Preexisting Levels of CD4 T Cells Expressing PD-1 Are Related to Overall Survival in Prostate Cancer Patients Treated with Ipilimumab

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Abstract

Cytotoxic T-lymphocyte–associated antigen-4 (CTLA-4) blockade can induce tumor regression and improved survival in cancer patients. This treatment can enhance adaptive immune responses without an exogenous vaccine, but the immunologic biomarkers associated with improved clinical outcome in cancer patients are not fully established. A phase Ib trial in patients with metastatic, castration-resistant prostate cancer was performed combining ipilimumab with sargramostim (GM-CSF). In addition to evaluating ipilimumab dose, patients were followed clinically for response and overall survival, and for immunomodulation of circulating T cells. PSA declines of ≥50% and radiographic responses were observed at doses of ≥3 mg/kg/dose. Timing of clinical responses could be either immediate or delayed. Durable responses were also observed off treatment. A subset of patients experienced long-term survival with or without objective clinical responses. The relationship between T-cell phenotype in peripheral blood and overall survival was examined retrospectively. We found that the treatment induced an increase in the levels of CD4+ effector T (Teff) cells, regulatory T cells, PD-1+ CD4+ Teff cells, and PD-1+ CD8 T cells. However, these increased levels were not associated with overall survival. Instead, low pretreatment baseline levels of PD-1+ CD4+ Teff cells were found to correlate with longer overall survival. Furthermore, baseline levels of PD-1+ CD4+ Teff cells from patients with shorter overall survival were higher than from cancer-free male control subjects. These results suggest that preexisting expression of immunologic checkpoint marker PD-1 on CD4 Teff cells may help identify patients that may benefit from ipilimumab treatment. Cancer Immunol Res; 1:9. ©2015 AACR.

Introduction

Cytotoxic T-lymphocyte–associated antigen-4 (CTLA-4) is an immune checkpoint receptor expressed on T cells that provides inhibitory signaling following activation of naïve and memory T cells to maintain immune homeostasis (1, 2). Blocking CTLA-4 may serve to remove this inhibition of T-cell responses in the setting of an immunosuppressive tumor environment, thereby leading to immune responses against the tumor. In animal models, CTLA-4 blockade with monoclonal antibodies can enhance T-cell responses and may also deplete intratumoral regulatory T cells (Treg) enabling tumor regression (3, 4).

Ipilimumab is a fully humanized monoclonal antibody targeting CTLA-4 that is FDA approved for the treatment of unresectable or metastatic melanoma at 3 mg/kg/dose (5). In two phase III studies in advanced melanoma, ipilimumab was shown to significantly prolong overall survival (OS; refs. 5, 6). In the pivotal clinical trial, melanoma patients were treated with ipilimumab plus gp100 (a melanoma peptide vaccine), ipilimumab alone, or gp100 alone (5). The median OS durations were 10.0, 10.1, and 6.4 months, respectively. Although improvement in median OS was modest, a subset of patients was observed in these and other melanoma clinical trials to have a durable long-term survival benefit (7, 8). Notably, long-term survival can occur without accompanying objective tumor response. Improved OS was also observed with ipilimumab in combination with dacarbazine versus dacarbazine plus placebo in a phase III clinical trial of patients with metastatic melanoma who received no prior treatment (11.2 months vs. 9.1 months; ref. 6). In addition, treatment with ipilimumab plus sargramostim (GM-CSF) resulted in improved median OS and lower toxicity compared with ipilimumab alone (17.5 months vs. 12.7 months) in a phase II clinical trial in patients with unresectable melanoma (9).

In a phase III clinical trial for patients with metastatic castration-resistant prostate cancer (mCRPC) who had received prior chemotherapy, the results showed no significant difference in OS between treatments with 10 mg/kg of ipilimumab versus placebo following local radiotherapy to a metastatic site (10). The median OS was 11.2 months for the ipilimumab-treated group and 10.0 months for the placebo group. However, it was observed that the HR decreased over time favoring the ipilimumab arm, suggesting that ipilimumab treatment is associated with better survival at later time points. HR was 1.46 (95% confidence

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

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interval (CI), 1.10–1.95) for 0 to 5 months and 0.6 (95% CI, 0.43–0.86) for beyond 12 months.

