# Intratumoral Immune Cell Infiltrates, FoxP3, and Indoleamine 2,3-Dioxygenase in Patients with Melanoma Undergoing CTLA4 Blockade

Antoni Ribas,[1,2,3] Begoña Comin-Anduix,[2] James S. Economou,[2,3,4] Timothy R. Donahue,[2] Pilar de la Rocha,[2] Lilah F. Morris,[2] Jason Jalil,[2] Vivian B. Dissette,[2] Itsushi Peter Shintaku,[5] John A. Glaspy,[1,3] Jesus Gomez-Navarro,[6] and Alistair J. Cochran[2,3,5]

**Abstract**

**Purpose:** CTL-associated antigen 4 (CTLA4)-blocking monoclonal antibodies induce long-term regression of metastatic melanoma in some patients, but the exact mechanism is unknown. In this study, biopsies of selected accessible tumor lesions from patients treated with tremelimumab were examined to further elucidate the mechanism of its antitumor activity.

**Experimental Design:** Fifteen tumor biopsies from 7 patients who had been treated with tremelimumab (CP-675,206) were collected. Samples were analyzed for melanoma markers, immune cell subset markers, the presence of the T regulatory-specific transcription factor FoxP3 and the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO).

**Results:** Clinically responding lesions had diffuse intratumoral infiltrates of $CD8^+$ T cells that were markedly increased in cases where comparison with a baseline biopsy was available. Nonregressing lesions had sparse, patchy $CD8^+$ intratumoral infiltrates. Patients with regressing lesions had an increased frequency of $CD8^+$ cells with or without a concomitant increase in $CD4^+$ cells. Two of 3 responding patients with paired samples showed a slight increase in the number of $FoxP3^+$ cells in the postdosing biopsies. In patients with regressing lesions who had paired samples, the intensity of IDO staining in macrophages and/or melanoma cells showed no clear pattern of change postdosing.

**Conclusions:** Administration of tremelimumab was associated with massive intratumoral infiltrates of $CD8^+$ CTLs in patients with regressing tumors but had varying effects on intratumoral infiltrates of $CD4^+$ and $FoxP3^+$ cells or intratumoral expression of IDO.

CTL-associated antigen 4 (CTLA4)-blocking monoclonal antibodies can induce regression of tumors in mice (1–8) and humans (9–12). Two fully human CTLA4-blocking monoclonal antibodies are currently in clinical development, ipilimumab (formerly known as MDX-010 and BMS 184024) and tremelimumab (CP-675,206; formerly ticilimumab). Clinical data indicate that a subset of patients with metastatic melanoma respond to these CTLA4-blocking antibodies (13). CTLA4 is expressed on the surface of recently activated T cells, both $CD4^+$ T helper and $CD8^+$ CTLs (14). CTLA4 engagement by its ligands (the costimulatory molecules CD80 and CD86) decreases interleukin-2 transcription and T-cell activation (15). In addition, surface expression of CTLA4 on T cells results in restless cells that engage in shorter interactions with cells expressing their cognate antigen (16). The presumptive effect is suboptimal triggering of T-cell receptor signaling. Blockade of negative signaling with CTLA4-blocking antibodies may result in increased activation and proliferation of activated T cells (1) and/or allow longer interactions between activated T cells and cancer cells, thereby lowering the threshold of T-cell receptor signaling and enhancing the cytotoxic effects of activated CTLs on cancer cells (16). Based on these mechanisms, it would be anticipated that immune effector cells, particularly $CD8^+$ CTLs, would accumulate at increased frequency in the tumors of responding patients.

CTLA4 is also constitutively expressed by $CD4^+/CD25^+$ T regulatory cells (Treg). Tregs are dominant immunosuppressor cells with a critical role in controlling autoimmune reactions in peripheral tissues (17). Preclinical models have shown that CTLA4 on Tregs can provide back signaling to $CD80^+$ or $CD86^+$ cells, including activated T cells (18) and indoleamine 2,3-dioxygenase (IDO)-competent plasmacytoid

**Authors' Affiliations:** [1]Division of Hematology/Oncology, Department of Medicine, [2]Division of Surgical Oncology, Department of Surgery, [3]Jonsson Comprehensive Cancer Center, and [4]Department of Microbiology, Immunology and Molecular Genetics, and [5]Department of Pathology and Laboratory Medicine, University of California-Los Angeles, Los Angeles, California; and [6]Pfizer Global Research and Development, New London, Connecticut

Received 3/26/08; revised 8/28/08; accepted 9/1/08.

**Grant support:** U54 CA119347, Melanoma Research Foundation, Harry J. Lloyd Charitable Trust, and Jonsson Comprehensive Cancer Center at University of California-Los Angeles (A. Ribas).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

**Requests for reprints:** Antoni Ribas, Division of Hematology/Oncology, Department of Medicine, University of California-Los Angeles Medical Center, 11-934 Factor Building, 10833 Le Conte Avenue, Los Angeles, CA 90095-1782. Phone: 310-206-3928; Fax: 310-206-0914; E-mail: aribas@mednet.ucla.edu.



doi:10.1158/1078-0432.CCR-08-0783

### Translational Relevance

Administration of anti-CTLA4-blocking antibodies to patients with metastatic melanoma results in durable response rates in a small subset of patients. Immune monitoring analysis of peripheral blood has failed to differentiate clinical responders from nonresponders. This study suggests that biopsies of tumor lesions may be more appropriate for studying the mechanism of action and resistance to anti-CTLA4 in patients with metastatic melanoma.

dendritic cells (19–21). IDO is a rate-limiting enzyme in tryptophan catabolism, and IDO-expressing plasmacytoid dendritic cells acquire potent and dominant T-cell-suppressive properties, resulting in the inhibition of the cytotoxic effects of antigen-specific T cells (22). Blockade of the CTLA4 reverse signaling using anti-CTLA4 antibodies would be expected to deplete or inhibit the function of Tregs and decrease the expression of the immunosuppressive IDO enzyme as suggested in preclinical models (23).

Tremelimumab is a CTLA4-blocking monoclonal antibody of the human $IgG_2$ subtype. This very high affinity antibody blocks binding of CTLA4 to its natural receptor CD80 or CD86 at subnanomolar concentrations. *In vitro* studies showed that blocking the activity of CTLA4 results in markedly increased interleukin-2 production by peripheral blood mononuclear cells and whole blood that are simultaneously exposed to strong T-cell receptor signaling (24). When administered to humans, the pharmacokinetics of tremelimumab is exactly like that of endogenous immunoglobulins, with a half-life exceeding 3 weeks (12). At doses of 10 or 15 mg/kg, sustained plasma levels are achieved in the 10 to 30 μg/mL range for up to 3 months (12), which is the minimal antibody concentration defined in preclinical models to be associated with a biological effect on CTLA4. Administration of tremelimumab at doses of 3 to 15 mg/kg has resulted in long-lived objective responses in a subset of patients with metastatic melanoma (12).

The most common site to analyze immune responses after cancer immunotherapy has been the peripheral blood. Sensitive immune monitoring assays allow quantitative assessment of the phenotype of immune effector cells in peripheral blood, their ability to bind to defined MHC-peptide determinants, and their functional responses on exposure to cognate antigen (25–27). However, the analysis of peripheral blood samples from patients receiving CTLA4-blocking monoclonal antibodies has yielded nonimpressive results, with no clear evidence of increased frequency of melanoma antigen-specific T cells evaluated by MHC tetramer assays (11, 28–31). There have been conflicting reports on the effects of CTLA4-blocking antibodies on Tregs in peripheral blood. Two groups have suggested a decrease in Tregs (30, 32), whereas other groups, including ours, have provided evidence that no change in the frequency or function of circulating Tregs occurs after CTLA4 blockade (29, 31). The only consistent finding has been a slight increase in expression of T activation human leukocyte antigen (HLA)-DR and memory (CD45RO) markers on peripheral blood T cells (11, 29, 30, 32).

In an effort to elucidate the mechanism of action of CTLA4 blockade in humans, we report here findings from the evaluation of intratumoral changes in patients with locally advanced and metastatic melanoma who were treated at full therapeutic doses of tremelimumab. We hypothesized that the tumor may be an ideal site to test if CTLA4 blockade leads to effective antitumor infiltration by immune effector cells and/or depletion of intratumor immunosuppressor cells such as Tregs or IDO-competent plasmacytoid dendritic cells. To date, analysis of tumor biopsies has only been undertaken in a few select patients treated with the CTLA4-blocking antibodies, and these studies have revealed marked intratumoral changes and the presence of inflammatory infiltrates (9, 12, 30, 33). We studied accessible tumor lesions from patients with melanoma who were treated with tremelimumab on two study protocols being conducted at the University of California-Los Angeles (UCLA).

