCs led to a detectable increase in ICOS^+ CD4 T cells (Supplementary Fig. S1), which was similarly observed for 10 different donors (Supplementary Table S1).

To determine whether our flow cytometric assay was reproducible, we obtained blood samples from 3 healthy donors on 3 different days and evaluated frequency of ICOS^+ CD4 T cells before and after *in vitro* activation for both fresh and frozen

Table 1. Evaluation of six different immunologic parameters in patients treated with anti-CTLA-4

Patients with bladder cancer who received anti-CTLA-4 therapy			
Patient	Pretreatment	Posttreatment (dose 1)	Posttreatment (dose 2)
% ICOS⁺ CD4 T cells			
1	1.4	13.5	8.7
2	1.9	9.7	21.7
3	2.8	9.7	40.1
4	1.9	14.2	6.4
5	4.2	16.6	27.8
6	2.9	13.5	6.6
% HLA-DR⁺ CD4 T cells			
1	3.5	6.5	3.4
2	1.2	3.0	1.2
3	1.9	8.3	5.2
% CD25⁺ CD4 T cells			
1	6.8	10.9	18.8
2	30.7	31.3	38.5
3	43.0	37.4	38.5
% CD4 T cells			
1	52.7	51.3	51.5
2	32.1	40.5	43.5
3	41.0	37.1	23.7
4	46.9	45.0	45.4
5	22.4	41.7	35.3
6	29.6	35.7	34.3
% CD8 T cells			
1	11.5	6.7	10.8
2	12.0	15.3	22.7
3	11.5	10.5	5.8
4	16.1	13.2	26.0
5	6.1	6.8	5.8
6	9.3	8.9	7.5
Absolute lymphocyte count (k/μL)			
1	2.34	3.17	1.86
2	3.51	4.63	5.13
3	1.88	1.91	0.95
4	3.94	2.82	4.49
5	0.76	1.13	1.08
6	0.90	2.83	3.21

samples. Blood was obtained from donors on 3 separate days, and samples were evaluated by two independent operators before and after activation either immediately (fresh) or after being frozen for 24 hours (Fig. 1, representative donor). Blood samples from 2 additional healthy donors were also obtained on 3 separate days and underwent similar analyses (Supplementary Table S2). Each donor sample was tested in triplicate, and the coefficient of variation was found to be less than 20% for the two operators (Supplementary Table S2). In addition, we evaluated the intra-assay variability on triplicate samples studied at each timepoint for 6 patients who received

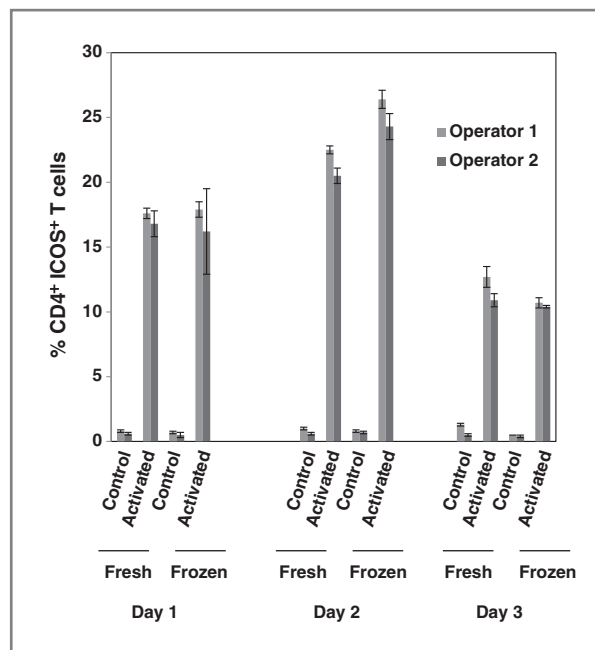


Figure 1. Flow cytometric studies can reproducibly measure frequency of ICOS⁺ CD4 T cells. Peripheral blood from a single individual was drawn on 3 different days, and cells were unactivated (control) or activated *in vitro* before two different operators performed flow cytometry to evaluate frequency of ICOS⁺ CD4 T cells on fresh or frozen samples.

treatment with anti-CTLA-4 and found that the coefficient of variation was less than 10% (Supplementary Table S3). These data indicate that ICOS expression on CD4 T cells could be reproducibly detected by flow cytometric analyses.