Here, we present survival outcomes along with an updated ipilimumab dose evaluation of 42 mCRPC patients treated with a combination of ipilimumab and sargramostim in a phase Ib trial (11). As of censor date of the trial on October 21, 2014, all except 2 patients have died. Clinical responses, designated as ≥50% PSA declines from the level at start of treatment or objective tumor responses, were not observed at dose levels less than 3 mg/kg of ipilimumab. A subset of patients experienced long-term survival with and without clinical responses. The relationship between survival and immune subsets was evaluated in an exploratory level with patients from the 3-mg/kg and above dose groups. We found that improved OS was correlated with baseline expression levels of programmed death-1 (PD-1) on CD4 effector T (Teff) cells.

Materials and Methods

Clinical trial

Results for the lower-dose levels up to 3 mg/kg/dose for this phase 1b trial have been described (11). Inclusion criteria for patients were histologically proven metastatic castration-resistant adenocarcinoma of the prostate with progression as defined by the PSA Working Group Consensus Criteria (12), and no prior treatment with steroids, chemotherapy, or immunotherapy. For patients with measurable disease, progressive CRPC was defined as a 50% rise in PSA above the nadir or back to baseline, whichever was lower, on at least two consecutive measurements at least 2 weeks apart, or the appearance of one or more new lesions occurring more than 1 month after the initiation of therapy. Best PSA decline was the maximum percentage (%) decline from initial PSA levels before treatment. OS was calculated from date of first treatment to date of death (n = 40) or censor date of trial on October 21, 2014 (n = 2).

Flow cytometry

Staining for flow cytometry was carried out on cryopreserved peripheral blood mononuclear cells (PBMC). In addition to study participants, PBMCs were also obtained from men undergoing prostate cancer screening without a subsequent diagnosis of cancer (cancer-free male controls). Cells were incubated with DNAse I (15 U/mL; Roche Diagnostics) for 30 minutes at 30˚C and washed twice with FACS buffer (PBS with 2% FBS and 2 mmol/L EDTA). Cell surface staining was performed in FACS buffer for 30 minutes at 4˚C. Intracellular FoxP3 was performed using the FoxP3 fix/perm buffer set (Biolegend, Inc.) according to the manufacturer’s protocol. The following anti-human antibodies were used: (Alexa Fluor 700)-CD3 (clone HIT3a), (Brilliant violet 570)-CD4 (clone RPA-T4), (Brilliant violet 650)-CD25 (clone BC96), (Alexa Fluor 647)-CD127 (clone A019D5), (Alexa Fluor 488)-FoxP3 (clone 206D), and (Brilliant violet 421)-PD-1 (clone EH12.2H7). Stained cells were fixed with FluoroFix buffer (Biolegend, Inc.) according to the manufacturer’s instructions and analyzed with an LSRII (BD Biosciences) flow cytometer. Data analysis was performed with Flowjo software (Treestar). Percentage (%) of positive cells was gated based on appropriate isotype control. Absolute count (per μL of blood) for each immune subset was calculated by multiplying the percentage of each subset with the preceding parent subset and with the absolute lymphocyte count quantified on the day of blood drawn.

Table 1. Clinical responses per cohort

<table>
<thead>
<tr>
<th>Dose level* (mg/kg × 4)</th>
<th>≥50% PSA response (best decline %)</th>
<th>Objective tumor response§</th>
<th>TTPt (months)</th>
<th>Median OS (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (0.5 mg/kg × 4)</td>
<td>0/3</td>
<td>0/3</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>2 (0.5 mg/kg × 3, 1.5 mg/kg × 1)</td>
<td>0/7</td>
<td>0/7</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>3 (1.5 mg/kg × 4)</td>
<td>0/5</td>
<td>0/5</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>4 (1.5 mg/kg × 3, 3 mg/kg × 1)</td>
<td>0/3</td>
<td>0/3</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>5 (3 mg/kg × 4)</td>
<td>3/6 (79, 95, 97)</td>
<td>2/6</td>
<td>20, 25.75, 89.25</td>
<td>56</td>
</tr>
<tr>
<td>6 (5 mg/kg × 2)</td>
<td>0/6</td>
<td>0/6</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>7 (10 mg/kg × 4)</td>
<td>2/6 (50, 80)</td>
<td>0/6</td>
<td>9.75, 18</td>
<td>19</td>
</tr>
<tr>
<td>5A (3 mg/kg × 4)</td>
<td>0/6</td>
<td>0/6</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Cumulative</td>
<td>5/42</td>
<td>2/42</td>
<td>20 (median)</td>
<td>23.6</td>
</tr>
</tbody>
</table>