## Materials and Methods

***Study design and assessments.*** Fifteen tumor samples were collected from 7 patients receiving the anti-CTLA4 monoclonal antibody tremelimumab administered intravenously either once every month at 10 mg/kg (5 patients) or once every 3 months at 15 mg/kg (2 patients) under institutional review board-approved protocols (03-01-059 and 05-11-036). Biopsies were obtained after signing the tissue banking UCLA institutional review board protocol 02-08-067. This study was conducted in accordance with the Declaration of Helsinki and its amendments and relevant International Conference on Harmonization Good Clinical Practice guidelines. Tumor samples were obtained for diagnostic or therapeutic purposes in eight instances and solely for research purposes in four cases. Because of the proposed mechanism of action of this antibody (which may require tumor inflammation to induce an objective response; ref. 12), some patients were considered to have a tumor response whether they fulfilled the standard criteria for response following Response Evaluation Criteria in Solid Tumors (RECIST; ref. 34). Responses that did not meet RECIST are noted.

***Immunohistochemistry staining and evaluation.*** Paraffin sections were cut at 2 μm and baked for 4 h at 60°C. Slides were deparaffinized in xylene, and endogenous peroxidase activity was quenched with 0.5% hydrogen peroxide in methyl alcohol for 10 min. Heat-induced epitope retrieval was done on the slides using 0.001 mol/L EDTA (pH 8.0) for 3 min or 0.01 mol/L citrate buffer (pH 6.0) for 25 min. After heating, cooling, and washing in 0.01 mol/L PBS, slides were placed on a DAKO Autostainer (DAKOCytomation). For CD4, CD8, S100, MART-1, and HMB45, slides were incubated sequentially in primary antibody for 30 min, in rabbit anti-mouse immunoglobulins for 15 min, then followed by Envision$^+$ (rabbit, peroxidase; DAKOCytomation) for 30 min. For FoxP3 and IDO, slides were incubated in the primary anti-FoxP3 antibody ab20034-250 (Abcam) or anti-IDO antibody MAB 5412 (Chemicon-Millipore) for 45 min and then in MACH2 (anti-mouse, peroxidase polymer) for 30 min. Diaminobenzidine and hydrogen peroxide were used as the substrates for the peroxidase enzyme. Slides were counterstained with hematoxylin. All stains included a negative control using mouse isotype IgG1 (DAKOCytomation), and a positive control was obtained from prior samples with known positive staining (tonsils for FoxP3 and placenta for IDO). Samples were analyzed by one pathologist (A.J.C.) and scored based on frequency (0-3+) of reactive cells and their distribution (diffuse or patchy) throughout the biopsy specimen.

***Tumor sample processing to obtain tumor-associated lymphocytes.*** To generate a single-cell suspension for analysis of tumor-infiltrating lymphocytes (TIL), tumor samples were decapsulated, minced with sterile surgical blades, and enzymatically digested for 1 to 2 h with DNase I (0.1 mg/mL; Sigma) and collagenase D (1 mg/mL; Boehringer

Mannheim) in 100 mL serum-free AIM-V medium (Life Technologies). Cells were plated in tissue culture flasks in RPMI and allowed to adhere for at least 2 h and up to 18 h. Nonadherent cells, enriched for TILs, were collected and cryopreserved in the vapor phase of a liquid nitrogen tank in sterile cryogenic vials after resuspension in RPMI (Life Technologies) supplemented with 20% (v/v) heat-inactivated human AB or FCS (Omega Scientific) and 10% DMSO (Sigma-Aldrich).

***Multicolor surface flow cytometry.*** Cryopreserved aliquots of nonadherent intratumoral cell aliquots were thawed by brief (1-3 min) submersion in a 37°C water bath and immediately diluted with RPMI supplemented with 10% human AB serum and 1% penicillin, streptomycin, and amphotericin (Omega Scientific). Cells were washed and subjected to enzymatic treatment with DNase (0.002%; Sigma) for 1 h at 37°C to avoid cell clumping caused by released DNA. Cells were washed again and stained using a panel of fluorescein-labeled antibodies against the following T-cell surface antigens: Alexa Fluor 405/Pacific Blue-conjugated S4.1 (anti-CD3) and FITC-conjugated UCHL1 (anti-CD45RO; Invitrogen); antigen-presenting cells-Cy7-conjugated SK3 (anti-CD4), PE-conjugated M-T271 (anti-CD27), and antigen-presenting cells-Alexa Fluor 647-3D12 (anti-CCR7; BD Biosciences); and ECD-conjugated Immu-357 anti-HLA-DR (Beckman Coulter). A dump channel was generated with cells expressing PC5-conjugated anti-CD56 and anti-CD19. Cells were fixed with 0.5% paraformaldehyde. Immediately before flow cytometric analysis, 5 μL 7-amino-actinomycin D was added to gate out dead cells. The seven-color flow cytometry staining was acquired by a FACSAria (BD Biosciences) using Fluorescence Minus One approach (35). Analysis was done with FCS Express (De Novo Software) software.

***FoxP3 intracellular staining.*** Thawed nonadherent intratumoral cells were first labeled with the following surface antibodies: Pacific Blue-conjugated S4.1 (anti-CD3), Alexa Fluor 467-conjugated RPA-T4 (anti-CD4), and antigen-presenting cells-Cy7-conjugated M-A251 (anti-CD25). Intracellular staining for FoxP3 protein was done following the manufacturer's instructions using the PE-conjugated PCH101 anti-FoxP3 antibody (eBioscience). Flow cytometric analysis was done as described above.

***MHC tetramer assay.*** Nonadherent intratumoral cell aliquots were thawed as described above and stained with commercially available MHC tetramers (Beckman Coulter) loaded with three HLA-A*0201 immunodominant peptides derived from tumor rejection antigens: MART-$1_{26-35}$ (ELAGIGILTV), tyrosinase$_{368-376}$ (YMDGTMSQV), and gp$100_{209-217}$ (ITDQVPFSV). The assay was done following the manufacturer's instructions with minor modifications as described previously (36).

## Results

***Patients and clinical response.*** Table 1 provides detailed information on patient characteristics and tremelimumab administration for the 7 patients with biopsies available for study. These patients were selected from 89 patients who received tremelimumab at UCLA between February 2002 and December 2006 because they had tumor biopsies available for analysis and provided written informed consent for analysis of biopsy material. The samples are skewed to patients with an objective tumor response, because these patients were more likely to agree to undergo a research tumor biopsy. Three of these patients had a partial response (PR) per RECIST and remained alive and melanoma free for 31 to 47 months after the initial dose of tremelimumab. One patient had stable

**Table 1.** Patient characteristics and intratumoral changes in $CD8^+$ and $CD4^+$ TILs

| Case no. | Age (y) | Sex | Regimen | Response | PFS/OS | Toxicity | Timing of biopsy | No. doses before biopsy | Months from last dose | CD8 | Change | CD4 | Change |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 39 | M | 15 mg/kg every 3 mo | PR | 47+/47+ | | Pre | 0 | - | 0 | | NA | |
| | | | | | | | Post | 1 | 3 mo | +++ diffuse | ↑ | ++ diffuse | NA |
| 2 | 78 | M | 10 mg/kg every 1 mo | PR | 9/31+ | Grade 2 arthritis | Pre | 0 | - | 0 | | 0 | |
| | | | | | | | Post | 3 | 1 mo | ++ diffuse | ↑ | + diffuse | ↑ |
| | | | | | | | Post* | 3 | 7 mo | +/- patchy | ↓ | +/- patchy | ↓ |
| 3 | 64 | M | 10 mg/kg every 1 mo | pPR | 36/39+ | Grade 2 asthenia | Pre | 0 | - | ++ patchy | | + patchy | |
| | | | | | | | Post | 9 | 1 mo | ++ diffuse | ↑ | +++ diffuse | ↑ |
| 4 | 90 | M | 10 mg/kg every 1 mo | PR | 8/40+ | | Post† | 8 | 1 mo | + patchy | | +++ diffuse | |
| | | | | | | | Post‡ | 8 | 1 mo | + patchy | | +++ diffuse | |
| | | | | | | | Post§ | 8 | 1 mo | +++ diffuse | ↑ | ++ diffuse | ↓ |
| 5 | 68 | F | 15 mg/kg every 3 mo | PD | 3/18 | | Pre | 0 | - | +/- patchy | | +/- patchy | |
| | | | | | | | Post | 1 | 3 mo | +/- patchy | = | +/- patchy | = |
| 6 | 57 | F | 10 mg/kg every 1 mo | PD | 1/7 | | Post | 4 | 1 mo | +/- patchy | | ++ diffuse | |
| 7 | 62 | M | 10 mg/kg every 1 mo | SD | 8/22 | Grade 3 diarrhea | Post* | 2 | 6 mo | + diffuse | | +++ diffuse | |

