Analysis of the Cellular Mechanism of Antitumor Responses and Autoimmunity in Patients Treated with CTLA-4 Blockade

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Analysis of the Cellular Mechanism of Antitumor Responses in Patients Treated with CTLA-4 Blockade

Ajay V. Maker, Peter Attia, and Steven A. Rosenberg

We have demonstrated previously that the administration of CTLA-4 blockade has mediated objective cancer regression and autoimmunity in patients with metastatic melanoma. To explore the mechanism of these in vivo effects, we have studied the changes in lymphocyte phenotype and function in patients receiving anti-CTLA-4 Ab (MDX-010). Patients with stage IV melanoma or renal cell cancer were treated every 3 wk with an anti-CTLA-4 Ab with or without peptide immunization. Pheresis samples were analyzed using flow cytometry to determine lymphocyte cell surface markers. Gene expression analyses and proliferation assays were conducted on purified T cell subsets. Anti-CTLA-4 Ab did not inhibit the suppressive activity of CD4+/CD25+ cells in vitro or in vivo. In addition, there was no decrease in the expression of CD4+CD25+ cells in whole PBMC, nor a decrease in Foxp3 gene expression in the CD4+ or CD4+CD25+ purified cell populations posttreatment. The percentage of CD4+, CD8+, CD4+CD25+, and CD4+CD25- T cells in PBMC expressing the activation marker HLA-DR increased following anti-CTLA-4 Ab administration. Therefore, our results suggest that the antitumor effects of CTLA-4 blockade are due to increased T cell activation rather than inhibition or depletion of T regulatory cells. The Journal of Immunology, 2005, 175: 7746–7754.

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was defined as the disappearance of all lesions for $\geq 1$ mo. Patients not achieving either a partial or complete response were nonresponders. Treatment, autoimmunity, and response characteristics for patients treated with anti-CTLA-4 Ab are summarized in Table I. Patients F and G provided cells for study but were not enrolled in anti-CTLA-4 treatment protocols.

**Lymphocyte preparation**

CD4+ cells were isolated from whole PBMC by negative selection according to manufacturer’s instructions (CD4-negative isolation kit; Dynal Biotech). For suppression assays, CD4+ cells were further purified into CD25+ and CD25− subpopulations with the Dynal T regulatory kit, and accessory cells were T cell depleted from autologous PBMC using the Dynal CD3+ depletion kit (Dynal Biotech) according to the manufacturer’s instructions. For RT-PCR gene analysis of CD4+ subpopulations, CD4+-purified cells were sorted using a FACSVantage (BD Biosciences) into CD4+CD25+ “high” and CD4+CD25− “low” cells, defined as the upper and lower 10% of cells staining for CD25 (CD25-PE; BD Biosciences).

**Flow cytometry**

Flow cytometry was used to evaluate surface expression of selected T cell markers. Whole PBMC were washed in ice-cold PBS with 0.5% BSA and incubated with appropriate fluorochrome-labeled Abs and relevant isotype controls (CD4-FITC (clone SK3), CD8-FITC (clone SK1), CD25-PE (clone 2A3), CD69-APC (clone L78), and HLA-DR-APC (clone L243); BD Biosciences). Sample fluorescence was acquired and analyzed with a FACSCalibur and CellQuest software (BD Biosciences).

**RNA isolation, cDNA synthesis, and real-time PCR**

RNA isolation and cDNA synthesis were performed in batches containing pre- and posttreatment samples to minimize variability. Total RNA was isolated using an RNAeasy minikit (Qiagen) and was reverse transcribed to cDNA using the ThermoScript RT-PCR system (Invitrogen Life Technologies) according to the manufacturers’ instructions.

