

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

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**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<sup>+</sup> T cells that were markedly increased in cases where comparison with a baseline biopsy was available. Nonregressing lesions had sparse, patchy CD8<sup>+</sup> intratumoral infiltrates. Patients with regressing lesions had an increased frequency of CD8<sup>+</sup> cells with or without a concomitant increase in CD4<sup>+</sup> cells. Two of 3 responding patients with paired samples showed a slight increase in the number of FoxP3<sup>+</sup> 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<sup>+</sup> CTLs in patients with regressing tumors but had varying effects on intratumoral infiltrates of CD4<sup>+</sup> and FoxP3<sup>+</sup> 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<sup>+</sup> T helper and CD8<sup>+</sup> 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<sup>+</sup> CTLs, would accumulate at increased frequency in the tumors of responding patients.

CTLA4 is also constitutively expressed by CD4<sup>+</sup>/CD25<sup>+</sup> 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<sup>+</sup> or CD86<sup>+</sup> cells, including activated T cells (18) and indoleamine 2,3-dioxygenase (IDO)-competent plasmacytoid

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## 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<sub>2</sub> 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 (DAKO Cytomation). 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; DAKO Cytomation) 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 (DAKO Cytomation), 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 FACSria (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<sub>26-35</sub> (ELAGIGILTV), tyrosinase<sub>368-376</sub> (YMDGTSQV), and gp100<sub>209-217</sub> (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<sup>+</sup> and CD4<sup>+</sup> 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 Post	0 1	- 3 mo	0 +++ diffuse	↑	NA ++ diffuse	NA
2	78	M	10 mg/kg every 1 mo	PR	9/31+	Grade 2 arthritis	Pre Post Post*	0 3 3	- 1 mo 7 mo	0 ++ diffuse +/- patchy	↑ ↓	0 + diffuse +/- patchy	↑ ↓
3	64	M	10 mg/kg every 1 mo	pPR	36/39+	Grade 2 asthenia	Pre Post	0 9	- 1 mo	++ patchy ++ diffuse	↑	+ patchy +++ diffuse	↑
4	90	M	10 mg/kg every 1 mo	PR	8/40+		Post † Post ‡ Post §	8 8 8	1 mo 1 mo 1 mo	+ patchy + patchy +++ diffuse	↑	+++ diffuse +++ diffuse +++ diffuse	↓
5	68	F	15 mg/kg every 3 mo	PD	3/18		Pre Post	0 1	- 3 mo	+/- patchy +/- patchy	=	+/- 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  $\mu\text{g/mL}$  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<sup>+</sup> or CD4<sup>+</sup> cell infiltrate, whereas the postdosing biopsy had a marked CD8<sup>+</sup> infiltrate percolated between tumor cells and a less abundant CD4<sup>+</sup> infiltrate (Table 1; Fig. 1A and B). Immunohistochemistry staining showed that the CD8<sup>+</sup> infiltrating cells had intracellular vacuoles loaded with the toxic granule, granzyme B, suggesting that these were true CTLs (Fig. 1C). The predominant CD8<sup>+</sup> infiltrate in case 2 was confirmed by multicolor flow cytometry of nonadherent TILs (Fig. 1D). This analysis revealed that >40% of the nonadherent CD3<sup>+</sup> TILs were CD3<sup>+</sup>/CD4<sup>-</sup> (by exclusion mostly CD8<sup>+</sup>), whereas only 6.5% were CD3<sup>+</sup>/CD4<sup>+</sup>. Extended surface analysis of the tumor-infiltrating CD3<sup>+</sup>/CD4<sup>-</sup> T cells in the regressing lesion showed that the cells had a HLA-DR<sup>+</sup>, CD45RO<sup>+++</sup>, CD27<sup>++</sup>, and CCR7<sup>-</sup> 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 [<sup>18</sup>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<sup>+</sup> and CD4<sup>+</sup> infiltrate mostly in peritumoral areas in marked contrast with the massive diffuse intratumoral CD8<sup>+</sup> 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<sup>+</sup> and CD4<sup>+</sup> 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 [<sup>18</sup>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<sup>+</sup> and CD4<sup>+</sup> mononuclear cells, with a slight predominance of CD4<sup>+</sup> cells (Table 1).