*Dosage of ipilimumab and the number of doses are given in parentheses ( ).
§Objective tumor response defined by RECIST.
†TTP is time to progression calculated from the time of initial response.
Statistical analysis

Distributions of percentage of paired immune subsets at week 0 (pretreatment) were compared with that at week 4 (cycle 1) or at week 8 (cycle 2) using Wilcoxon matched-pairs signed rank test using Prism (GraphPad) software. The number of patients with PBMCs at the various time points differed based on availability.

Distributions of categorical patient characteristics, such as Eastern Cooperative Oncology group (ECOG) status, Gleason score, prior radical prostatectomy, prior radiation, subsequent therapies, and clinical responses between long-term survivors (LTS; OS range, 25.4 months – 99.7 months; n = 11) and short-term survivors (STS; OS range, 1.9 months – 22.4 months; n = 12), were compared using the Fisher exact test with Prism (GraphPad) software.

Distributions of continuous patient characteristics, such as age, baseline PSA levels, lactate dehydrogenase (LDH) levels, months on study, and percentage of immune subsets between LTS and STS as described above, were compared using the Mann–Whitney U test with Prism (GraphPad) software. Distributions of percentage of immune subsets between cancer-free male controls (n = 7) and LTS or STS were similarly compared using the Mann–Whitney U test.

Statistical significance was declared based on alpha level of 0.05 with Bonferroni correction to adjust for multiple testing as needed. Due to the small sample size, all significant outcomes should be considered as hypothesis generating, and confirmation with a larger sample size is needed.

Results

Patient characteristics

A total of 42 patients underwent treatment. Patient characteristics for each cohort (media and range) are presented in Supplementary Table S1. For all 42 patients, the median age was 70.5 years (range, 47–82). Eight patients had a Gleason score of ≤6, 12 patients had a Gleason score of 7, and 20 patients had a Gleason score of 8 to 10. Gleason scores were not

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available for 2 patients. Thirty-one and 11 patients had an ECOG performance status of 0 and 1, respectively. At pretreatment, the median LDH was 172 U/L (range, 136–557), and the median alkaline phosphatase was 92 U/L (range, 28–1,725). The median PSA at entry was 37.45 ng/mL (range, 6.72–435.10). Twenty-five patients had bone-only disease, 5 patients had soft tissue–only disease, and 12 patients had both bone and soft tissue disease.

Toxicity
Consistent with other studies of ipilimumab, toxicity was primarily immune in nature with the most common adverse events being diarrhea and rash. All adverse events are delineated by cohort in Supplementary Table S2. Seven patients experienced diarrhea, with 3 of these being grade 1 and 4 being grade 3. One of these events required steroids, which is defined as a DLT. Eight patients experienced a rash; one was grade 1, three were grade 2, and four were grade 3. Two of the patients required steroids, making them DLTs. Other immune-related adverse events included adrenal insufficiency (grade 2), panhypopituitarism (grade 3), pneumonitis (grade 2), and temporal arteritis (grade 3). Aside from the expected immune-related adverse events, cardiovascular events were also observed, with 2 occurrences of atrial fibrillation (both grade 3), two cerebrovascular incidences (one grade 3 and one grade 4), and one grade 3 deep venous thrombosis. One patient died from pulmonary embolism and not from disease progression. The MTD was not established for this trial as the 2 DLTs were not observed in the highest dose level (10 mg/kg/dose).