Levels of $\beta$-actin and Foxp3 gene expression were assessed with the ABI Prism 7700 Sequence Detection System (PerkinElmer). For $\beta$-actin, the forward primer, 5′-GGCACCAAGCACATTGAAG-3′, reverse primer, 5′-GCCGATCCACAGCTTACACT-3′, and probe, 5′-6-FAM TCAAGATCATGTCCTCCTGACGAGC-TAMRA-3′, were used. For Foxp3, the combined probe reagent (Assay-on-Demand gene expression assay; Applied Biosystems) was used. cDNA was analyzed in a 25-μl mixture containing TaqMan 2× Universal MasterMix (Applied Biosystems) and respective primers and probes at optimized concentrations. All samples were run in duplicate. Foxp3 copy numbers were calculated from a linear regression of known standards that were included in each RT-PCR run. All samples were run with the same set of standards, except samples from patients 5–10, where a different set of internal standards were used. Thermal cycler parameters were 2 min at 50°C, 10 min at 95°C, and 40 cycles of denaturation at 95°C for 15 s with annealing/extension at 60°C.

**Suppression assay**

The proliferative potential of CD4+CD25+ subpopulations were assessed in coculture assays, CD4+CD25−, CD4+CD25+ (1.0 × 105), or both cell subpopulations (1:1; 1.0 × 106 each per well) were cultured with T cell-depleted irradiated accessory cells (5 × 105/well). For polyclonal activation, cells were cultured with 1.0 μg/ml soluble anti-CD3. One microcurie of [3H]thymidine per well was added during the final 18 h of a 5- or 6-day culture, and proliferation was measured using a scintillation counter. These culture conditions and reagent concentrations were optimized for sensitivity in prior experiments. Cultures were performed in 96-well round-bottom plates in sextuplets. Suppression of CD4+CD25− proliferation by CD4+CD25+ cells was calculated using the formula: percentage of suppression = (1 − (1/1 – CD25+/CD25−)) × 100%.

**Results**

**Effects of in vitro CTLA-4 blockade on the function of T regulatory cells**

To test whether CTLA-4 blockade could abrogate the suppressive activity of CD4+CD25+ regulatory cells in vitro, coculture assays were performed using pretreatment cells in the presence and in the absence of anti-CTLA-4 Ab. Zero, 10, or 100 μg/ml anti-CTLA-4 Ab was added on day 0 to cultures for all patients, except patient G where Ab was added on days 0, 2, and 4. A typical example of the coculture suppression assay (patient F) is shown in Table II, and a summary of the suppression results on all five patients is shown in Fig. 1. The suppression of CD4+CD25+ proliferation by coculture with CD4+CD25− cells was not significantly affected by the addition of anti-CTLA-4 Ab in vitro.

**Effects of in vivo CTLA-4 blockade on the expression of T regulatory cells**

Pheresis samples from four patients treated in vivo with escalating doses of anti-CTLA-4 Ab every 3 wk were evaluated using flow cytometry for phenotype expression before treatment and after receiving two doses each at 3, 5, and 9 mg/kg. The percentage of PBMC expressing CD4+CD25+ was analyzed in the total CD4+ population by gating on the isotype control (Fig. 2, lower panel). In addition, the CD4+CD25+ “high” population, thought to contain a higher proportion of regulatory cells (27, 33), was analyzed by gating the upper 10% of CD4+CD25+ cells in the pretreatment sample and using that gate to evaluate subsequent posttreatment samples (Fig. 2, upper panel). The administration of anti-CTLA-4 Ab in vivo did not appear to cause a consistent change in the percentage of CD4+ lymphocytes expressing CD4+CD25+ post-treatment compared with pretreatment when analyzed in both the total CD4+ and the “high” CD25+ subpopulations (Fig. 2).

