Tumor immunosurveillance is thought to operate through the differentiation of antigen-specific CD4+ and CD8+ T lymphocytes capable of recognizing and killing tumor cells (1). In numerous cancers, the presence of CD4+ and CD8+ tumor-infiltrating T lymphocytes (TIL) is a favorable prognostic factor and correlate with increased survival (2–4). The efficacy of therapy based on the adoptive transfer of melanoma-specific TIL or CTL brought up the proof of principle that T lymphocytes can play a role in the control of melanoma (5–7). However, the local presence of CD4+CD25+ regulatory T cells (Treg) in tumor beds predicts reduced patient survival (8, 9). These data support the existence of a tumor immunosurveillance system that is locally suppressed by Treg cells. Accumulating evidence now points out to a deleterious role of Treg in the success of the current tumor immunotherapy and vaccination protocols (10). Therefore, the concept of reversing immunosuppression in cancer has merit as a therapeutic approach, and few studies targeting Tregs have been reported (11–16).

One of the most recognized strategies to harness T-cell hyporesponsiveness or anergy against tumor cells has been the development of CTL antigen-4 (CTLA-4) blocking antibodies. CTLA-4 is a closely related homologue of CD28, the key costimulatory pathway to T-cell receptor ligation. CTLA-4 is a negative regulator of CD28-dependent T-cell responses (17–19). Mouse studies indicated that the inhibitory effects of CTLA-4 on both CD4+ and CD8+ T-cell responses are more pronounced during secondary rather than primary responses (20, 21). Mouse studies indicated that the inhibitory effects of CTLA-4 on both CD4+ and CD8+ T-cell responses are more pronounced during secondary rather than primary responses (20, 21). CTLA-4 also plays an essential role in the function of Treg (22, 23) and human tumor Treg express high levels of CTLA-4 (9).

Two human anti–CTLA-4 monoclonal antibodies (mAb) are being developed, including ipilimumab (Medarex, BMS) and tremelimumab (Pfizer). Both have been tested in phase I/II trials and have led to encouraging antitumor activity associated with frequent immune-related adverse events in patients.

CTLA-4 Blockade Confers Lymphocyte Resistance to Regulatory T-Cells in Advanced Melanoma: Surrogate Marker of Efficacy of Tremelimumab?

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Abstract

Purpose: Anti–CTLA antigen-4 (CTLA-4) monoclonal antibody (mAb) has led to encouraging antitumor activity associated with immune-related adverse events in patients with heavily pretreated melanoma. However, mechanisms of action and surrogate immunologic markers of efficacy have not been reported thus far.

Experimental Design: We monitored the immune responses of 10 melanoma patients included in a phase I clinical trial, which evaluated the efficacy of a second line of therapy of tremelimumab anti–CTLA-4 mAb in patients with metastatic melanoma. The frequency of blood leukocyte populations in association with Tcell and regulatory Tcell (Treg) functions were evaluated.

Results: Prior to therapy, patients with advanced melanoma presented with a severe CD4+ and CD8+ T cell lymphopenia associated with blunted T-cell proliferative capacities that could be assigned to Treg. Tremelimumab rapidly restored the effector and memory CD4+ and CD8+ T-cell pool and TCR-dependent T-cell proliferation that became entirely resistant to Treg-mediated suppression. Progression-free survival and overall survival was directly correlated with the acquisition of a biological response defined as the resistance of peripheral lymphocytes to Treg-inhibitory effects (obtained in 7 of 10 patients).