Clinical outcomes
A waterfall plot of nadir PSA values (Fig. 1A) shows that 23 of 42 patients (54%) had some decline in PSA. Five of 42 patients (11.9%) experienced a 50% or greater decline in PSA (Table 1). The median time to PSA nadir was 5.9 weeks (range, 1.9–39.1 weeks) for patients with any PSA decline and 15.9 weeks (range, 11.9–39.1 weeks) for patients with ≥50% PSA decline (Fig. 1B). Objective tumor response and ≥50% PSA decline were not observed in cohorts treated at <3 mg/kg/dose level. Three of 12 patients treated at 3 mg/kg/dose experienced ≥50% PSA decline, and 2 of these 3 patients had objective responses with regression of liver metastasis in 1 patient and of bone metastasis in the other (cohort 5). One patient in the expansion cohort at 3 mg/kg experienced a 49% decline (cohort 5A). In the cohort treated at 5 mg/kg/dose, none of 6 patients showed ≥50% PSA decline or objective tumor response. Of the 6 patients treated at the 10-mg/kg level, 2 had ≥50% PSA decline. There was no accompanying objective tumor response.

As of the censor date of the trial, all patients had come off study. One patient came off treatment by choice. Thirteen patients came off treatment for PSA progression before the first set of scans, 16 patients came off treatment with PSA progression following the first set of scans, 6 patients came off treatment for tumor progression by scans, and 6 patients came off treatment for immune-related adverse toxicities. However, 2 patients from the 3-mg/kg dose group that came off treatment due to immune-related toxicities showed durable responses, with their PSA levels remaining less than 50% of their pretreatment levels for 19 and 85 months after being off treatment without any new treatment (Fig. 1C). One patient from the 5-mg/kg/dose group came off treatment due to an initial disease progression, but a delayed response was observed with his PSA decline attaining 50% at 7 months without any new treatment.

Long-term follow-up
This was a phase Ib study, and survival analysis was not a planned protocol endpoint. Nevertheless, because immunotherapies are now known to induce improvements in OS even in the
absence of objective responses (8, 14), survival analysis was carried out post hoc. The median OS for all patients (n = 42) was 23.6 months (95% CI, 16.2–39.3; Fig. 1D).

Analysis on the censor date showed that 2 of the 42 patients were still living and 40 patients had died. Four of the 5 patients who had clinical responses had OS greater than the median for the group (OS range, 25–100 months). For all patients who had clinical responses described above as defined by objective tumor response and/or PSA decline of ≥50% from baseline, OS ranged from 14 months to 100 months. For the remainder of patients from the same dose cohorts (3 mg/kg/dose to 10 mg/kg/dose) who did not show clinical responses, OS ranged from 2 months to 86 months. Long OS was observed in patients without clinical responses.

Distribution of patient baseline characteristics with OS
Because ipilimumab is FDA approved for treatment of unresectable or metastatic melanoma at the 3 mg/kg/dose and clinical responses were not observed at less than 3 mg/kg/dose in this trial, we chose to evaluate further patients that were treated with at least 3 mg/kg/dose of ipilimumab. Patient characteristics and clinical responses for individual patients treated with ≥3 mg/kg/dose and sargramostim at 250 µg/m²/dose are presented in Supplementary Table S3. These patients were divided into two groups using median survival of 23.6 months as the cutoff. Baseline characteristics of patients with long overall survival (LTS; OS range, 25.4 months–99.7 months; n = 11) were compared with those of patients with short overall survival (STS; OS range, 1.9 months–22.4 months; n = 12; Supplementary Fig. S1). Patients’ age, baseline PSA levels, LDH levels, or months on study did not correlate with OS (P = 0.193, 0.311, 0.277, and 0.100, respectively). The numbers of patients with ECOG status of 0 or 1, Gleason scores grouped as 3 to 6 or 7 to 9, prior radical prostatectomy, and prior radiation were not significantly different between the two groups (P = 1.00, 0.90, 1.00, and 0.67, respectively). The number of patients with clinical responses as described above and the number of patients who went on to subsequent therapies also did not correlate with OS (P = 0.16 and 0.38, respectively).