An increase in the frequency of ICOS⁺ CD4 T cells is specific for anti-CTLA-4 therapy

To determine whether there were any differences in the frequency of ICOS⁺ CD4 T cells in samples obtained from normal healthy donors as compared with baseline (pretherapy) samples obtained from patients with cancer, we compared data from a cohort of normal healthy donors (*n* = 10) to pretherapy data obtained from patients with localized bladder cancer (*n* = 12) and patients with metastatic melanoma (*n* = 34; 24 patients who would receive treatment with ipilimumab and 10 patients who would receive treatment with gp100 DNA vaccine). As shown in Table 2, there was not a statistically significant difference in the frequency of ICOS⁺ CD4 T cells at baseline among these cohorts. However, after treatment with anti-CTLA-4, the frequency of ICOS⁺ CD4 T cells significantly increased in patients with localized bladder cancer and metastatic melanoma (*P* ≤ 0.004). Patients who were treated with a different immunomodulatory agent, gp100 DNA vaccine, did not show a statistically significant change in the frequency of ICOS⁺ CD4 T cells (*P* = 0.92; Table 3). We used the upper bound of 95% CI obtained from the baseline samples (*n* = 56) to define a positive test for the frequency of ICOS⁺ CD4 T cells as more than 5.63. On the basis of this threshold, flow cytometric analysis for the frequency of ICOS⁺ CD4 T cells as a pharmacodynamic biomarker for ipilimumab therapy provided an

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Table 2. Frequency of ICOS⁺ CD4 T cells from normal healthy donors and baseline blood samples from cancer patients

	All samples	Healthy donors	Patients with cancer (localized bladder cancer, will receive ipilimumab therapy)	Patients with cancer (metastatic melanoma, will receive ipilimumab therapy)	Patients with cancer (metastatic melanoma, will receive gp100 vaccine therapy)
N	56	10	12	24	10
Median	2	2	2.5	1	2
Mean	2.22	1.90	2.36	2.08	2.7
SD	1.39	0.99	1.18	1.67	1.25
P	—	reference	0.323	0.737	0.128

Table 3. Frequency of ICOS⁺ CD4 T cells in pre- and posttherapy samples after treatment with anti-CTLA-4

	Patients with localized bladder cancer (ipilimumab therapy)			Patients with metastatic melanoma (ipilimumab therapy)			Patients with metastatic melanoma (gp100 DNA vaccine)		
	Mean	SD	P	Mean	SD	P	Mean	SD	P
Pretreatment	2.36	1.18	Ref.	2.08	1.67	Ref.	2.70	1.25	Ref.
Posttreatment (dose 1)	13.33	4.14	0.003	6.13	5.27	0.001	2.70	0.95	0.92
Posttreatment (dose 2)	16.27	10.29	0.004	7.46	6.18	0.0001			

average specificity of 96% (95% CI, 88–100) and sensitivity of 71% (95% CI, 54–85) after administration of two doses of anti-CTLA-4 (Table 4).

Discussion

Pharmacodynamic biomarkers are markers of drug effect that usually encompass the pathway associated with the molecular target or represent downstream consequences of target and pathway modulation. In the case of anti-CTLA-4 therapy, the mAb blocks the inhibitory CTLA-4 molecule and prevents interactions between CTLA-4 and its ligand (B7), which subsequently enables B7 to be free for interactions with CD28, a critical costimulatory molecule for T-cell activation (21, 22). Therefore, anti-CTLA-4 therapy leads to enhanced T-cell activation. Upon T-cell activation, ICOS expression is upregulated (18, 23); therefore, ICOS can serve as a marker of T-cell activation. We were the first to report that anti-CTLA-4 therapy (ipilimumab) led to an increase in the frequency of

ICOS⁺ T cells in patients with cancer (13–15). Subsequently, another group found an increase in the frequency of ICOS⁺ T cells after patients with cancer were treated with a different anti-CTLA-4 antibody, tremelimumab (17). In this study, we tested the hypothesis that an increased frequency of ICOS⁺ CD4 T cells detected by flow cytometry can be used as a pharmacodynamic biomarker for anti-CTLA-4 therapy. We showed 96% specificity and 71% sensitivity for flow cytometric measurement of an increased frequency of ICOS⁺ CD4 T cells after treatment with anti-CTLA-4.