Conclusion: CTLA-4 blockade seems to be a valuable strategy to revive reactive memory T cells energized in the context of stage IV melanoma, and our work suggests that memory T-cell resistance to Treg resulting from anti–CTLA-4 treatment might be a biological activity marker for tremelimumab in patients with melanoma.
Translational Relevance

There are few published data concerning the biological effects of anti–CTLA-4 in humans, and no biological signals of activity have been identified thus far. The rate of objective response in patients with metastatic melanoma is not very high (>10%), nevertheless, CTLA-4 blockade remains a very promising therapeutic approach because of the long-lasting responses observed in responding patients. It is therefore critical to find ways to identify this subgroup of patients and this can be done only by understanding precisely how these drugs work in vivo in patients. We found that anti–CTLA-4 mAb restored the effector and memory T-cell pool and TCR-dependent T-cell proliferation. These cells became entirely resistant to Treg-mediated suppression. Progression-free survival and overall survival was directly correlated with the acquisition of a biological response.

(13–15, 24–26). Understanding the mechanism of tumor regression following therapy with anti–CTLA-4 antibodies would be helpful to design their therapeutic potential.

Two groups performed immunomonitoring studies on 10 to 12 melanoma patients treated with anti–CTLA-4 mAb and reported conflicting results. Therapy with ipilimumab induced a significant increase in circulating effector/memory CD4+ T cells but Treg numbers and functions remained unchanged (13, 27). By contrast, in tremelimumab-injected patients, immune-related adverse events and clinical objective responses correlated with a decrease in the percentage of Treg and a reduction of interleukin-10 secretion by peripheral blood mononuclear cells (PBMC; ref. 28). Therefore, we analyzed the modulation of the phenotype and function of both conventional and regulatory T cells in melanoma patients treated with tremelimumab. Our study shows for the first time that CTLA-4 blockade not only restored TCR-driven T-cell proliferative potential in immunosuppressed advanced melanoma patients but also confers lymphocyte resistance to Treg. The biological criteria defined as “the resistance of TCR-mediated T-cell proliferation to the inhibitory effects of Treg” obtained after a single injection correlated with time to progression. Furthermore, both resistance to Treg returned to pretherapeutic levels 2 months after tremelimumab injection.

Altogether, our results suggest that the biological markers reported in our study may serve as prognostic indicators of responses for future clinical trials with tremelimumab and that the quarterly schedule of tremelimumab injections might not be optimal.

Materials and Methods

Patients. The immunomonitoring was done on all patients enrolled in the international multicentric phase II trial (at the Gustave Roussy Institute) evaluating the efficacy of tremelimumab in patients with advanced melanoma (protocol no. A3671008, U.S. IND number BB-10096; Pfizer) after at least one systemic therapy. Informed consent was obtained for all patients and the study was approved by the local ethic committee. Heparinized blood (60 mL) was drawn before tremelimumab injection (day 0), 30 days and 60 days after. Age-matched and sex-matched normal volunteers were chosen as controls. Patients’ characteristics are described in Table 1. Response to the treatment was assessed by computed tomography scan according to Response Evaluation Criteria in Solid Tumors parameters. We considered patients with partial response or stable disease using Response Evaluation Criteria in Solid Tumors guidelines as clinical responders.

Lymphocyte isolation and cellular assays. PBMC were isolated from patients’ or healthy donors’ heparinized blood by Ficoll density separation. In some experiments, depletion of Treg was done using magnetic beads. Briefly, PBMC were incubated with anti-CD25 MicroBeads (Miltenyi Biotec) at a final ratio of 1 μL per 10^6 PBMC for 15 min on ice, and then washed. The CD25^{low/neg}- fraction was eluted over a magnetic field and expressed >10-fold less CD25 and 5-fold less Foxp3 protein than nondepleted cells as assessed by flow cytometry (data not shown). In other experiments using fluorescence-activated cell sorting with MoFlow (Dako Cytomation), we purified Treg using the classical labeling CD3, CD4, CD25, and CD127 as previously described (29, 30). More than 90% of sorted fraction expressed Foxp3 (data not shown).

For T-cell proliferation assays, carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled PBMC (2 μmol/L) depleted or not of CD25+ T cells were cultured with anti-CD3 and anti-CD28–coated beads (Dynabeads T cell expander; Dynal Biotech) in 96-well plates according to the manufacturer’s instructions. Proliferation was monitored by fluorescence-activated cell sorting analysis assessing the dilution of the CFSE dye at day 4.