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**Table I. Patient treatments, autoimmunity, and responses**

<table>
<thead>
<tr>
<th>Anti-CTLA-4 Ab Treatment</th>
<th>Patient</th>
<th>Dose level (mg/kg) (no. of doses)</th>
<th>Grade III/IV Autoimmunity</th>
<th>Response</th>
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<tbody>
<tr>
<td></td>
<td>A</td>
<td>3.0 (12)</td>
<td></td>
<td>NR</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>3.0 (2), 5.0 (2)</td>
<td></td>
<td>NR</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>5.0 (6)</td>
<td></td>
<td>PR</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>3.0 (2), 5.0 (2), 9.0 (6)</td>
<td></td>
<td>PR</td>
</tr>
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<td>E</td>
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<tr>
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<td>1</td>
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<td></td>
<td>PR</td>
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<tr>
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<td>2</td>
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<td>Hypophysitis</td>
<td>PR</td>
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<td>3.0 (2), 5.0 (2), 9.0 (2)</td>
<td>Hypophysitis</td>
<td>NR</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3.0 (2), 5.0 (2), 9.0 (2)</td>
<td></td>
<td>PR</td>
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<td>Colitis, dermatitis</td>
<td>PR</td>
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<td>6</td>
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<td>Hypophysitis</td>
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<td></td>
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<td>Colitis</td>
<td>PR</td>
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<td></td>
<td>8</td>
<td>3.0 (1), 1.0 (1)</td>
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<td></td>
<td>9</td>
<td>3.0 (1), 1.0 (1)</td>
<td></td>
<td>PR</td>
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<tr>
<td></td>
<td>10</td>
<td>3.0 (1), 1.0 (1)</td>
<td></td>
<td>NR</td>
</tr>
</tbody>
</table>

* Patients A and E presented with metastatic renal cell cancer. All other patients treated had metastatic melanoma. Patients received the fully human anti-CTLA-4 Ab as an i.v. bolus over 90 min every 3 wk. Pheresis samples were obtained 3 wk after each dose course. CR, complete response; NR, no response; and PR, partial response.

**Table II. In vitro effect of CTLA-4 blockade on the suppressive function of CD4+CD25+ cells**

<table>
<thead>
<tr>
<th>CD25−</th>
<th>CD25+</th>
<th>% suppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accessory cells</td>
<td>103 ± 17</td>
<td>101 ± 12</td>
</tr>
<tr>
<td>CD25−</td>
<td>5936 ± 1130</td>
<td>3708 ± 1592</td>
</tr>
<tr>
<td>CD25+</td>
<td>330 ± 25</td>
<td>356 ± 77</td>
</tr>
<tr>
<td>1:1</td>
<td>2608 ± 686</td>
<td>1890 ± 489</td>
</tr>
</tbody>
</table>

* The effect of CTLA-4 blockade on CD4+CD25− cell inhibition of CD4+CD25+ cell proliferation was measured in a suppression assay. Cell populations were cocultured in a 1:1 ratio for 5 days and pulsed with [3H]thymidine 18 h prior to harvest in representative patient F.
Effects of in vivo CTLA-4 blockade on Foxp3 gene expression

Foxp3 is highly expressed in CD4<sup>+</sup>CD25<sup>+</sup> T regulatory cells and has been described as a lineage specification factor for these cells (34). Thus, CD4<sup>+</sup> cells were purified from the PBMC of the four patients from whom data were obtained for Fig. 2, and Foxp3 gene expression was determined relative to the expression of β-actin. There was no consistent difference in Foxp3 expression in CD4<sup>+</sup> cells as a function of Ab dose in patients treated with 3, 5, or 9 mg/kg anti-CTLA-4 Ab, although there was a trend toward higher relative Foxp3 levels at some anti-CTLA-4 doses compared with pretreatment levels in each patient (relative Foxp3 levels at 3, 5, and 9 mg/kg: +2.96, p = 0.24; +3.26, p = 0.18; +4.61, p = 0.05; not corrected for multiple analyses). (Fig. 3).

CD4<sup>+</sup> lymphocyte populations from an additional six patients treated at 3 mg/kg anti-CTLA-4 Ab were studied before and after treatment to determine relative Foxp3 gene expression in purified CD4<sup>+</sup> cells. Table III shows Foxp3 levels relative to β-actin in PBMC from all 10 patients before and after receiving anti-CTLA-4 Ab. Eight of these 10 patients revealed an increase in relative Foxp3 gene expression posttreatment, again suggesting that this Ab may have an agonistic effect in vivo on Foxp3-expressing cells.