Although we present data to show that the frequency of ICOS⁺ CD4 T cells can serve as a pharmacodynamic biomarker for anti-CTLA-4 therapy, it should be noted that ICOS⁺ T cells also play an important role in the antitumor response mediated by anti-CTLA-4 therapy. We have previously shown that mice lacking ICOS or its ligand (ICOSL) have impaired antitumor responses after treatment with anti-CTLA-4, as compared with wild-type mice (16). Therefore, based on the clear relationship between T-cell

Table 4. Sensitivity and specificity of ICOS⁺ CD4 T cells as a pharmacodynamic biomarker of anti-CTLA-4 therapy

	N	More than 5.63 ^a (+)	Sensitivity (95% CI)	Specificity (95% CI)	
Pretreatment	All samples	56	2	—	96.4% (87.7–99.6)
After dose 2 of ipilimumab	Patients with cancer	35	25	71% (53.7–85.4)	—

^aUpper bound of 95% CI from baseline samples.

activation and upregulation of ICOS expression, as well as the biologic role of the ICOS/ICOSL pathway in mediating antitumor immune responses, we propose that ICOS expression on CD4 T cells represents a valid biomarker to be further developed in the setting of anti-CTLA-4 therapy.

Anti-CTLA-4 (ipilimumab) is currently approved by the U.S. Food and Drug Administration for the treatment of patients with metastatic melanoma. In addition, because anti-CTLA-4 targets a T-cell-specific molecule, as opposed to a tumor-specific molecule, it is being tested in multiple clinical trials in patients with different malignancies, which will require appropriate development of biomarkers for anti-CTLA-4 therapy. Also, because preclinical data suggest that combination therapies with agents that prime a T-cell response plus anti-CTLA-4 are more effective at eliciting antitumor responses as compared with monotherapy (24–26), clinical trials are under way with anti-CTLA-4 in various combination strategies, which will require appropriate biomarker development to evaluate whether the combination maintains biologic efficacy of anti-CTLA-4. Given the number of clinical trials that are ongoing or are expected to be conducted with anti-CTLA-4 antibody, appropriate biomarkers are clearly needed to enable rational decisions regarding dose, schedule, and combinations. Here, we define an increased frequency of ICOS⁺ CD4 T cells measured by flow cytometry as a pharmacodynamic biomarker that can be measured in blood samples, which can potentially be used for making decisions regarding different doses, schedules, and/or combinations tested in clinical trials with anti-CTLA-4. Our work represents a small retrospective analysis and

warrants further prospective studies in a larger cohort of patients.

Disclosure of Potential Conflicts of Interest

J.D. Wolchok has a commercial research grant and is a consultant/advisory board member for Bristol-Myers Squibb. J.P. Allison has ownership interest (including patents) in Bristol-Myers Squibb and is a consultant/advisory board member for Kite Pharmaceuticals and Jounce Therapeutics. P. Sharma has ownership interest (including patents) in Jounce and is a consultant/advisory board member for Bristol-Myers Squibb and MedImmune. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: P. Sharma
Development of methodology: D.N. Tang, J. Yuan, P. Sharma
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.N. Tang, J.D. Wolchok, J. Yuan, P. Sharma
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.N. Tang, Y. Shen, J. Sun, S. Wen, J. Yuan, J.P. Allison, P. Sharma
Writing, review, and/or revision of the manuscript: D.N. Tang, Y. Shen, J. Sun, S. Wen, J.D. Wolchok, J. Yuan, J.P. Allison, P. Sharma
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.N. Tang, Y. Shen
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