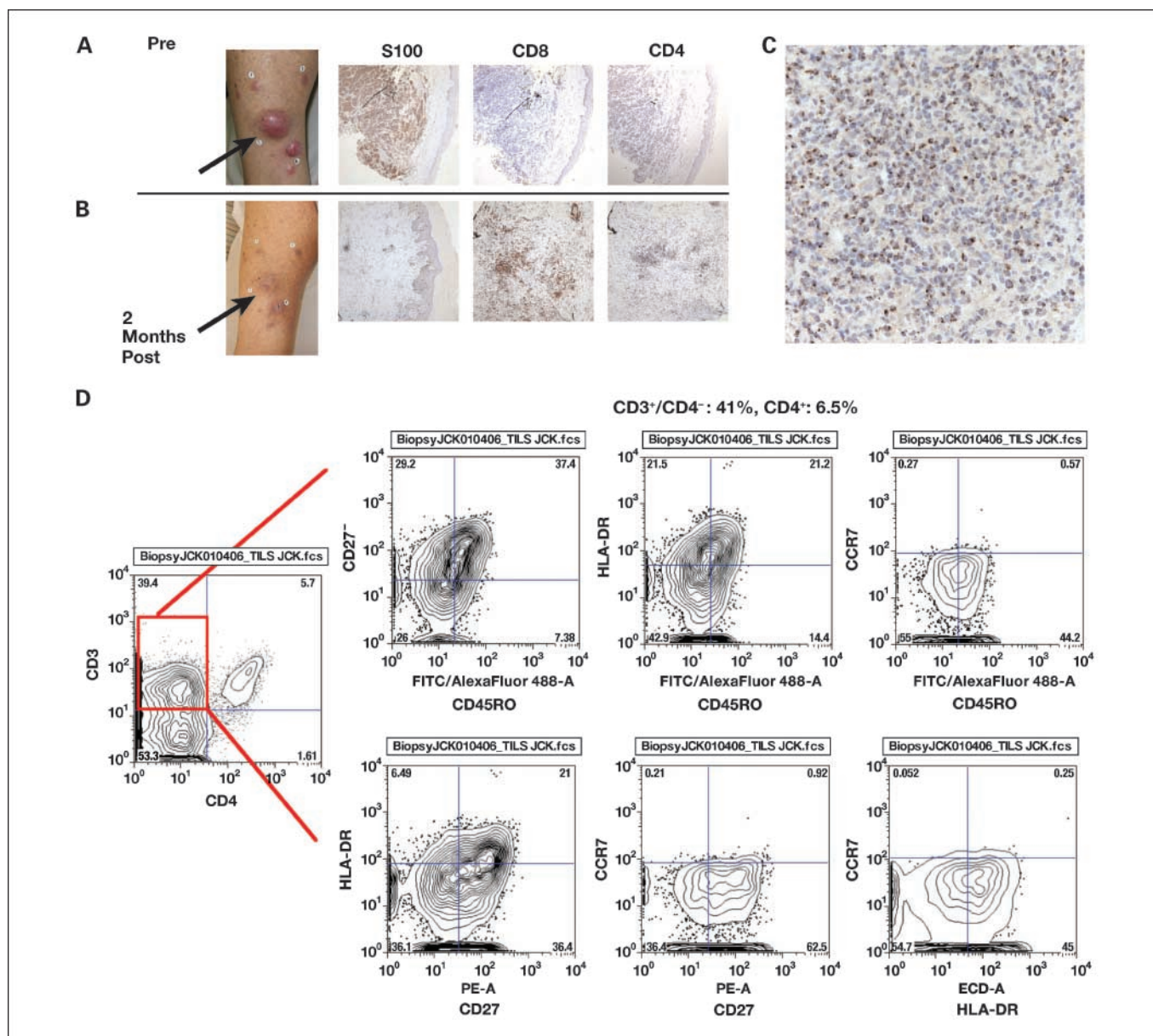
Case 4 had a major PR following tremelimumab treatment (Fig. 2A). An initial biopsy of a regressing in-transit lesion