### Table 1. Patients’ characteristics

<table>
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<tr>
<th>Patient</th>
<th>Sex</th>
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<th>Prior therapy</th>
<th>Clinical outcome</th>
<th>Toxicity</th>
<th>Biological response</th>
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</table>

Abbreviations: M/F, male/female; s.c., subcutaneous; DTIC, dacarbazine; PD, progressing disease; SD: stable disease; PR, partial response.

* Tumor response assessed by computed tomography scan and clinical observation before and 1, 2, and 3 mo after tremelimumab injection.

↑ Biological response is the ability of T cells to resist Treg inhibition following tremelimumab therapy.

↑ Serum IP-10 levels were assessed before and 1 mo after tremelimumab injection and compared (↑, increase; =, stable).
For natural killer (NK) cell cytotoxicity assay, $2 \times 10^4$ purified NK cells sorted using Human NK Cell Enrichment kit (StemCell Technologies) were incubated for 4 h with $^{51}$Cr labeled K562 cells at 10:1 E/T ratio. Patient Tregs ($10^4$) sorted using MoFlow were added in some wells. NK cell cytotoxicity was assessed in standard 4-h $^{51}$Cr release assay at 37°C. Spontaneous release was measured in wells that contained labeled target cells alone, and maximum $^{51}$Cr release was obtained by the addition of 2% cetrimide (Sigma Aldrich). Specific cytotoxicity was calculated as:

$$\text{percentage of } ^{51}\text{Cr release} = 100 \times \frac{\text{cpm experimental} - \text{cpm spontaneous release}}{\text{cpm maximum release} - \text{cpm spontaneous release}}.$$

For allogenic proliferation assay, Treg and CD4+CD45RO+ T-cells were sorted from PBMC with a MoFlow cell sorter. Cells were then seeded in 96-well plates and incubated with anti-CD3/CD28 beads (one bead for five effector cells) in the presence or absence of Treg or conventional T cells (Tconv). Proliferation of cells was assessed by adding tritiated thymidine for the last 18 h of a 72-h culture.
Flow cytometry analysis. The following antibodies were used for cytometry analysis: CD69-FITC, CD45-FITC, HLA-DR-FITC, CD3-PE, CD4-PerCp, CD45RO-APC, CD127-PE, CD25-APC, CD152-PC5 (purchased from BD PharMingen); NKp30-PE, NKp46-PE, CD56-PE (from Beckman-Coulter); and CD8-FITC, CD25-PE, CD62L-PE (from Miltenyi Biotec). FoxP3 intracellular staining was done on thawed PBMC with the Human Regulatory T cell staining kit (anti–FoxP3-APC; eBioscience). All labelings were made using four-color fluorescence-activated cell sorting analysis. Fluorescence was acquired on a FACScalibur cytometer with the CellQuest Pro software and analyzed with FlowJo software.

ELISA tests on serum. Serum samples were drawn at day 0 and day 30 post-tremelimumab injection and stored at -80°C before being assayed in a Luminex bioassay investigating a variety of different human cytokines and chemokines.

Statistical analysis. To compare two groups of values, Kruskall-Wallis or Wilcoxon-matched tests were done as appropriate. The correlation between two qualitative variables was assessed by $\chi^2$ test. Survival analyses used the Cox model with the log-rank count. Results were considered significant at 95% confidence when $P < 0.05$. All the statistical analyses were done with the GraphPad Prism software version 5.