Abbreviations: PFS, progression-free survival; OS, overall survival; pPR, pathologic PR; NA, not available; PD, progressive disease.
*Delayed.
†Progressing.
‡Stable.
§Regressing.

disease by RECIST but a pathologic PR at biopsy with disease progression 36 months after dosing, 1 patient had stable disease (SD) as best response on therapy followed by disease progression at 8 months, and 2 patients had progressive disease (PD) at the first restaging scans. These patients underwent 15 biopsies, 13 within 1 month of the last dose of tremelimumab at 10 mg/kg or within 3 months at 15 mg/kg. During this period, the trough plasma levels would be expected to be in the 10 to 30 μg/mL range, well within therapeutic levels (12).

***Characterization of intratumoral cell infiltrates.*** Cases 1 and 2 had objective responses to therapy per RECIST and had paired predosing and postdosing biopsies available for analysis. In both cases, the predosing biopsy had no detectable intratumoral $CD8^+$ or $CD4^+$ cell infiltrate, whereas the postdosing biopsy had a marked $CD8^+$ infiltrate percolated between tumor cells and a less abundant $CD4^+$ infiltrate (Table 1; Fig. 1A and B). Immunohistochemistry staining showed that the $CD8^+$ infiltrating cells had intracellular vacuoles loaded with the toxic granule, granzyme B, suggesting that these were true CTLs (Fig. 1C). The predominant $CD8^+$ infiltrate in case 2 was confirmed by multicolor flow cytometry of nonadherent TILs (Fig. 1D). This analysis revealed that >40% of the nonadherent $CD3^+$ TILs were $CD3^+/CD4^-$ (by exclusion mostly $CD8^+$), whereas only 6.5% were $CD3^+/CD4^+$. Extended surface analysis of the tumor-infiltrating $CD3^+/CD4^-$ T cells in the regressing lesion showed that the cells had a HLA-$DR^+$, $CD45RO^{+++}$, $CD27^{++}$, and $CCR7^-$ phenotype consistent with early memory T cells (37). This patient later developed inflammatory arthritis, which was treated with methotrexate. All but one in-transit metastasis had completely regressed before starting methotrexate. This lesion, which remained stable in size after major regression and persisted as a [$^{18}$F]2-deoxyglucose positron emission tomography-positive lesion (data not shown), was eventually resected 7 months after the last dose of tremelimumab. This lesion (labeled as postdosing delayed biopsy in Table 1) was obtained, whereas the patient was being treated with methotrexate and showed persistent melanoma cells with a patchy $CD8^+$ and $CD4^+$ infiltrate mostly in peritumoral areas in marked contrast with the massive diffuse intratumoral $CD8^+$ infiltrate observed in the immediate postdosing biopsy presumably at the peak of the antitumor immune response (Table 1).

Case 3 entered the study with a 4 cm metastatic lesion in the left psoas muscle and several smaller pelvic lymph node metastases persisting after five previous surgical resections. The predosing biopsy revealed patchy peritumoral $CD8^+$ and $CD4^+$ infiltrates in the tumor margins. Tumor response was assessed by predosing and postdosing positron emission tomography and computed tomography scans. The psoas muscle lesion lost [$^{18}$F]2-deoxyglucose uptake by positron emission tomography ~4 months after dosing, but it did not change in size by computed tomography scan and therefore does not meet the criteria for PR by RECIST. This lesion was resected after this patient had received nine monthly doses of tremelimumab at 10 mg/kg. Pathologic analysis showed that the resected psoas muscle melanoma was 90% regressed and therefore was labeled as a pathologic PR. The remaining 10% of the lesion showed melanoma infiltrated by $CD8^+$ and $CD4^+$ mononuclear cells, with a slight predominance of $CD4^+$ cells (Table 1).

Case 4 had a major PR following tremelimumab treatment (Fig. 2A). An initial biopsy of a regressing in-transit lesion 1 month after receiving the first dose revealed a massive diffuse intratumoral infiltrate of both $CD8^+$ and $CD4^+$ cells (data not shown). Two months later, the patient underwent a skin biopsy, as the peritumoral area was tender and inflamed. This revealed an organized lymphocytic infiltrate in the dermis, with central $CD1a^+$ and $CD20^+$ cells surrounded by a mantle of $CD4^+$ and $CD8^+$ cells (Supplementary Fig. S1). Seven months after the first dose of tremelimumab, while on continuous monthly treatments, a protuberance appeared in a lesion that was regressing more slowly than the majority of the patient's other lesions (Fig. 2B). A decision was made to resect the residual in-transit metastasis, which contained concomitant regressing, stable, and progressive lesions. Pathologic analysis showed that the macroscopically pigmented regressing lesion had nearly absent melanoma cells with a massive $CD8^+$ and $CD4^+$ infiltrate, whereas the stable and progressing lesions had tumor cells positive for the MART-1 and HMB45 melanoma antigens, with a dense $CD4^+$ infiltrate and nearly absent $CD8^+$ infiltrate (Fig. 2C).

Case 5 entered the study with in-transit metastasis localized in the right breast that recurred after surgical resection and treatment with temozolomide and sorafenib. These lesions progressed while on therapy with tremelimumab at 15 mg/kg. Both predosing and postdosing biopsies revealed nearly absent $CD8^+$ and $CD4^+$ infiltrates (Table 1; Fig. 3). Cases 6 and 7 had PD and SD, respectively, and only postdosing biopsies were available. Both showed evidence of melanoma by immunohistochemistry. In case 6, the biopsy had minimal to absent $CD8^+$ and $CD4^+$ infiltrates, whereas the biopsy from case 7 showed evidence of diffuse intratumoral $CD8^+$ and $CD4^+$ infiltrates with a predominance of $CD4^+$ cells (Table 1; Supplementary Fig. S2). We found no significant intratumoral infiltration by CD1a dendritic cells, $CD20^+$ B cells, or $CD57^+$ natural killer cells in any of the staining samples analyzed. Staining for $CD68^+$ macrophages was difficult to evaluate, as melanoma cells from some patients were positive for this marker (data not shown).

Given the presence of $CD8^+$ T cells in both regressing and nonregressing lesions, we explored their antigen specificity using the MHC tetramer assay. Nonadherent cells obtained from biopsies in cases 4 and 7, both HLA-$A^*0201^+$, were subjected to staining with commercially available tetramers folded with the MART-$1_{26\text{-}35}$, tyrosinase$_{368\text{-}376}$, and gp$100_{209\text{-}217}$ epitopes as well as a negative control tetramer (36). In a prior methodologic study of the tetramer assay, we defined the low limit of detection of the assay at 0.03% of $CD8^+$ T cells (36). Both the regressing and the progressing lesions in case 4 had accumulation of a population of $CD8^+$ T cells specific for gp$100_{209\text{-}217}$ above the low limit of detection and at approximately the same frequency (Supplementary Fig. S2); the stable lesion had a higher percentage of gp$100_{209\text{-}217}$-specific $CD8^+$ T cells, whereas none of these lesions had detectable $CD8^+$ T cells with MART-$1_{26\text{-}35}$ or tyrosinase$_{368\text{-}376}$ specificity. There were no cells in either lesion that were positive for the negative control tetramer (data not shown). In all three lesions, the intratumoral melanoma antigen-specific $CD8^+$ T-cell population was enriched four to seven times compared with their frequency in peripheral blood. The lesion in case 7 also had a detectable population of melanoma antigen-specific T cells above the low limit of detection (Supplementary Fig. S2), in this case specific for MART-$1_{26\text{-}35}$, but not for tyrosinase$_{368\text{-}376}$ or gp$100_{209\text{-}217}$. This was in contrast to the