1 month after receiving the first dose revealed a massive diffuse intratumoral infiltrate of both CD8<sup>+</sup> and CD4<sup>+</sup> 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<sup>+</sup> and CD20<sup>+</sup> cells surrounded by a mantle of CD4<sup>+</sup> and CD8<sup>+</sup> 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<sup>+</sup> and CD4<sup>+</sup> infiltrate, whereas the stable and progressing lesions had tumor cells positive for the MART-1 and HMB45 melanoma antigens, with a dense CD4<sup>+</sup> infiltrate and nearly absent CD8<sup>+</sup> 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<sup>+</sup> and CD4<sup>+</sup> 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<sup>+</sup> and CD4<sup>+</sup> infiltrates, whereas the biopsy from case 7 showed evidence of diffuse intratumoral CD8<sup>+</sup> and CD4<sup>+</sup> infiltrates with a predominance of CD4<sup>+</sup> cells (Table 1; Supplementary Fig. S2). We found no significant intratumoral infiltration by CD1a dendritic cells, CD20<sup>+</sup> B cells, or CD57<sup>+</sup> natural killer cells in any of the staining samples analyzed. Staining for CD68<sup>+</sup> macrophages was difficult to evaluate, as melanoma cells from some patients were positive for this marker (data not shown).

Given the presence of CD8<sup>+</sup> 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<sup>+</sup>, were subjected to staining with commercially available tetramers folded with the MART-1<sub>26-35</sub>, tyrosinase<sub>368-376</sub>, and gp100<sub>209-217</sub> 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<sup>+</sup> T cells (36). Both the regressing and the progressing lesions in case 4 had accumulation of a population of CD8<sup>+</sup> T cells specific for gp100<sub>209-217</sub> above the low limit of detection and at approximately the same frequency (Supplementary Fig. S2); the stable lesion had a higher percentage of gp100<sub>209-217</sub>-specific CD8<sup>+</sup> T cells, whereas none of these lesions had detectable CD8<sup>+</sup> T cells with MART-1<sub>26-35</sub> or tyrosinase<sub>368-376</sub> 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<sup>+</sup> 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<sub>26-35</sub>, but not for tyrosinase<sub>368-376</sub> or gp100<sub>209-217</sub>. 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<sup>+</sup>, with no detectable CD8<sup>+</sup> and CD4<sup>+</sup> infiltrates. *B*, regression of in-transit metastasis 2 mo after the first dose of tremelimumab. Immunohistochemistry staining showed near-complete disappearance of S100<sup>+</sup> melanoma cells and a diffuse immune infiltrate, with predominance of CD8<sup>+</sup> versus CD4<sup>+</sup> cells replacing the tumor. *C*, high-power ( $\times 40$ ) 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<sup>+</sup> T lymphocytes, of which only 6.5% were CD4<sup>+</sup>. Among the CD3<sup>+</sup>/CD4<sup>-</sup> population, which should contain a majority of CD8<sup>+</sup> CTL, cells had a surface phenotype of HLA-DR<sup>+</sup>CD45RO<sup>+</sup>CD27<sup>+</sup>CCR7<sup>-</sup>, consistent with early memory T cells. CD4<sup>+</sup>, CD8<sup>+</sup>, FoxP3<sup>+</sup>, and IDO<sup>+</sup> cells were detected in a delayed biopsy from case 2.

peripheral blood, where populations of gp100<sub>209-217</sub><sup>-</sup> and tyrosinase<sub>368-376</sub>-specific CD8<sup>+</sup> T cells were detected by tetramer assay, whereas MART-1<sub>26-35</sub>-specific cells were below the low limit of detection (Fig. 4; data not shown).