Treatment increased the levels of Tregs, CD4 T_{eff} cells, and PD-1^{+}CD4 T_{eff} and PD-1^{+}CD8 T_{cells}
Where possible, analyses for treatment-induced changes in levels of immune subsets for patients treated with ≥3 mg/kg/dose and sargramostim at 250 µg/m²/dose were performed. Distribution and levels of immune subsets from week 4 (cycle 1) or week 8 (cycle 2) of treatment were compared with those of pretreatment levels at week 0.

The absolute lymphocyte counts were significantly higher compared with those of pretreatment levels after cycle 1 but not after cycle 2 of treatment (P = 0.002 and 0.119, respectively; Fig. 2A).

We have shown previously that ipilimumab and sargramostim expanded the levels of circulating Tregs (CD4^{+}CD3^{+}FoxP3^{+}CD127^{−}CD25^{+}; ref. 16). This was also observed for patients who were treated with ≥3 mg/kg/dose of ipilimumab and 250 µg/m²/dose of sargramostim after cycle 1 and after cycle 2 of treatment (Fig. 3A–C).

The percentages of total lymphocytes and absolute counts of CD4 T_{eff} cells (CD4^{+}CD3^{+}FoxP3^{−}) were significantly higher after one cycle of treatment (Fig. 2C and E). However, for CD8 T cells, only the absolute counts and not the percentage of total lymphocytes were significantly higher after one cycle of treatment (Fig. 2G and I). This difference could be due to the higher levels of absolute lymphocyte counts after one cycle of treatment as described above.
The percentages of CD4 T_{eff} cells that express surface PD-1 were significantly higher after cycle 1 ($P = 0.0001$) and continued to be significantly higher after cycle 2 compared with pretreatment levels ($P = 0.0002$; Fig. 4A, B, and D). The absolute counts of PD-1^{+} CD4 T_{eff} cells were also significantly higher after both cycles ($P = 0.0001$ and 0.0002, respectively). The percentages of CD8 T cells that express PD-1 were also significantly higher from pretreatment levels after cycle 1 ($P = 0.004$) and after cycle 2 of treatment ($P = 0.005$; Fig. 4A, C, and E). The absolute counts of PD-1^{+} CD8 T cells were also significantly higher after both cycles ($P = 0.005$ and 0.022, respectively).

Lower levels of preexisting PD-1^{+} CD4 T_{eff} cells correlated with longer survival

Next, we investigated in an exploratory manner if immune subsets were related to survival duration with available samples. The levels of immune subsets of patients treated with 3 mg/kg dose and sargramostim at 250 μg/m²/dose with long overall survival (LTS, OS range, 25.4 months–99.7 months) were compared with those of patients with short overall survival (STS, OS range, 1.9 months–22.4 months).

Distribution of the absolute lymphocyte counts did not differ between LTS and STS at pretreatment ($P = 0.201$), after cycle 1 ($P = 0.670$), and after cycle 2 of treatment ($P = 0.779$; Fig. 2B).
**Table 2. Comparison of T-cell subsets between LTS and STS**