CD4<sup>+</sup> lymphocytes obtained before and after in vivo anti-CTLA-4 treatment from patients 5–10 were further sorted using flow cytometry to purify the upper 10% of cells expressing CD4<sup>+</sup>CD25<sup>+</sup> ("high") and the lower 10% of cells expressing CD4<sup>+</sup>CD25<sup>-</sup> ("low") (Fig. 4, right panel). Patient 7 was not evaluated because of insufficient cell yield postseparation. Foxp3 gene expression relative to β-actin in these highly purified CD25<sup>+</sup> and CD25<sup>-</sup> subpopulations was determined. As expected, very low levels of Foxp3 gene expression were seen in CD25<sup>-</sup> low populations compared with the CD25<sup>+</sup> high populations. There was a significant increase in relative Foxp3 expression in CD25<sup>+</sup> high population (p = 0.05, paired t test) (Fig. 4).

Results using direct staining for Foxp3 or GITR are inconsistent
in the literature, and we were not able to obtain reproducible results with commercially available Abs.

Effects of in vivo CTLA-4 blockade on the function of T regulatory cells

To test whether CD4+CD25+ cells from patients treated with CTLA-4 blockade maintained suppressive function posttreatment, lymphocytes from patients treated with anti-CTLA-4 Ab in vivo were evaluated in coculture suppression assays. Lymphocytes from five patients for whom fresh pheresis samples were available were bead purified into CD4+CD25+ and CD4+CD25− cell populations. All patients had received multiple doses of anti-CTLA-4 Ab and were apheresed after the last dose (Table I). These posttreatment CD4+CD25+ cells displayed a persistent ability to suppress proliferation of CD4+CD25− cells, ranging from 49 to 84%, thus indicating that CTLA-4 blockade in vivo did not eliminate circulating lymphocytes with suppressive function (Fig. 5).

Effects of in vivo CTLA-4 blockade on the activation of T cells

To evaluate the influence of CTLA-4 blockade on the activation state of lymphocytes, whole PBMC from four patients who received the highest doses of anti-CTLA-4 Ab and were apheresed after the last dose (Table I). These posttreatment CD4+CD25+ cells displayed a persistent ability to suppress proliferation of CD4+CD25− cells, ranging from 49 to 84%, thus indicating that CTLA-4 blockade in vivo did not eliminate circulating lymphocytes with suppressive function (Fig. 5).

Table III. Foxp3 gene expression relative to β-actin in CD4+ cells from patients treated at 3 mg/kg anti-CTLA-4 Ab

<table>
<thead>
<tr>
<th>Patient</th>
<th>Pretreatment</th>
<th>After 3 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.80 ± 0.26</td>
<td>6.40 ± 0.01</td>
</tr>
<tr>
<td>2</td>
<td>4.10 ± 0.27</td>
<td>5.56 ± 0.02</td>
</tr>
<tr>
<td>3</td>
<td>5.08 ± 4.51</td>
<td>13.90 ± 0.90</td>
</tr>
<tr>
<td>4</td>
<td>15.33 ± 0.41</td>
<td>17.29 ± 0.96</td>
</tr>
<tr>
<td>5</td>
<td>0.16 ± 0.01</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td>6</td>
<td>0.10 ± 0.00</td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td>7</td>
<td>0.17 ± 0.01</td>
<td>0.23 ± 0.01</td>
</tr>
<tr>
<td>8</td>
<td>0.08 ± 0.01</td>
<td>0.17 ± 0.00</td>
</tr>
<tr>
<td>9</td>
<td>0.17 ± 0.01</td>
<td>0.27 ± 0.00</td>
</tr>
<tr>
<td>10</td>
<td>0.26 ± 0.02</td>
<td>0.25 ± 0.00</td>
</tr>
</tbody>
</table>