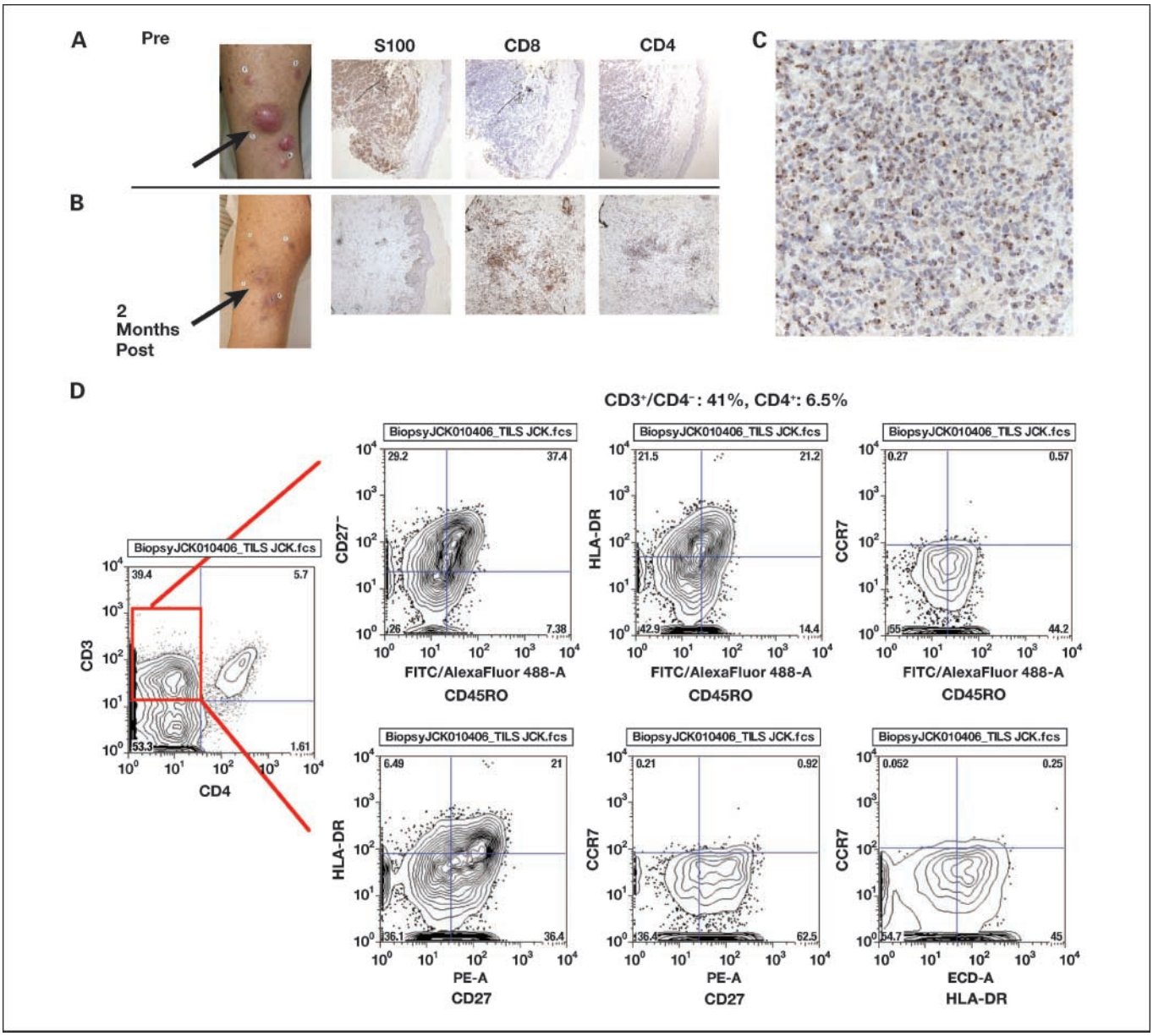


**Fig. 1.** T-cell infiltrates in case 2 (PR). *A,* predosing in-transit lesions in the left calf area, $S100^+$, with no detectable $CD8^+$ and $CD4^+$ infiltrates. *B,* regression of in-transit metastasis 2 mo after the first dose of tremelimumab. Immunohistochemistry staining showed near-complete disappearance of $S100^+$ melanoma cells and a diffuse immune infiltrate, with predominance of $CD8^+$ versus $CD4^+$ cells replacing the tumor. *C,* high-power (×40) image of immunohistochemistry staining for granzyme B in a postdosing regressing melanoma lesion. *D,* seven-color flow cytometry surface staining of mononuclear cells obtained from the postdosing biopsy. The majority of nonadherent cells were $CD3^+$ T lymphocytes, of which only 6.5% were $CD4^+$. Among the $CD3^+/CD4^-$ population, which should contain a majority of $CD8^+$ CTL, cells had a surface phenotype of $HLA\text{-}DR^+CD45RO^{+++}CD27^{++}CCR7^-$, consistent with early memory T cells. $CD4^+$, $CD8^+$, $FoxP3^+$, and $IDO^+$ cells were detected in a delayed biopsy from case 2.

peripheral blood, where populations of $gp100_{209\text{-}217}$- and $tyrosinase_{368\text{-}376}$-specific $CD8^+$ T cells were detected by tetramer assay, whereas MART-$1_{26\text{-}35}$-specific cells were below the low limit of detection (Fig. 4; data not shown).

***Intratumoral infiltration by FoxP3$^+$ cells.*** We then explored the possibility that the antitumor activity of tremelimumab may be mediated by the removal of cells expressing the transcription factor FoxP3, which has been reported to be preferentially expressed in human $CD4^+$ Tregs (38) and also in $CD8^+$ T suppressor cells (39). In the four cases with available predosing and postdosing biopsies (cases 1-3 and 5), there were very few changes observed in the frequency or pattern of infiltration by Tregs with nuclear FoxP3 protein expression (Table 2). In addition, the delayed biopsy of a residual melanoma lesion excised while the patient was being treated with methotrexate (case 2; Table 2) showed little difference in the presence of $FoxP3^+$ cells compared with regressing lesions biopsied while the patient was being treated with tremelimumab.

Case 4 showed patchy $FoxP3^+$ infiltrates in the concurrent regressing, stable, and progressive lesions. Detailed review of these areas showed that FoxP3 positivity represented coexistence of melanoma cells and a mononuclear cell infiltrate

including cells with FoxP3 expression (Fig. 2D). This pattern suggests that the FoxP3$^+$ cells are enriched in the areas of interaction between melanoma cells and immune effector cells, which agrees with preclinical observations of immune responses to viral pathogens (40).

Postdosing biopsies of progressive lesions from cases 6 and 7 showed markedly different patterns of FoxP3 expression. Case 6 had nearly absent FoxP3$^+$ cells, whereas case 7 had frequent and diffusely infiltrating FoxP3$^+$ cells (Table 2; Fig. 3D). Because the latter case had frequent CD4$^+$ and FoxP3$^+$ cells diffusely infiltrating the tumor, we sought to confirm that the immunohistochemistry staining represented a CD4$^+$/FoxP3$^+$ double-positive population. Nonadherent intratumorally infiltrating cells were surface stained for CD4 and CD25 and intracellularly stained for FoxP3. More than 90% of the CD4$^+$ cells with high expression of CD25 also expressed intracellular FoxP3, a phenotype consistent with Tregs (Supplementary Fig. S2).

***Lack of major effect of tremelimumab on intratumoral infiltration by IDO$^+$ cells.*** Tremelimumab might block the back signaling provided by CTLA4 expressed on Tregs that leads to the induction of the immunosuppressive IDO enzyme (19, 23, 41). In the four cases with available predosing and postdosing biopsies, there were very few changes observed in the frequency or pattern of infiltration by cells expressing IDO (Table 2). The lowest expression was found in the patient with progressive lesions (case 5; Fig. 3D), with nearly complete absence of IDO$^+$ cells both before and after treatment with tremelimumab. Analysis of the sample from the delayed resection of a resistant melanoma lesion in case 2 again showed little difference in the presence of IDO$^+$ cells despite treatment with immunosuppressive therapy and having no active treatment with tremelimumab for 7 months (Table 1). The regressing lesion in case 4 had patchy areas of denser IDO$^+$ infiltrates compared with the stable and progressing lesions (Table 2; Fig. 2D). In all three cases, these IDO$^+$ areas coincided with areas of FoxP3$^+$ cells intercalated between melanoma cells, forming granuloma-like accumulations of inflammatory and neoplastic cells. The stable and progressive lesions lacked IDO$^+$ cells in adjacent areas of melanoma cells. Postdosing biopsies of progressive lesions from cases 6 and 7 had patchy areas with IDO$^+$ cells after receiving tremelimumab (Table 2).