<table>
<thead>
<tr>
<th>T-cell subsets*</th>
<th>LTS*</th>
<th>Median† (range)</th>
<th>STS*</th>
<th>Median† (range)</th>
<th>p‡</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Week 0 (pretreatment)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total CD4 T cells (CD4⁺CD3⁺)</td>
<td>41.0 (32.8–59.6)</td>
<td>48.3 (33.6–71.8)</td>
<td></td>
<td></td>
<td>0.203</td>
</tr>
<tr>
<td>CD4 Teff cells (CD4⁺CD3⁺FoxP3⁻)</td>
<td>38.9 (30.1–56.2)</td>
<td>45.2 (30.4–66.8)</td>
<td></td>
<td></td>
<td>0.263</td>
</tr>
<tr>
<td>PD-1⁺ (PD-1⁺CD4⁺CD3⁺FoxP3⁻)</td>
<td>10.1 (5.4–14.5)</td>
<td>22.0 (12.8–42.3)</td>
<td></td>
<td></td>
<td>0.0007</td>
</tr>
<tr>
<td>Treg (CD4⁺CD3⁺FoxP3⁺CD127⁺CD25⁺)</td>
<td>1.6 (0.7–2.8)</td>
<td>2.0 (1.23–3.9)</td>
<td></td>
<td></td>
<td>0.113</td>
</tr>
<tr>
<td>Total CD8 T cells (CD4⁻CD8⁺)</td>
<td>24.5 (5.0–42.2)</td>
<td>18.3 (6.7–50.7)</td>
<td></td>
<td></td>
<td>0.461</td>
</tr>
<tr>
<td>PD-1⁺ (PD-1⁺CD8⁺)</td>
<td>15.0 (5.31–28.0)</td>
<td>22.2 (8.4–33.0)</td>
<td></td>
<td></td>
<td>0.246</td>
</tr>
<tr>
<td><strong>Week 4 (cycle 1)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total CD4 T cells (CD4⁺CD3⁺)</td>
<td>58.1 (43.3–62.3)</td>
<td>58.3 (45.0–62.0)</td>
<td></td>
<td></td>
<td>0.942</td>
</tr>
<tr>
<td>CD4 Teff cells (CD4⁺CD3⁺FoxP3⁻)</td>
<td>53.3 (41.1–54.7)</td>
<td>51.9 (32.1–59.0)</td>
<td></td>
<td></td>
<td>0.805</td>
</tr>
<tr>
<td>PD-1⁺ (PD-1⁺CD4⁺CD3⁺FoxP3⁻)</td>
<td>18.8 (7.12–35.4)</td>
<td>28.1 (13.6–49.5)</td>
<td></td>
<td></td>
<td>0.055</td>
</tr>
<tr>
<td>Treg (CD4⁺CD3⁺FoxP3⁺CD127⁺CD25⁺)</td>
<td>3.0 (1.9–4.2)</td>
<td>2.7 (1.7–6.0)</td>
<td></td>
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<td>0.980</td>
</tr>
<tr>
<td>Total CD8 T cells (CD4⁻CD8⁺)</td>
<td>20.6 (5.1–32.2)</td>
<td>16.1 (8.6–22.0)</td>
<td></td>
<td></td>
<td>0.555</td>
</tr>
<tr>
<td>PD-1⁺ (PD-1⁺CD8⁺)</td>
<td>16.5 (6.49–33.0)</td>
<td>26.7 (14.1–38.7)</td>
<td></td>
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<td>0.246</td>
</tr>
<tr>
<td><strong>Week 8 (cycle 2)</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Total CD4 T cells (CD4⁺CD3⁺)</td>
<td>54.5 (38.5–65.6)</td>
<td>55.4 (29.8–70.1)</td>
<td></td>
<td></td>
<td>0.931</td>
</tr>
<tr>
<td>CD4 Teff cells (CD4⁺CD3⁺FoxP3⁻)</td>
<td>50.8 (36.1–61.1)</td>
<td>50.4 (26.8–63.1)</td>
<td></td>
<td></td>
<td>0.920</td>
</tr>
<tr>
<td>PD-1⁺ (PD-1⁺CD4⁺CD3⁺FoxP3⁻)</td>
<td>18.3 (8.91–34.5)</td>
<td>31.1 (17.2–51.2)</td>
<td></td>
<td></td>
<td>0.054</td>
</tr>
<tr>
<td>Treg (CD4⁺CD3⁺FoxP3⁺CD127⁺CD25⁺)</td>
<td>2.5 (1.4–3.6)</td>
<td>3.1 (1.5–4.1)</td>
<td></td>
<td></td>
<td>0.367</td>
</tr>
<tr>
<td>Total CD8 T cells (CD4⁻CD8⁺)</td>
<td>15.4 (5.8–31.7)</td>
<td>18.6 (15.2–52.6)</td>
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<td></td>
<td>0.671</td>
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<tr>
<td>PD-1⁺ (PD-1⁺CD8⁺)</td>
<td>21.2 (5.93–30.3)</td>
<td>26.5 (14.7–40.4)</td>
<td></td>
<td></td>
<td>0.228</td>
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</table>

* T-cell subsets are defined by immune markers as indicated in the table.