CD4+ cells is shown in Fig. 6. A trend toward an increase in HLA-DR expression was seen on CD4+ lymphocytes in patients tested after receiving 3 mg/kg (t = 8.55, p = 0.07, paired t test), 5 mg/kg (t = 12.78, p = 0.08, paired t test), and 9 mg/kg (t = 13.12, p = 0.11, paired t test) anti-CTLA-4 Ab. A trend toward increase in HLA-DR expression was also seen on CD8+ lymphocytes in patients tested after receiving 3 mg/kg (t = 9.69, p = 0.12, paired t test), 5 mg/kg (t = 14.35, p = 0.02, paired t test), and 9 mg/kg (t = 15.46, p = 0.03, paired t test) anti-CTLA-4 Ab, with the exception of patient 1, in whom CD8+HLA-DR+ levels remained stable at 3 mg/kg before increasing (Fig. 7).

Analysis of HLA-DR expression on the CD4+CD25+ and CD4+CD25− subpopulations in the same patients was also performed. Although there was no clear dose-response effect in the patients tested, there was a trend toward increased HLA-DR expression on both CD4+CD25+ and CD4+CD25− subpopulations (Fig. 8). There was no significant change in CD69 expression on CD4+ or CD8+ lymphocytes after treatment with CTLA-4 blockade (data not shown).

These studies involved a small number of patients. Thus, we reviewed phenotypic markers of activation that were analyzed previously in 30 patients before and after treatment with anti-CTLA-4 Ab in two separate dose cohorts in the Surgery Branch, National Cancer Institute. A small but significant increase in the percentage of HLA-DR expression on CD4+ (p = 0.0001 and p = 0.0004, paired t test) and CD8+ (p = 0.015 and p = 0.04, paired t test) lymphocytes was seen in posttreatment samples compared with pretreatment in both studies. There was also a significant increase in CD45RO expression (p = 0.009 and p = 0.04, paired t test) and a trend toward a decrease in CD25 expression (p = 0.047 and p = 0.13, paired t test) on CD4+ cells after in vivo treatment with anti-CTLA-4 Ab (23, 24) (Table IV).

Discussion

The administration of anti-CTLA-4 Ab to patients with metastatic melanoma induced durable objective clinical responses that were highly associated with the induction of autoimmune side effects (23, 24). However, the mechanism responsible for tumor regression and autoimmunity was unclear, although two dominant hypotheses have been proposed. CTLA-4 is constitutively expressed...
FIGURE 4. Relative Foxp3 levels in CD4⁺CD25⁺ and CD4⁺CD25⁻ cells from patients treated with CTLA-4 blockade. Lymphocytes from patients treated with at least one dose of anti-CTLA-4 Ab at 3 mg/kg were highly purified by flow cytometry into CD4⁺CD25⁺ high and CD4⁺CD25⁻ low populations. Cell acquisition was accomplished by flow sorting the upper 10% of CD4⁺CD25⁺ cells and the lower 10% of CD4⁺CD25⁻ cells (right panel). Foxp3 gene expression relative to β-actin was higher in CD25⁺ compared with CD25⁻ cells and significantly increased in CD4⁺CD25⁺ cell subpopulations posttreatment with anti-CTLA-4 Ab compared with pretreatment (p = 0.05, paired t test).