Results

Patient characteristics and clinical outcome. Immunomonitoring was done on 10 melanoma patients enrolled at the Institut Gustave Roussy in a multicentric phase II trial aimed at evaluating the efficacy of tremelimumab in advanced melanoma. All patients presented with progressive stage IV melanoma (Table 1), Karnofsky performance status >60%, and no evidence of autoimmune or immunodeficiency disease. Seven of the patients had visceral metastases. All had undergone excision of the primary lesion and received at least one prior chemotherapy but no immunotherapy. Patients received one i.v. injection of 15 mg/kg of anti–CTLA-4 mAb (tremelimumab) every 3 months until progression. Tremelimumab therapy started at least 4 weeks after the last chemotherapy after full hematologic recovery. Upon evaluation at 90 days, two patients with previously progressive visceral lesions experienced nonsignificant tumor shrinkage followed by stable disease lasting more than 3 months (patients 2 and 7); whereas another patient had a partial response with dramatic shrinkage of subcutaneous lesions (patient 8). The other patients presented with progressive disease (Table 1). Concomitantly, grades 1 and 2 adverse events (immune-related adverse events) were observed in four patients, including diarrhea (patient 3), thyroiditis (patient 2) associated with antithyroglobulin antibodies, and skin rashes (patient 7 and 8) associated with pathologic infiltrates of lymphocytes into the superficial dermis (Table 1).

Thus, tremelimumab therapy induced immune-related adverse events in 4 out of 10 patients, associated with clinical benefit as shown by tumor shrinkage or stabilization in three cases.

Tremelimumab-induced amplification of the effector memory T-cell pool. To evaluate the influence of CTLA-4 blockade on lymphocyte activation, we analyzed the number of CD4+ and CD8+ T-cells and their phenotypic expression of HLA-DR and CD45RO molecules. First, patients with stage IV melanomas presented profound lymphopenia (Fig. 1A) in both the CD4 and CD8 compartments (Fig. 1B and C). Decreased
numbers of circulating NK cells were also found in this cohort (Fig. 1D). This lymphopenia is not a consequence of previous chemotherapy as we could observe the same level of lymphopenia in a cohort of patients with previously untreated stage IV melanoma (data not shown). However, although tremelimumab did not promote a significant increase in NK cell numbers (Fig. 1D), tremelimumab markedly induced the augmentation of the memory T-cell pool by day 30 postinjection, up to a normal level (Fig. 1E and F), as well as HLA-DR expression on CD4+ and CD8+ peripheral T-cells (Fig. 1G). In accordance with previous reports (27), patients with melanoma bore higher proportions of Treg defined either as CD3+CD4+CD25+Foxp3+ cells, or as CD3+CD4+CD127- cells (Fig. 2; refs. 29, 30) and higher staining of CTLA-4 (data not shown) than normal volunteers, but tremelimumab did not influence Treg percentages and induced a small but significant increase in their total number (Fig. 2).

Thus, tremelimumab therapy rapidly restored the circulating memory T-cell pool in patients with advanced melanoma but did not influence the Treg nor the NK cell compartment.

**Tremelimumab-induced restoration of TCR-driven T-cell proliferative potential.** We next assessed the effects of in vivo CTLA-4 blockade on the ability of PBMC to undergo TCR-driven proliferation in vitro in the absence of exogenous stimulus. We first observed a drastic reduction of the proliferative potential of PBMC derived from all melanoma patients before tremelimumab therapy compared with healthy volunteers (28 ± 9% cells entered in the cycle after 4 days of stimulation versus 54 ± 10% in healthy volunteers; P < 0.05; Fig. 3). This impaired proliferative function could be mainly assigned to the presence of Treg in the cultured PBMC because depletion of CD4+CD25+ T-cells from melanoma patients’ PBMC restored their TCR-driven proliferative capacities in all cases (Fig. 3). Interestingly, one injection of tremelimumab in vivo was sufficient to enhance TCR-mediated proliferation of PBMC in all individuals (Fig. 3). In addition, the TCR-driven proliferation of PBMC was resistant to the inhibitory effects of autologous Treg in 7 out of 10 patients. These protective effects on conventional T lymphocytes were transient because (a) the proliferation index dropped at day 60 returning to baseline pretherapeutic levels (in the absence of a second tremelimumab injection), (b) the TCR-driven proliferation became subverted by autologous Treg as observed prior to therapy.