† Median values of total CD4 T cells, total CD8 T cells, CD4 Teff cells, and Tregs are percentage of total lymphocytes. Median values of PD-1⁺-positive cells are percentage of the respective parent gate.

‡ Values in parentheses () are range of each data set.

Distribution of the percentages of total lymphocytes and the absolute counts of Tregs did not differ between LTS and STS at pretreatment (P = 0.113 and 0.504, respectively), after cycle 1 (P = 0.980 and 0.348, respectively), and after cycle 2 of treatment (P = 0.387 and 0.752, respectively; Fig. 3D and E; Table 2 and Supplementary Table S4).

Distribution of the percentages of total lymphocytes and absolute counts of CD4 Teff cells also did not differ between LTS and STS at pretreatment (P = 0.263 and 0.841, respectively), after cycle 1 (P = 0.805 and 0.745, respectively), and after cycle 2 of treatment (P = 0.920 and 0.845, respectively; Fig. 2D and F; Table 2 and Supplementary Table S4). The percentages of total lymphocytes and absolute counts of CD8 T cells did not correlate with survival at pretreatment (P = 0.461 and 0.304, respectively), after cycle 1 (P = 0.355 and 0.670, respectively), and after cycle 2 of treatment (P = 0.671 and 0.835, respectively; Fig. 2H and I; Table 2 and Supplementary Table S4).

However, distribution of the percentages of surface PD-1⁺ CD4 T eff cells and absolute counts of PD-1⁺ CD4 T eff at pretreatment was significantly lower in LTS compared with that in STS (P = 0.0007 and 0.003, respectively; Fig. 2F and H; Table 2 and Supplementary Table S4).

After treatment, the distribution of the percentages of surface PD-1⁺ CD4 T eff cells and absolute counts of PD-1⁺ CD4 T eff were not significantly different between STS or LTS after cycle 1 (P = 0.055 and 0.090, respectively) and after cycle 2 (P = 0.054 and 0.150, respectively; Fig. 4F and H).

Distributions of the percentages and absolute counts of surface PD-1⁺ CD8 T cells between STS and LTS did not differ at pretreatment (P = 0.246 and > 0.999, respectively) or at any time point after treatment (Fig. 4A, G, and I; Table 2 and Supplementary Table S4).

Comparison of the distribution of percentages of surface PD-1⁺ CD4 T cells in mCRPC patients with cancer-free male controls revealed that STS has significantly higher levels of PD-1⁺ CD4 T cells compared with those in cancer-free male controls (P = 0.002). There was no significant difference in the distribution of the percentages of PD-1⁺ CD8 T-cell levels between cancer-free male controls and patients with STS or LTS (Table 3).

**Table 3. Comparison of pretreatment T-cell subsets between cancer-free male controls and LTS or STS**

<table>
<thead>
<tr>
<th>T-cell subsets*</th>
<th>Groups‡</th>
<th>Median† (range)</th>
<th>p‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD-1⁺ CD4 T eff cells</td>
<td>Cancer-free controls</td>
<td>11.7 (6.67–14.30)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LTS week 0</td>
<td>10.1 (5.4–14.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>STS week 0</td>
<td>22.2 (8.4–33.0)</td>
<td></td>
</tr>
<tr>
<td>PD-1⁺ CD8 T cells</td>
<td>Cancer-free controls</td>
<td>13.7 (8.7–27.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LTS week 0</td>
<td>15.0 (5.31–28.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>STS week 0</td>
<td>22.2 (8.4–33.0)</td>
<td></td>
</tr>
</tbody>
</table>

* T-cell subsets are defined by immune markers as indicated in the table.

‡ Cancer-free male controls, n = 7; LTS, n = 8; STS, n = 12.

† Median values of PD-1⁺-positive cells are percentage of the respective parent gate.

‡ Values in brackets () are range of each data set.