**Intratumoral infiltration by FoxP3<sup>+</sup> 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<sup>+</sup> Tregs (38) and also in CD8<sup>+</sup> 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<sup>+</sup> cells compared with regressing lesions biopsied while the patient was being treated with tremelimumab.

Case 4 showed patchy FoxP3<sup>+</sup> 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<sup>+</sup> 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).

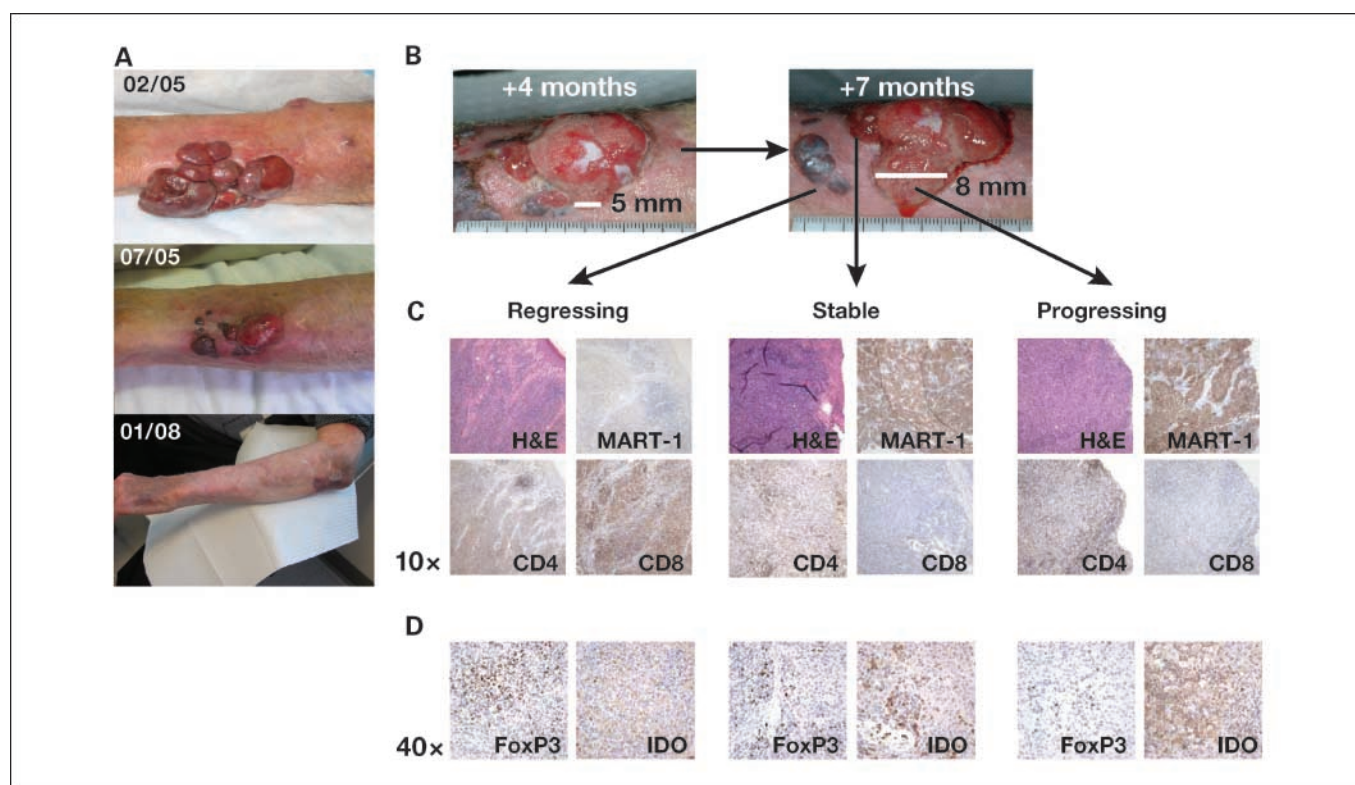
Postdosing biopsies of progressive lesions from cases 6 and 7 showed markedly different patterns of FoxP3 expression. Case 6 had nearly absent FoxP3<sup>+</sup> cells, whereas case 7 had frequent and diffusely infiltrating FoxP3<sup>+</sup> cells (Table 2; Fig. 3D). Because the latter case had frequent CD4<sup>+</sup> and FoxP3<sup>+</sup> cells diffusely infiltrating the tumor, we sought to confirm that the immunohistochemistry staining represented a CD4<sup>+</sup>/FoxP3<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> infiltrates compared with the stable and progressing lesions (Table 2; Fig. 2D). In all three cases, these IDO<sup>+</sup> areas coincided with areas of FoxP3<sup>+</sup> cells intercalated between melanoma cells, forming granuloma-like accumulations of inflammatory and neoplastic cells. The stable and progressive lesions lacked IDO<sup>+</sup> cells in adjacent areas of melanoma cells. Postdosing biopsies of progressive lesions from cases 6 and 7 had patchy areas with IDO<sup>+</sup> cells after receiving tremelimumab (Table 2).