## Discussion

In this study, we provide evidence that tumor regression after administration of a CTLA4-blocking antibody to patients with metastatic melanoma is accompanied by a dense tumor infiltration by CD8$^+$ CTL. These postdosing infiltrates were

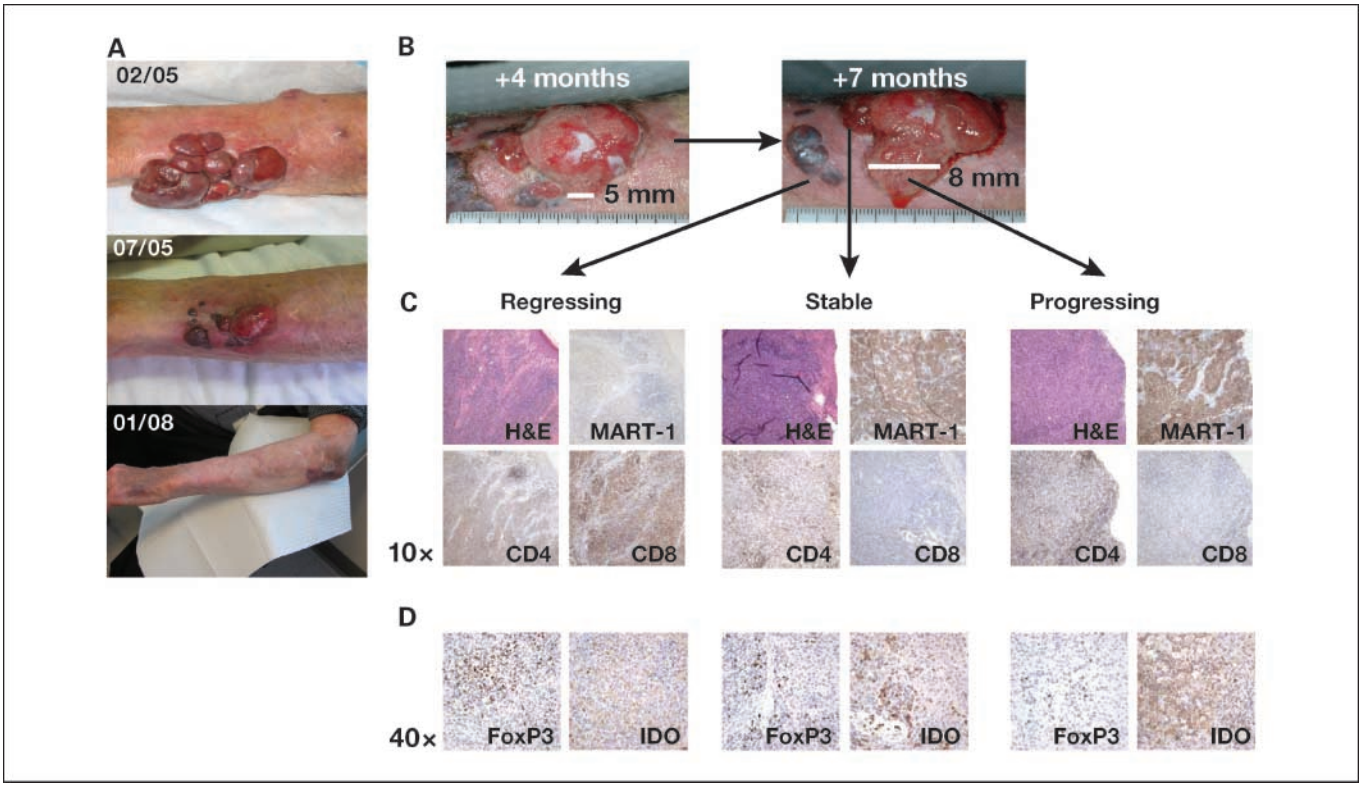


**Fig. 2.** T-cell infiltrates, skin biopsy, and responses in different lesions from case 4 (PR). *A,* regression of in-transit metastasis over a period of 5 mo. *B,* low-power immunohistochemistry staining images of an early biopsy taken 1 mo after the first dose of tremelimumab. There were residual HMB45$^+$ melanoma cells and a diffuse and dense intratumoral infiltrate by CD3$^+$ T lymphocytes, both CD4$^+$ and CD8$^+$, but no B cells. *C,* close-up pictures taken at 4 and 7 mo after starting monthly dosing with tremelimumab at 10 mg/kg, showing regression of lesions on the left side, a stable mass in the center, and a protuberance with progressive growth under it. *D,* immunohistochemistry was done on T-cell infiltrates in regressing, stable, and progressing lesions from case 4. Immunohistochemistry of a regressing lesion, with near-absence of MART-1$^+$ melanoma cells and a diffuse and dense infiltrate by T cells, most prominent by CD8$^+$ cells. Immunohistochemistry of the stable and progressing lesions, with persistence of MART-1$^+$ melanoma cells and little CD8$^+$ infiltrate but dense CD4$^+$ intratumoral infiltrate. Immunohistochemistry staining was used to detect FoxP3 and IDO in regressing, stable, and progressing lesions. Images labeled as melanoma have areas of monotonous malignant cells devoid of FoxP3$^+$ and IDO$^+$ cells. Images labeled as granuloma-like have areas of accumulation of immunohistochemistry-positive cells.