Tremelimumab could rapidly but transiently compensate the impaired T-cell responsiveness observed in patients with melanoma but not the innate arm of immune responses.

**Tremelimumab-induced resistance of lymphocytes to Treg inhibitory effects.** To analyze whether tremelimumab curtailed the suppressive functions of Treg or alternatively promoted lymphocyte resistance to the inhibitory effects of Treg, we set up the following experiments. First, patients’ Treg were assessed at day 0, day 30, or day 60 post-tremelimumab injection for their capacity to inhibit NK cell killing of K562 (as previously described in refs. 12, 31). In all patients tested, Treg remained fully inhibitory and functional for at least 60 days despite tremelimumab injection (Fig. 4A). As a control, the same level of inhibition could be obtained when using Treg from healthy volunteers instead of patients’ Treg (data not shown). However, although healthy volunteer and patients’ CD4+CD45RO+ memory T cells were sensitive to the inhibitory effects of allogeneic Treg in the TCR-driven proliferation assay in vitro, 1 month after tremelimumab injection, patients’ CD4+CD45RO+ memory T cells became highly resistant (Fig. 4B).

Thus, tremelimumab was capable of triggering the resistance of patients’ PBMC to the inhibitory effects of autologous Treg in 7 of 10 cases without affecting Treg functions.

**Biological markers correlating with clinical outcome.** We were intrigued to investigate the prognostic value of the tremelimumab-induced resistance of PBMC to the suppressive effects of Treg yet unaltered in their numbers or inhibitory functions in vivo. Therefore, we compared the clinical outcome of the seven patients that became biological responders at 1 month to that of the three patients (patients 1, 6, and 9) who, despite a modest restoration of their T-cell proliferative capacities, remained Treg-sensitive. This latter cohort of three patients exhibited a significantly shorter progression-free survival and overall survival compared with the seven biological responders (Fig. 5A, median progression-free survival was 10.6 in biological responders versus 5.2 in nonresponders; P = 0.001 log-rank test; Fig. 5B, median overall survival was 10.6 in biological responders versus 5.2 in nonresponders; P = 0.035, log-rank test). The search for a
simpler bioassay of prediction led us to the finding that TCR-stimulated T cells in vitro produced higher levels of CXCL10/IP-10 following CTLA-4 blockade. Therefore, we investigated the serum levels of effector cytokines/chemokines that could be produced by relieved or revived effector/memory T lymphocytes after tremelimumab therapy. Among the 29 cytokines and chemokines tested, the only one that was correlated to the biological response observed with tremelimumab was IP-10. Indeed, the IP-10 serum levels were significantly increased in the seven biological responders—patients for whom PBMC became resistant to the antiproliferative effects of Treg—while remaining stable for the three nonbiological responders. Although due to the low number of patients, this data needs to be confirmed in a larger trial.

Discussion

Our immunomonitoring shows an immunologic benefit of the CTLA-4 blockade in patients with advanced stage IV melanoma. We showed that tremelimumab could not only restore the numbers and proliferative functions of circulating effector memory T lymphocytes after tremelimumab therapy. Among the 29 cytokines and chemokines tested, the only one that was correlated to the biological response observed with tremelimumab was IP-10. Indeed, the IP-10 serum levels were significantly increased in the seven biological responders—patients for whom PBMC became resistant to the antiproliferative effects of Treg—while remaining stable for the three nonbiological responders. Although due to the low number of patients, this data needs to be confirmed in a larger trial.