FIGURE 5. Suppression of CD25⁻ cell proliferation by CD25⁺ cells in patients after in vivo CTLA-4 blockade. Fresh pheresis samples from five patients were bead purified into CD4⁺CD25⁺ and CD4⁺CD25⁻ subpopulations for use in suppression assays. Samples were taken from patients after receiving at least four doses of anti-CTLA-4 Ab. CD4⁺CD25⁺ cells displayed a persistent ability to suppress proliferation of CD4⁺CD25⁻ cells after in vivo treatment with CTLA-4 blockade. *, Cultures were pulsed with [³H]thymidine on day 5 and harvested on day 6. All other cultures were plated with 10,000 cells/population. †, A total of 5000 cells/population was cultured. All other cultures were plated with 10,000 cells/population.
on CD4+CD25+ T regulatory cells (25, 26); hence, the administration of an anti-CTLA-4 Ab may lead to the depletion or inhibition of these regulatory cells, with a resultant increase in the activity of effector T cells. Alternatively, CTLA-4 engagement imparts an inhibitory signal to the T cell (5), and the blockade of CTLA-4 may directly tip the balance toward T cell effector function. These two possibilities have been studied in this report.

We first addressed the impact of anti-CTLA-4 Ab on CD4+CD25+ T cell regulatory cell function in vitro using a coculture suppression assay. This proliferation assay was developed to functionally identify murine and human CD4+CD25+ T regulatory cells by their ability to suppress the proliferation of CD4+CD25− cells (26, 27, 33, 35, 36). Physiologic concentrations of anti-CTLA-4 Ab cocultured with purified populations of CD4+CD25+ lymphocytes did not abolish their ability to suppress the proliferation of CD4+CD25+ human lymphocytes (Table II and Fig. 1).

We next studied the phenotype and function of PBMC obtained from patients who received multiple doses of anti-CTLA-4 Ab in vivo. There was no consistent dose-response effect in the percentage of cells expressing CD4+CD25+ posttreatment compared with pretreatment in either the total CD4+ fraction or the CD25+ “high” fraction, which may be more representative of CD4+ cells with regulatory function (27, 33). These studies suggested that the administration of anti-CTLA-4 Ab did not deplete circulating CD4+CD25+ cells. This was further evaluated by assessing the effect of CTLA-4 blockade on a specific genetic marker of regulatory T cells. Foxp3 expression is restricted to CD4+CD25+ regulatory cells in mice, and forced gene expression of Foxp3 by viral transduction can impart regulatory activity to normal T cells (37–39). Furthermore, mutations in the murine foxp3 gene cause a fatal autoimmune disorder, termed scurvy, and a similar disease, immune dysregulation, polyendocrinopathy, X-linked syndrome, occurs in humans carrying a mutation in the human Foxp3 gene, which shares 86% similarity to the murine homologue (40–45). Although the role of Foxp3 in defining T regulatory cells in mice is clearer than in humans (34), its gene expression is a surrogate for identification of human T regulatory cells. In CD4+ cells, relative...
Foxp3 expression did not consistently change in patients treated with anti-CTLA-4 Ab at multiple dose levels and, in fact, increased at some dose levels. In patients evaluated after administration of 3 mg/kg Ab, there appeared to be an increase in Foxp3 gene expression that was evident in patients that showed a clinical antitumor response and in those that did not, regardless of whether the patient experienced grade III/IV autoimmunity. We then further purified this CD4+ population into CD4+CD25+ and CD4+CD25− subpopulations by flow sorting for the highest and lowest expressors of CD25 and evaluated Foxp3 gene expression in these subpopulations. Relative Foxp3 expression in the CD4+CD25+"high" population significantly increased in posttreatment compared with pretreatment CD4+ cells independent of patient clinical antitumor response or autoimmunity status. Thus, assessment of Foxp3 expression in either the CD4+ or the CD4+CD25− lymphocyte populations suggested that administration of Ab to CTLA-4 may be agonistic to T regulatory cells, rather than antagonistic. It should be mentioned that all measurements were made on cells taken 3 wk after the dose of anti-CTLA4 Ab.