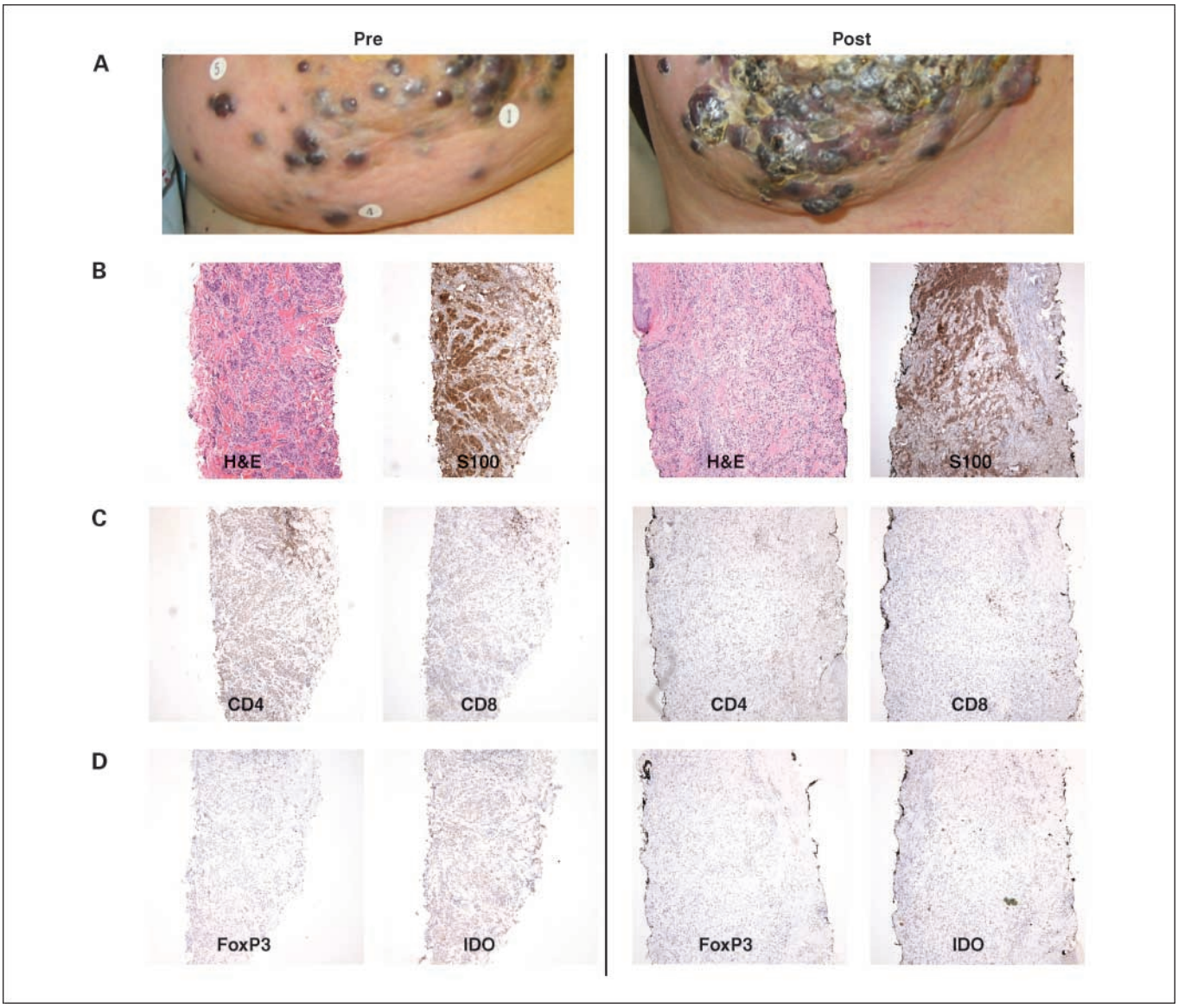


**Fig. 3.** Analysis of progressing lesions in case 5. *A,* predosing and 3-month postdosing pictures of progressive in-transit metastasis in a patient receiving tremelimumab at 15 mg/kg every 3 mo. *B,* H&E and S100 immunohistochemistry staining before and after dosing, showing dense melanoma lesions. *C,* scarce patchy infiltrates of $CD4^+$ and $CD8^+$ cells, with no major difference between predosing and postdosing biopsies. *D,* patchy FoxP3 and absent $IDO^+$ cells, with no difference between predosing and postdosing biopsies.

diffuse throughout the tumor or its remnant. In contrast, the T-cell infiltrates were commonly localized only in vascular stroma or peripheral areas of the tumor before treatment with tremelimumab. A decrease in intratumoral $CD8^+$ infiltration was also shown in 2 patients with persistent lesions despite regression of other lesions in the same patient (cases 2 and 4), suggesting that $CD8^+$ T-cell infiltration is associated with tumor regression. In some, but not all, cases of tumor regression, there were concomitant increases in $CD4^+$ T cells. However, in 1 patient (case 4), $CD4^+$ infiltration was reduced in a regressing lesion relative to stable or progressing lesions, suggesting that intratumoral $CD4^+$ T cells are not primary mediators of regression. Furthermore, some patients with progressive melanoma had diffuse infiltrates of $CD4^+$ cells. We cannot exclude that these differences reflect temporal variations resulting from the inconsistent timing of biopsies in different patients. The first published clinical report on the use of CTLA4-blocking antibodies provided evidence of T-cell infiltrations in tumors after dosing with ipilimumab (one case with both $CD4^+$ and $CD8^+$ infiltrates and a second case with only $CD8^+$ infiltrates), although none of the patients had objective responses or predominantly $CD8^+$ infiltrates (9). A recent report from this same group reported on 11 patients receiving sequential immunotherapy with a prior genetically modified granulocyte-macrophage colony-stimulating factor tumor vaccine (GVAX) followed 1 to 4 months later with administration of ipilimumab (33). There were three objective responses, and six cases underwent tumor biopsies. Pathologic analysis revealed extensive areas of necrosis in some cases, which is in marked contrast with the lack of necrosis in the regressing lesions reported herein. The cases with extensive necrosis had CD8 infiltrates. Another report provided evidence of both $CD4^+$ and

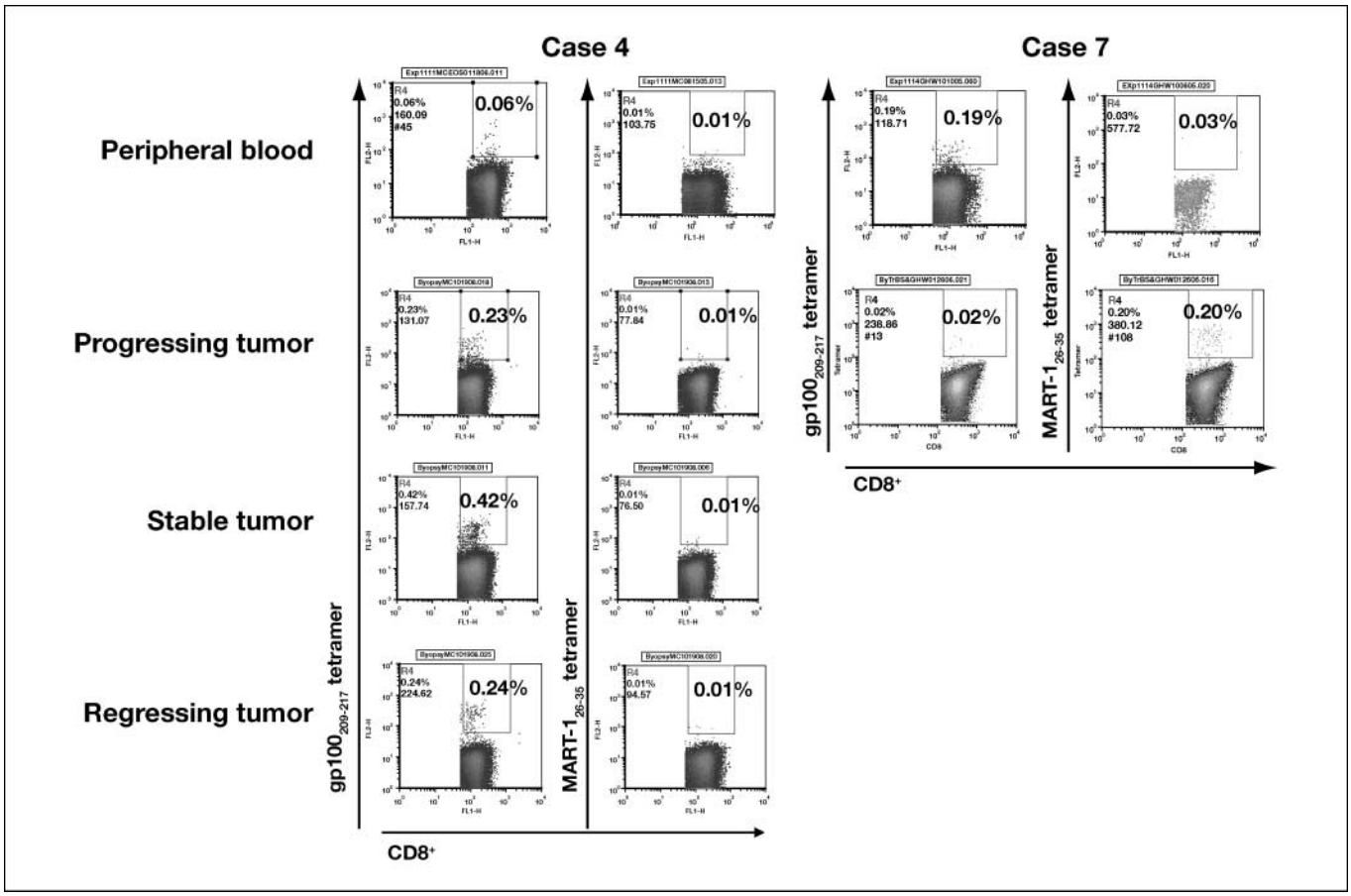


**Fig. 4.** MHC tetramer analysis comparing TILs with peripheral blood samples from responding and nonresponding lesions from HLA-A2.1$^+$ patients. Tetramer analysis was done on melanoma-specific CD8$^+$ T cells in peripheral blood mononuclear cells and TILs from cases 4 (PR) and 7 (stable disease). *Columns 1* and *2, row 1,* dot plots analyzing peripheral blood mononuclear cells for CD8$^+$/tetramer$^+$ cells specific for gp100$_{209\text{-}217}$ and MART-1$_{26\text{-}35}$ from case 4. *Columns 1* and *2, rows 2* to *4,* dot plots analyzing TILs from lesions for CD8$^+$/tetramer$^+$ cells specific for gp100$_{209\text{-}217}$ and MART-1$_{26\text{-}35}$. There is a clearly evident population of gp100$_{209\text{-}217}$-specific CD8$^+$ cells in TILs not present in peripheral blood. *Columns 3* and *4*, dot plots analyzing peripheral blood mononuclear cells and TILs for CD8$^+$/tetramer$^+$ cells specific for gp100$_{209\text{-}217}$ and MART-1$_{26\text{-}35}$ (data not shown for tyrosinase$_{368\text{-}376}$ tetramer reactivity) from case 7. There is a clearly evident population of MART-1$_{26\text{-}35}$-specific CD8$^+$ cells in TILs not present in peripheral blood, although there is a population of gp100$_{209\text{-}217}$-specific T cells in peripheral blood not accumulating in the tumor.