Fig. 4. PBMC becomes resistant to the inhibitory effects of Treg after CTLA-4 blockade. A, sorting of CD4+CD25bright T-cells from patients’ PBMC was done on days 0, 30, and 60 to test the inhibitory capacity of patients’ Treg on allogeneic NK cell lytic activity at various time points of the therapy (experimental setting, one representative experiment; left). B, sorting of CD4+CD25bright allogeneic T-cells from healthy volunteers’ PBMC allowed to purify Treg that were used to inhibit the TCR-driven proliferative potential of patients’ memory T cells (sorted as CD3+CD4+45RO+ cells) purified at various time points of the therapy (experimental setting, one representative experiment; left). All experiments were done twice in five biological responder patients with identical results. Statistical analyses used Kruskall-Wallis method.
signal necessary to fully activate T cells (34). Therefore, a direct link between attenuated CTLA-4 signals in Treg cells and improved tumor immunity remains to be established. Instead, Treg cells and the CTLA-4 signaling pathway may represent independent and synergistic regulatory mechanisms for the suppression of T-cell activation and antitumor immune responses in vivo (35). Moreover, chronic exposure to CTLA-4 mAb for 2 weeks in mice had no effect on the subsequent suppressive capacity of Treg ex vivo, this did not deplete the normal repertoire of Treg but rather contributed to the amplification of self-reactive Treg in the tumor-draining lymph node, presumably by removing the constraints on the production of interleukin-2 and cyclins (36–39).

Attempts at reversing immunosuppression in cancer patients have been actively pursued alone or in combination with immunotherapy or tumor vaccines. The use of denileukin difitox (Ontak), a fusion protein consisting of interleukin-2 fused to the enzymatically active domains of diphtheria toxin, had variable clinical efficacy and no biological effects on Foxp3 mRNA expression in CD4+ T-cells (16), although potentially toxic on CD25+ conventional effector T counterparts (40). Treatment with a low dosage of cyclophosphamide has been used for decades to reduce immunosuppression (11). Although current experimental data supports the idea that low doses of this alkylating agent could be clinically used to reduce Treg-mediated suppressive activity (41–43). We reported that a 1-month oral cyclophosphamide regimen induced a selective reduction of Treg numbers leading to the restoration on T cell and NK cell effector functions (12). Therefore, CTLA-4 blockade and metronomic cyclophosphamide may represent independent and synergistic strategies to revive chronically stimulated tumor/self-reactive T cells in cancer patients.

Our data showing a rapid but transient resistance of peripheral T cells to the inhibitory effects of Treg after one dose of tremelimumab prompted us to suggest that anti–CTLA-4 mAbs should be administered monthly instead of quarterly. Indeed, experimental studies using chronic CTLA-4 blockade in tumor-bearing hosts showed the cell autonomous intrinsic effects of anti–CTLA-4 mAb on Treg, i.e., an enhanced Treg proliferation and accumulation in tumor-draining lymph nodes as a result of the relief of the CTLA-4 inhibitory pathway (36). In such as much as the same authors could show that the balance between effector T cells and Treg in tumor beds became favorable for the host when chronic CTLA-4 blockade was combined with granulocyte macrophage colony-stimulating factor–based vaccination, one should assume that chronic CTLA-4 blockade should be associated with a strategy which will promote the differentiation of tumor-reactive T lymphocytes invading the tumors. Consequently, as suggested by Quezada et al., upon cessation of therapy, the regulatory activities of the amplified Treg will be retained to help in the control of possible adverse events induced by CTLA-4 blockade (36).

Finally, CTLA-4 blockade is not associated with a high immediate response rate (44, 45) but nevertheless seems to be a very promising therapeutic approach for patients with melanoma because of the frequent long-lasting response, even in patients with high tumor burden. If we are not able to identify early biological markers of activity for these drugs, we might fail to realize their true therapeutic value. Our findings allow us to postulate that patients who will most likely benefit from long-term exposure to anti–CTLA-4 mAb will be those whose T cells become resistant to Treg and produce IP-10 at 1 month.

The hypothesis that resistance of PBMC to Treg could be acquired following one dose of anti–CTLA-4 mAb may be a valuable prognostic factor predicting the clinical benefit of continuous therapy with tremelimumab opens new avenues for further investigations and warrants study in a larger cohorts of patients.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
References

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