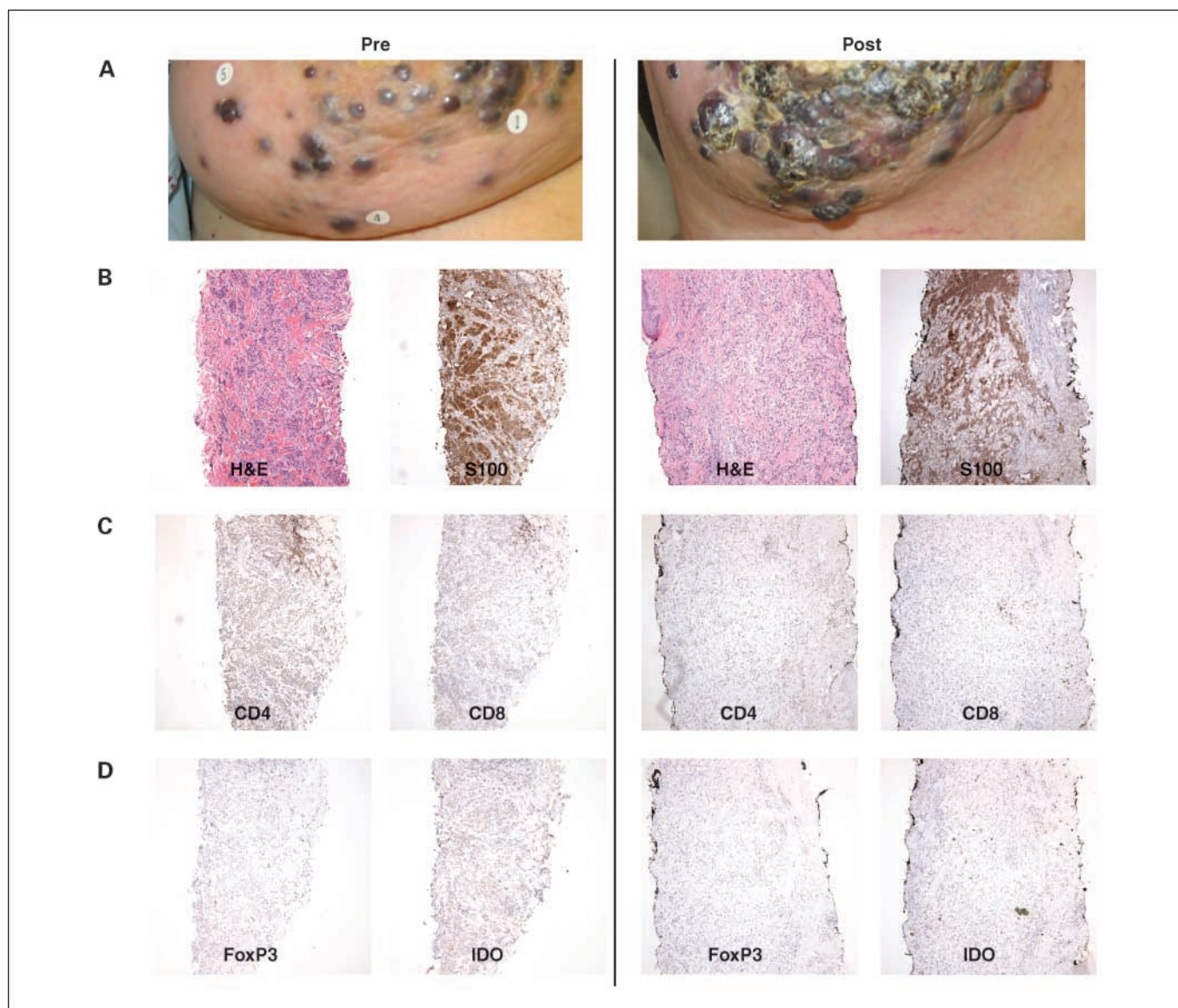
## Discussion

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<sup>+</sup> 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<sup>+</sup> melanoma cells and a diffuse and dense intratumoral infiltrate by CD3<sup>+</sup> T lymphocytes, both CD4<sup>+</sup> and CD8<sup>+</sup>, 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<sup>+</sup> melanoma cells and a diffuse and dense infiltrate by T cells, most prominent by CD8<sup>+</sup> cells. Immunohistochemistry of the stable and progressing lesions, with persistence of MART-1<sup>+</sup> melanoma cells and little CD8<sup>+</sup> infiltrate but dense CD4<sup>+</sup> 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<sup>+</sup> and IDO<sup>+</sup> cells. Images labeled as granuloma-like have areas of accumulation of immunohistochemistry-positive cells.