CD8$^+$ infiltrates with predominance of CD4$^+$ cells in a patient with follicular non-Hodgkin's lymphoma that was responding to ipilimumab (30).

We then explored the possibility that the antitumor activity of tremelimumab may be mediated by the removal of cells expressing the transcription factor FoxP3 (most frequently Tregs), which has been reported to be preferentially expressed in human CD4$^+$ Tregs (38) and also in CD8$^+$ T suppressor cells (39). Our data argue against two previously postulated hypotheses of the mechanism of action of CTLA4-blocking

**Table 2.** Intratumoral changes in FoxP3$^+$ and IDO$^+$ cells

| Case no. | Response | Timing of biopsy | FoxP3 | Change | IDO | Change |
|---|---|---|---|---|---|---|
| 1 | PR | Pre | 0 | | ++ diffuse | |
| | | Post | + patchy | ↑ | + patchy | ↓ |
| 2 | PR | Pre | 0 | | + patchy | |
| | | Post | + patchy | ↑ | + patchy | = |
| | | Post (delayed) | + patchy | ↑ | +/- patchy | ↓ |
| 3 | PR | Pre | + patchy | | + patchy | |
| | | Post | ++ patchy | ↑ | + patchy | = |
| 4 | PR | Post (progressing) | + patchy | | ++ patchy | |
| | | Post (stable) | + patchy | | ++ patchy | |
| | | Post (regressing) | + patchy | = | +++ patchy | ↑ |
| 5 | PD | Pre | + patchy | | - | |
| | | Post | + patchy | = | - | = |
| 6 | PD | Post | +/- patchy | | ++ patchy | |
| 7 | SD | Post (delayed) | +++ diffuse | | + patchy | |

antibodies. We did not find evidence of clearance of FoxP3$^+$ or IDO$^+$ cells. Tregs constitutively express CTLA4, and it has been proposed that anti-CTLA4 antibodies may deplete or modulate the activity of these cells (23, 32). Tremelimumab, a monoclonal antibody of IgG2 subtype, is unlikely to fix complement or to induce antibody-dependent cellular cytotoxicity (42). In fact, it was selected from a panel of antibodies because of its ability to activate cells with surface expression of CTLA4, and it does not deplete CTLA4$^+$ cells (24). A detailed analysis of Treg number and function in peripheral blood after administration of anti-CTLA4 monoclonal antibody ipilimumab did not show a depletion or inhibition of Treg function (29). In the experience of sequential administration of GVAX and ipilimumab, analysis of metastatic lesions suggested that the degree of tumor necrosis is inversely related to the number of intratumoral FoxP3$^+$ cells (33), but FoxP3$^+$ intratumoral infiltrates were evident in all samples. Further evidence that the antitumor activity of CTLA4-blocking monoclonal antibodies is not mediated by depletion of FoxP3$^+$ Treg cells is provided by two articles showing that antibodies that block CTLA4 in fact expand Tregs in mice (43) and in humans (44). In any case, depletion of Tregs does not appear to be a major mechanism associated with the antitumor activity of CTLA4-blocking monoclonal antibodies. We cannot exclude that tremelimumab modulates Treg function, because we could not collect sufficient TILs from the available biopsies to conduct functional studies. The need for functional assays to define Tregs is further exemplified by a report that tremelimumab may not deplete Tregs in peripheral blood but can induce resistance of T effector cells to the function of Tregs (45). Overall, our morphologic analysis of FoxP3$^+$ cells in regressing lesions after treatment with tremelimumab is in marked discordance with the preconceived hypothesis of their role as immunosuppressors, as our analysis shows an enrichment of FoxP3$^+$ cells in areas of active interaction between immune effector T cells and melanoma cells. This observation is more in line with FoxP3 cells acting as orchestrators of an effective adaptive immune response as shown in animal models of viral pathogenesis, where the depletion of Tregs resulted in worsening infections with herpes simplex virus and lymphocytic choriomeningitis virus (40). In these models, Tregs were shown to orchestrate timely homing of immune effector cells to the site of infection, and it is certainly possible that these cells may have the same role in tumor responses to tremelimumab.

IDO has been proposed as an immunosuppressive enzyme triggered by CTLA4 reverse signaling toward professional antigen-presenting cells (19, 41). In such a mechanism, blocking CTLA4 with monoclonal antibodies would be expected to inhibit IDO expression. We did not observe this pattern in our series; biopsies obtained at pharmacologic plasma levels of tremelimumab did not exhibit any evidence of inhibited IDO expression by immunohistochemistry. In fact, expression was markedly increased in the patient who had concurrent regressing, stable, and progressing lesions while on continued monthly dosing with tremelimumab, indicating that IDO expression was independent of the levels of circulating anti-CTLA4 antibody. These data are in contrast to a report from a preclinical study in which ipilimumab was administered together with antiretroviral therapy to macaques infected with simian immunodeficiency virus (23). The authors showed that antiretroviral therapy decreased expression of IDO mRNA in lymph nodes, and this decrease may be more profound in monkeys that received both antiretroviral therapy and ipilimumab. However, the results were rather variable, and the differences were small among groups and derived from a small number of animals. No immunohistochemistry data were provided in support of the PCR findings.

In conclusion, the antitumor effects of the CTLA4-blocking monoclonal antibody tremelimumab appear to be accompanied by dense intratumoral infiltrates of CD8$^+$ T cells, confirming the immunologic nature of its mechanism of action. Intratumoral infiltrates of CD4$^+$ cells are less consistently observed, which in our study could be explained by the performance of biopsies at different time points of an ongoing immune response. We and others have not noted expansion of tumor antigen-specific T cells in peripheral blood in these patients (10, 29–31, 36), nor have we found evidence of an effect on the number or function of peripheral Tregs (29, 31). Among the hypotheses proposed to describe the mechanism of action of anti-CTLA4 antibodies, our data suggest that these antibodies most likely promote increased accumulation of CD8$^+$ CTL inside tumors by allowing better interaction with melanoma cells (16).

## Disclosure of Potential Conflicts of Interest

A. Ribas: research grants and honoraria from Pfizer; J.A. Glaspy: speaker's bureau/honoraria from Pfizer; J. Gomez-Navarro: employee of Pfizer. Financial support for medical editorial assistance for this article was provided by Pfizer.

## Acknowledgments

We thank Drs. Jonathan Said and Dorina Guo for providing access to image capture microscopes and the staff at the UCLA Immunopathology laboratory for diligent immunohistochemistry staining and Todd Parker Ph.D. (ProEd Communications) for medical editorial assistance with this article. Flow cytometry assays were done in the UCLA Jonsson Comprehensive Cancer Center and Center for AIDS Research Flow Cytometry Core Facility, which is supported by NIH awards CA-16042 and AI-28697 and by the Jonsson Comprehensive Cancer Center, UCLA AIDS Institute, and David Geffen School of Medicine at UCLA.

## References

1. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. Science 1996;271:1734–6.
2. Sotomayor EM, Borrello I, Tubb E, Allison JP, Levitsky HI. *In vivo* blockade of CTLA-4 enhances the priming of responsive T cells but fails to prevent the induction of tumor antigen-specific tolerance. Proc Natl Acad Sci U S A 1999;96:11476–81.
3. Kwon ED, Foster BA, Hurwitz AA, et al. Elimination of residual metastatic prostate cancer after surgery and adjunctive cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) blockade immunotherapy. Proc Natl Acad Sci U S A 1999;96:15074–9.
4. van Elsas A, Hurwitz AA, Allison JP. Combination immunotherapy of B16 melanoma using anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF)-producing vaccines induces rejection of subcutaneous and metastatic tumors accompanied by autoimmune depigmentation. J Exp Med 1999; 190:355–66.
5. Hurwitz AA, Foster BA, Kwon ED, et al. Combination immunotherapy of primary prostate cancer in a transgenic mouse model using CTLA-4 blockade. Cancer Res 2000;60:2444–8.
6. Sutmuller RP, van Duivenvoorde LM, van Elsas A, et al. Synergism of cytotoxic T lymphocyte-associated

antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. J Exp Med 2001;194:823–32.