Functional assays represent the most direct method for assessing the presence of T regulatory cell-suppressive ability. Therefore, we next analyzed lymphocytes from patients treated with in vivo anti-CTLA-4 Ab, for whom fresh pheresis samples were available, using in vitro suppression assays. These studies revealed that circulating CD4+CD25+ cells obtained after in vivo treatment with anti-CTLA-4 Ab maintained the ability to suppress CD4+CD25− cells by 49–84%. This suppression was evident in PBMC obtained from patients whether or not they experienced clinical response to

<table>
<thead>
<tr>
<th>Dose Schedule (mg/kg)</th>
<th>Reference</th>
<th>Patients Analyzed</th>
<th>% ΔHLA-DR</th>
<th>% ΔCD25</th>
<th>% ΔCD45RO</th>
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<tbody>
<tr>
<td>3 + 3</td>
<td>23</td>
<td>14</td>
<td></td>
<td></td>
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<tr>
<td>3 + 1</td>
<td>24</td>
<td>16</td>
<td>+16.8*</td>
<td>+10.0*</td>
<td>−5.4</td>
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<tr>
<td>3 + 3 + 5 + 5 + 9 + 9</td>
<td>Current study</td>
<td>4</td>
<td>+9.8*</td>
<td>+2.3*</td>
<td>−4.7*</td>
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<tr>
<td>3 + 3 + 5 + 5 + 9 + 9</td>
<td>Current study</td>
<td>4</td>
<td>+13.1</td>
<td>+15.5*</td>
<td>−7.2</td>
</tr>
</tbody>
</table>

* Lymphocytes from patients treated on various CTLA-4 blockade protocols in the Surgery Branch were analyzed by FACS for phenotypic markers of activation in posttreatment samples compared with pretreatment samples. ND, not done. *, p < 0.05, paired t test.
treatment. Therefore, we concluded that in vivo CTLA-4 blockade did not abolish the suppressive function of T regulatory cells. However, it should be emphasized that the T regulatory subset was incompletely defined at this time, and expression of the Foxp3 gene may not be an adequate marker. It remains possible that a subset of tumor-specific T regulatory cells were depleted. Because CTLA-4 blockade did not inhibit the expression or function of CD4+CD25+ cells, it seemed very unlikely that the antitumor and autoimmune effects seen in patients were due to enhanced activity of effector T cells secondary to the absence of T regulatory-mediated suppression. Therefore, we turned our attention to an examination of the activation status of T cells from patients that responded to Ab treatment and/or experienced autoimmunity. HLA-DR expression, a T cell activation marker (46, 47), appeared to increase in CD4+ and CD8+ cells obtained from patients after treatment with escalating doses of anti-CTLA-4 Ab. HLA-DR expression also tended to increase in both the CD4+CD25+ and CD4+CD25− cell populations, implying a pan-lymphocytic activation. CD25 and CD69 expression did not change significantly in these patients, although the window in which these phenotypic markers of activation would be up-regulated may have been missed at the time points measured (46, 47).

This data was consistent with our prior analyses of patients treated with anti-CTLA-4 Ab and tested at similar time points, where we observed an increase in HLA-DR expression on CD4+ and CD8+ lymphocytes with antitumor activity. The low levels of antitumor T cells in these patients precluded our ability to measure DR expression specifically on these cells. As reported earlier there was a correlation between increased levels of autoreactive CD4+CD25− T cells after Ab administration in both responders and nonresponders with or without autoimmunity (23, 24).

It should be emphasized that the measurements of T regulatory function, Foxp3 expression, and phenotypic analyses reported here were performed on circulating lymphocytes and not specifically on lymphocytes with antimicrobial activity. The low levels of antimicrobial T cells in these patients precluded our ability to measure DR expression specifically on these cells. As reported earlier there was no apparent impact of anti-CTLA-4 Ab administration on the ability to generate antimicrobial precursors following peptide immunization (23).

Thus, the present data suggest that the antitumor and autoimmune effects seen after CTLA-4 blockade using an anti-CTLA-4 Ab are not due to inhibition or depletion of T regulatory cells but rather appear to act through direct activation of CD4+ and CD8+ effector cells.

**Disclosures**

The authors have no financial conflict of interest.

**References**