Mann–Whitney test; bold-faced characters highlight P < 0.017 (Bonferroni correction).
Discussion

Treatment of mCRPC patients with ipilimumab and sargramostim in this study revealed several findings. First, delayed response by PSA decline can be observed. Second, a small number of patients continued to experience durable responses off treatment without additional treatment. Third, a subset of patients had longer OS than expected for this disease with or without a clinical response to study treatment. Durable benefit and the potential for long-term survival were similarly observed for treatment of melanoma with ipilimumab and treatment of prostate cancer with sipuleucel-T and PROSTVAC-VF (8, 14). Treatment kinetics of ipilimumab differ from those observed with radiation and chemotherapy and could be explained by the mechanism of action of immunotherapy (17). Immunotherapy targets the immune system, which subsequently targets the tumor. Presumably, the immune system is capable of generating long-lived memory cells to sustain clinical response beyond the duration of treatment. The immune system may also slow tumor growth without reducing tumor size, resulting in longer OS without accompanying clinical responses.

Identifying patients most likely to benefit before start of treatment is a significant unmet clinical need. We hypothesized that expression of immune checkpoint markers in T cells could differ between patients with long and short overall survival. In cancer patients, higher levels of immune checkpoint markers in T cells could be indicative of endogenous T-cell activation and perhaps tumor-induced immune suppression. We found that study treatment induced increased levels of Tregs, CD4 $^{+}$ T cells, and surface PD-1 expression on CD4 $^{+}$ T cells and CD8 T cells, which are consistent with activating T cells in vivo. These observations may also explain the increased effectiveness of combined CTLA-4 and PD-1 blockade therapies (18), as CTLA-4 blockade would increase the levels of PD-1 on CD4 and CD8 $^{+}$ T eff cells during treatment. However, the increased levels of these cells examined after one and two cycles of ipilimumab and sargramostim were not associated with clinical outcome. Although the absolute lymphocyte counts of melanoma patients were reported to correlate with OS after two ipilimumab doses (19), this relationship was not observed with the subset of prostate cancer patients that were analyzed.

We found that lower pretreatment levels of surface PD-1 on CD4 $^{+}$ T eff cells (PD-1$^{+}$CD4$^{+}$CD3$^{+}$FoxP3$^{-}$) were correlated positively with longer survival in this study. Consistent with our data, lower baseline PD-1$^{-}$Tim-3$^{-}$ CD4 memory T cells (CD45RA$^{+}$CD62L$^{-}$CCR7$^{-}$) were reported to correlate significantly with longer survival in a phase I trial combining ipilimumab and PROSTVAC vaccine (20, 21). We also observed that levels of PD-1$^{+}$CD4 $^{+}$ T eff cells in patients with short OS were higher than those in male controls who did not have a diagnosis of prostate cancer. Therefore, higher baseline levels of immune checkpoint molecules in circulating lymphocytes from cancer patients may reflect the presence of tumor-reactive T cells that also serve to maintain the lymphocytes in a tolerant state (22). Notably, the ipilimumab plus PROSTVAC vaccine study and our study both revealed associations of inhibitory immune markers with survival on CD4 rather than CD8 T cells. This suggests that there may be differential effects of PD-1 in CD4 and CD8 T cells, and further studies of PD-1$^{+}$CD4 $^{+}$ T cells in cancer patients are required.

Because all patients received ipilimumab and sargramostim treatment, it is unclear at present whether these findings are specific to this combined regimen and/or to prostate cancer. It is also unclear whether expression levels of PD-1 on CD4 $^{+}$ T eff cells are prognostic or predictive biomarkers. It will be interesting to see if the same or different observations would be found in clinical trials with ipilimumab with or without sargramostim. The data reported in this study are intended to be hypothesis generating. Prospective clinical trials will be needed to examine formally whether low PD-1 levels in circulating CD4 $^{+}$ T eff cells could be potential biomarkers for CTLA-4 blockade and GM-CSF combination immunotherapy.

Disclosure of Potential Conflicts of Interest

L. Fong reports receiving commercial research support from Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

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 Study supervision: S.S. Kwek, L. Fong

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