**7.** Ko K, Yamazaki S, Nakamura K, et al. Treatment of advanced tumors with agonistic anti-GITR mAb and its effects on tumor-infiltrating $Foxp3^+CD25^+CD4^+$ regulatory T cells. J Exp Med 2005;202:885–91.

**8.** van Elsas A, Sutmuller RP, Hurwitz AA, et al. Elucidating the autoimmune and antitumor effector mechanisms of a treatment based on cytotoxic T lymphocyte antigen-4 blockade in combination with a B16 melanoma vaccine: comparison of prophylaxis and therapy. J Exp Med 2001;194:481–9.

**9.** Hodi FS, Mihm MC, Soiffer RJ, et al. Biologic activity of cytotoxic T lymphocyte-associated antigen 4 antibody blockade in previously vaccinated metastatic melanoma and ovarian carcinoma patients. Proc Natl Acad Sci U S A 2003;100:4712–7.

**10.** Phan GQ, Yang JC, Sherry RM, et al. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. Proc Natl Acad Sci U S A 2003;100:8372–7.

**11.** Sanderson K, Scotland R, Lee P, et al. Autoimmunity in a phase I trial of a fully human anti-cytotoxic T-lymphocyte antigen-4 monoclonal antibody with multiple melanoma peptides and Montanide ISA 51 for patients with resected stages III and IV melanoma. J Clin Oncol 2005;23:741–50.

**12.** Ribas A, Camacho LH, Lopez-Berestein G, et al. Antitumor activity in melanoma and anti-self responses in a phase I trial with the anti-cytotoxic T lymphocyte-associated antigen 4 monoclonal antibody CP-675,206. J Clin Oncol 2005;23:8968–77.

**13.** Korman A, Yellin M, Keler T. Tumor immunotherapy: preclinical and clinical activity of anti-CTLA4 antibodies. Curr Opin Investig Drugs 2005;6:582–91.

**14.** Chambers CA, Kuhns MS, Egen JG, Allison JP. CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy. Annu Rev Immunol 2001;19:565–94.

**15.** Wang XB, Giscombe R, Yan Z, Heiden T, Xu D, Lefvert AK. Expression of CTLA-4 by human monocytes. Scand J Immunol 2002;55:53–60.

**16.** Schneider H, Downey J, Smith A, et al. Reversal of the TCR stop signal by CTLA-4. Science 2006;313:1972–5.

**17.** Shevach EM. Certified professionals: CD4(+)CD25(+) suppressor T cells. J Exp Med 2001;193:F41–6.

**18.** Paust S, Lu L, McCarty N, Cantor H. Engagement of B7 on effector T cells by regulatory T cells prevents autoimmune disease. Proc Natl Acad Sci U S A 2004;101:10398–403.

**19.** Grohmann U, Orabona C, Fallarino F, et al. CTLA-4-Ig regulates tryptophan catabolism *in vivo*. Nat Immunol 2002;3:1097–101.

**20.** Fallarino F, Grohmann U, Hwang KW, et al. Modulation of tryptophan catabolism by regulatory T cells. Nat Immunol 2003;4:1206–12.

**21.** Munn DH, Sharma MD, Hou D, et al. Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. J Clin Invest 2004;114:280–90.

**22.** Mellor AL, Chandler P, Baban B, et al. Specific subsets of murine dendritic cells acquire potent T cell regulatory functions following CTLA4-mediated induction of indoleamine 2,3 dioxygenase. Int Immunol 2004;16:1391–401.

**23.** Hryniewicz A, Boasso A, Edghill-Smith Y, et al. CTLA-4 blockade decreases TGF-β, IDO, and viral RNA expression in tissues of SIVmac251-infected macaques. Blood 2006;108:3834–42.

**24.** Ribas A, Hanson DC, Noe DA, et al. Tremelimumab (CP-675,206), a cytotoxic T lymphocyte associated antigen 4 blocking monoclonal antibody in clinical development for patients with cancer. Oncologist 2007;12:873–83.

**25.** Herr W, Schneider J, Lohse AW, Meyer zum Buschenfelde KH, Wolfel T. Detection and quantification of blood-derived $CD8^+$ T lymphocytes secreting tumor necrosis factor α in response to HLA-A2.1-binding melanoma and viral peptide antigens. J Immunol Methods 1996;191:131–42.

**26.** Altman JD, Moss PA, Goulder PJ, et al. Phenotypic analysis of antigen-specific T lymphocytes. Science 1996;274:94–6.

**27.** Keilholz U, Weber J, Finke JH, et al. Immunologic monitoring of cancer vaccine therapy: results of a workshop sponsored by the Society for Biological Therapy. J Immunother 2002;25:97–138.

**28.** Phan GQ, Attia P, Steinberg SM, White DE, Rosenberg SA. Factors associated with response to high-dose interleukin-2 in patients with metastatic melanoma. J Clin Oncol 2001;19:3477–82.

**29.** Maker AV, Attia P, Rosenberg SA. Analysis of the cellular mechanism of antitumor responses and autoimmunity in patients treated with CTLA-4 blockade. J Immunol 2005;175:7746–54.

**30.** O'Mahony D, Morris JC, Quinn C, et al. A pilot study of CTLA-4 blockade after cancer vaccine failure in patients with advanced malignancy. Clin Cancer Res 2007;13:958–64.

**31.** Comin-Anduix B, Lee Y, Jalil J, et al. Detailed analysis of immunologic effects of the cytotoxic T lymphocyte-associated antigen 4-blocking monoclonal antibody tremelimumab in peripheral blood of patients with melanoma. J Transl Med 2008;6:22.

**32.** Reuben JM, Lee BN, Li C, et al. Biologic and immunomodulatory events after CTLA-4 blockade with ticilimumab in patients with advanced malignant melanoma. Cancer 2006;106:2437–44.

**33.** Hodi FS, Butler M, Oble DA, et al. Immunologic and clinical effects of antibody blockade of cytotoxic T lymphocyte-associated antigen 4 in previously vaccinated cancer patients. Proc Natl Acad Sci U S A 2008;105:3005–10.

**34.** Therasse P, Arbuck SG, Eisenhauer EA, et al.; European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. New guidelines to evaluate the response to treatment in solid tumors. J Natl Cancer Inst 2000;92:205–16.

**35.** Perfetto SP, Chattopadhyay PK, Roederer M. Seventeen-colour flow cytometry: unravelling the immune system. Nat Rev Immunol 2004;4:648–55.

**36.** Comin-Anduix B, Gualberto A, Glaspy JA, et al. Definition of an immunologic response using the major histocompatibility complex tetramer and enzyme-linked immunospot assays. Clin Cancer Res 2006;12:107–16.

**37.** Pages F, Berger A, Camus M, et al. Effector memory T cells, early metastasis, and survival in colorectal cancer. N Engl J Med 2005;353:2654–66.

**38.** Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003;299:1057–61.

**39.** Hahn BH, Singh RP, La Cava A, Ebling FM. Tolerogenic treatment of lupus mice with consensus peptide induces Foxp3-expressing, apoptosis-resistant, TGFβ-secreting $CD8^+$ T cell suppressors. J Immunol 2005;175:7728–37.

**40.** Lund JM, Hsing L, Pham TT, Rudensky AY. Coordination of early protective immunity to viral infection by regulatory T cells. Science 2008;320:1220–4.

**41.** Munn DH, Mellor AL. IDO and tolerance to tumors. Trends Mol Med 2004;10:15–8.

**42.** Bruggemann M, Williams GT, Bindon CI, et al. Comparison of the effector functions of human immunoglobulins using a matched set of chimeric antibodies. J Exp Med 1987;166:1351–61.

**43.** Tang AL, Teijaro JR, Njau MN, et al. CTLA4 expression is an indicator and regulator of steady-state $CD4^+$ $FoxP3^+$ T cell homeostasis. J Immunol 2008;181:1806–13.

**44.** Kavanagh B, O'Brien S, Lee D, et al. CTLA4 blockade expands $FoxP3^+$ regulatory and activated effector $CD4^+$ T cells in a dose-dependant fashion. Blood 2008;121:1175–83.

**45.** Menard C, Ghiringhelli F, Roux S, et al. CTLA-4 blockade confers lymphocyte resistance to regulatory T-cells in advanced melanoma: surrogate marker of efficacy of tremelimumab? Clin Cancer Res 2008;14:5242–9.