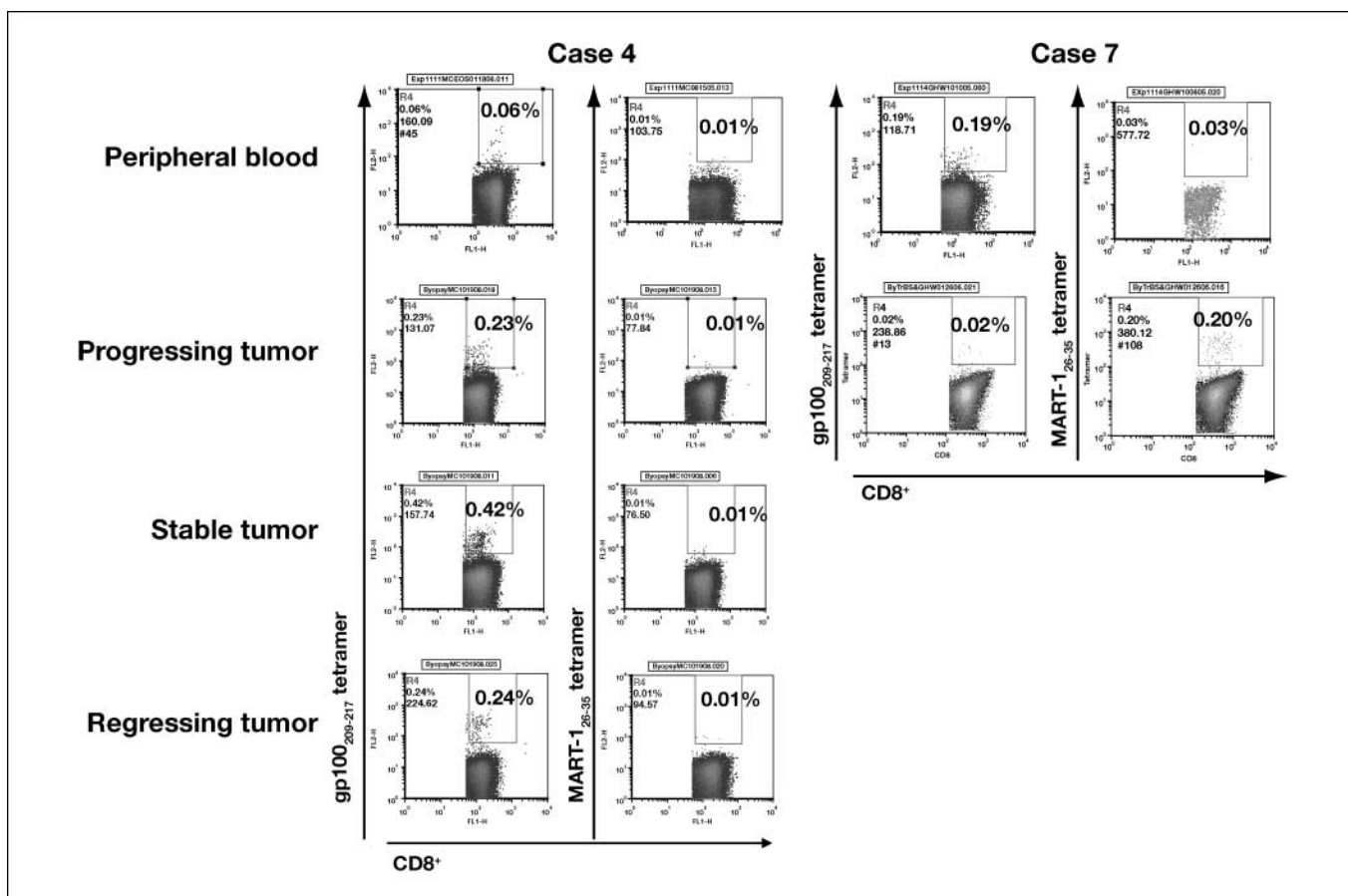




**Fig. 3.** Analysis of progressing lesions in case 5. *A*, predosing and 3-month postdosing pictures of progressive in-transit metastasis in a patient receiving tremelimumab at 15 mg/kg every 3 mo. *B*, H&E and S100 immunohistochemistry staining before and after dosing, showing dense melanoma lesions. *C*, scarce patchy infiltrates of CD4<sup>+</sup> and CD8<sup>+</sup> cells, with no major difference between predosing and postdosing biopsies. *D*, patchy FoxP3 and absent IDO<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T-cell infiltration is associated with tumor regression. In some, but not all, cases of tumor regression, there were concomitant increases in CD4<sup>+</sup> T cells. However, in 1 patient (case 4), CD4<sup>+</sup> infiltration was reduced in a regressing lesion relative to stable or progressing lesions, suggesting that intratumoral CD4<sup>+</sup> T cells are not primary mediators of regression. Furthermore, some patients with progressive melanoma had diffuse infiltrates of CD4<sup>+</sup> 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<sup>+</sup> and CD8<sup>+</sup> infiltrates and a second case with only CD8<sup>+</sup> infiltrates), although none of the patients had objective responses or predominantly CD8<sup>+</sup> 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<sup>+</sup> and



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

CD8<sup>+</sup> infiltrates with predominance of CD4<sup>+</sup> 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<sup>+</sup> Tregs (38) and also in CD8<sup>+</sup> 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<sup>+</sup> and IDO<sup>+</sup> 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	=
3	PR	Pre	+ patchy		+/- patchy	
		Post (delayed)	+ patchy	↑	+ patchy	↓
4	PR	Pre	++ patchy		+ patchy	
		Post	+ patchy	↑	++ patchy	=
5	PD	Pre	+ patchy		++ patchy	
		Post	+ patchy	=	++ patchy	↑
6	PD	Pre	+ patchy		++ patchy	
		Post	+/- patchy	=	++ patchy	=
7	SD	Post (delayed)	+++ diffuse		+ patchy	



antibodies. We did not find evidence of clearance of FoxP3<sup>+</sup> or IDO<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> cells (33), but FoxP3<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> T cells, confirming the immunologic nature of its mechanism of action. Intratumoral infiltrates of CD4<sup>+</sup> 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<sup>+</sup> 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.

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

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